RECEPTORY ZÚČASTNĚNÉ V REGULACÍCH ZÁNĚTLIVÉ REAKCE U RENÁLNÍCH ONEMOCNĚNÍ

DISERTAČNÍ PRÁCE

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SEZNAM POUŽITÝCH ZKRATEK

ACE	Angiotensin-converting enzyme
AT	Angiotensin
BMP7	Bone morphogenetic protein 7
BP	Blood pressure
DARC	Duffy antigen/chemokine receptor
DNA	Deoxyribonukleová kyselina
DOCA	Deoxycorticosterone Acetate
EGF	Epidermal growth factor
EMT	Epiteliální - mezenchymální přeměna
ET	Endothelin
FGF-2	Fibroblast growth factor 2
FSGS	Fokálně segmentální glomerulonefritida
FSP-1	Fibroblast-specific protein 1
GBM	Glomerulární bazální membrána
GISEN	Gruppo Italiano di Studi Epidemiologici in Nefrologia
GTP	Guanosintrifosfát
H_2O_2	Peroxid vodíku
HanSD	Hannover Sprague-Dawley
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
ICAM-1	Intercellular adhesion molecule 1
IGF-1	Insuline-like growth factor 1
IL	Interleukin
ILK	Integrin-linked kinase
INFγ	Interferon-gamma
kDa	Kilodalton
KDOQI	Kidney Disease Outcomes Quality Initiative
LPS	Lipopolysacharid
MAPK	Mitogen-activated protein kinase
MCP-1	Macrophages and monocyte chemoattractant protein 1
MIP-1	Macrophage inflammatory protein 1
MMP-9	Matrix metalloproteinase 9
mRNA	Messenger ribonukleová kyselina
NADPH	Nikotinamid adenin dinukleotid fosfát
NEOERICA	New Opportunities for Early Renal Intervention by Computerised Assessment
NF-κB	Nuclear factor kappa B
NHANES	The National Health and Nutrition Examination Survey
NO	Oxid dusnatý
PAF	Platelet-activating factor
PAI-1	Plasminogen activator inhibitor 1

PDGF	Platelet-derived growth factor
RAAS	Systém renin-angiotenzin-aldosteron
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RPGN	Rychle progredující glomerulonefritida
RT-PCR	Reverse transcription polymerase chain reaction
SLE	Systémový lupus erythematodes
TBM	Tubulární bazální membrána
TGFβ1	Transforming growth factor beta 1
TGR	Transgenní potkan
TIMP-1	Tissue inhibitor of metalloproteinase 1
TNF-α	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UUO	Jednostranná obstrukce ureteru
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
α-SMA	Aktin hladké svaloviny

1 ÚVOD DO PROBLEMATIKY

1.1 Chronické onemocnění ledvin

Chronické onemocnění ledvin je v současné době definováno dle Kidney Disease Outcomes Quality Initiative (KDOQI Guidelines, 2002, Levey et al 2009, Glassock et Winearls 2010), doporučení americké National Kidney Foundation, jako

1. poškození ledvin trvající déle než tři měsíce, definované strukturálními nebo funkčními abnormalitami ledvin, s nebo bez snížené glomerulární filtrace, které se manifestuje patologickými abnormalitami nebo markery poškození ledvin (detekovanými vyšetřením krve, moči, močového sedimentu nebo detekovanými na základě zobrazovacích metod)
2. pokles glomerulární filtrace pod 1,0 ml/s/1,73 m2 trvající déle než 3 měsíce, s nebo bez poškození ledviny.

KDOQI dělí chronické onemocnění ledvin do pěti stádií (1-5), kde stadium 3 a 4 znamená snížení glomerulární filtrace středního a těžkého stupně, stádium 5 pak selhání ledvin s nutností náhrady renálních funkcí dialýzou nebo transplantací ledviny. Podle americké studie NHANES (The National Health and Nutrition Examination Survey) (Coresh et al 2003) je chronické onemocnění ledvin časté (stadium 3–5 postihuje 4,7 % populace) a jeho výskyt roste s věkem. Britská studie NEOERICA (New Opportunities for Early Renal Intervention by Computerised Assessment) (Stevens et al 2007) zahrnovala dospělé osoby průměrného věku 58,1 ± 18,1 let. V této studii mělo celkem 8,5 % pacientů chronické onemocnění ledvin stadia 3–5. Významně častěji byly postiženy ženy (10,6 %) proti mužům (5,8 %), a to prakticky ve všech věkových skupinách. Zhruba polovina pacientů s chronickým onemocněním ledvin ve stadiu 3 a zhruba tři čtvrtiny pacientů ve stadiu 4–5 jsou starší než 70 let.

Hlavní příčiny chronického onemocnění ledvin jsou: diabetes mellitus, hypertenze, chronické glomerulonefritidy a renovaskulární onemocnění (zejména aterosklerotická stenóza renální arterie).

Vzhledem k tomu, že populace vyspělých zemí stárne, lze očekávat postupně se zvyšující prevalenci chronického onemocnění ledvin. Chronické onemocnění ledvin je spojeno s vysokou celkovou a kardiovaskulární mortalitou, navíc bývá spojeno s dalšími závažnými komorbiditami; léčba jeho pokročilých stádií představuje pro společnost poměrně značnou ekonomickou zátěž.

1.2 Schéma progrese chronického renálního onemocnění

Progrese celé řady nefropatií do chronického renálního selhání je jedním z hlavních problémů nefrologie, protože jen několik málo onemocnění ledvin vede po krátké akutní fázi k úplnému vyhojení a funkční restituci. Většina renálních onemocnění má chronický průběh, s pomalou postupnou ztrátou ledvinných funkcí a s rozvojem renální insuficience (Anders et al 2003). Jakmile jakékoli renální poškození dosáhne určité prahové úrovně, nezávisle na původním inzultu progreduje nezvratně a nepřetržitě do obrazu svraštělé ledviny konečného stádia – end-stage kidney. Progrese do tohoto stavu morfologicky koreluje s rozvojem tubulointersticiálního poškození a fibrózy renálního intersticia (Nath 1992, Müller et al 2000, Nangaku 2004), morfologicky charakterizovanými tubulární atrofií, zmnožením fibroblastů, produkcí zvýšeného množství extracelulární matrix a akumulací leukocytů (Nath 1992, Strutz et al 1999, Zeisberg et al 2000). Schematicky lze průběh progresivního renálního onemocnění rozdělit do čtyř fází - iniciální fáze, fáze amplifikace, fáze progrese a terminální fáze (Anders et al 2003).

1.2.1 Iniciální fáze

Primární poškození každého z typů buněk ledvinného parenchymu vede k uvolňování prozánětlivých mediátorů, cytokinů a chemokinů, které má za následek infiltraci poškozené tkáně leukocyty (Anders et al 2003). Pokud je zánětlivý proces ohraničen jen na některý z kompartmentů ledviny (glomeruly nebo tubulointersticium), infiltrují zánětlivé buňky pouze tuto oblast (Anders et al 2001, Vielhauer et al 2001). Různé kompartmenty ledviny jsou přitom osídlovány selektivně různými populacemi lymfocytů. Zatímco v tubulointersticiálním kompartmentu jsou obvyklou komponentou

zánětlivého infiltrátu CD3 pozitivní T lymfocyty (Segerer et al 2000b), v poškozených glomerulech se nevyskytují, dokud nedojde k poškození Bowmannova pouzdra. Naproti tomu v poškozených glomerulech nacházíme pravidelně monocytární/makrofagický zánětlivý infiltrát, zejména u proliferativních glomerulonefritid a glomerulonefritidy se srpky. Tato selektivita je dána různou expresí chemokinů a adhezivních molekul v těchto kompartmentech (jak je uvedeno níže).

1.2.2 Fáze amplifikace

Infiltrace leukocyty výrazně posiluje produkci cytokinů a chemokinů v ložisku poškození. Makrofágy jsou hlavním zdrojem růstových faktorů a cytokinů, jako je TGF-β1, EGF, PDGF a TNF-α, ET-1, PAI-1. V glomerulech stimulují tyto cytokiny proliferaci mesangiálních buněk a syntézu extracelulární matrix, vedoucí k obrazu mesangioproliferativní glomerulonefritidy (Daha 2000). Alterace podocytů vede k řadě strukturálních změn, ústících nakonec ve splývání podocytárních výběžků, s následnou proteinurií. Pokles počtu podocytů vede k denudaci GBM, se ztrátou mechanické podpory kapilární kličky, formací mikroaneurysmat a vznikem synechií s Bowmanovým pouzdrem – vznik synechií byl experimentálně prokázán jako první stádium rozvoje fokálně segmentální glomerulosklerózy (Kriz et al 1986, Mundel 2002, Schwartz et al 1985). Proliferace a aktivace fibroblastů v intersticiu doprovázená produkcí extracelulární matrix vede k fibróze intersticia ledviny. V tomto procesu se vedle proliferace rezidentních fibroblastů zásadním způsobem uplatňuje epiteliální - mezenchymální přeměna (transdiferenciace), kdy z epitelu tubulů vznikají fibroblasty. Tento komplexní proces je mimo jiné indukován cytokiny uvolňovanými makrofágy, jako je FGF-2 (Strutz et al 2002). Navíc makrofágy a neutrofilní granulocyty zánětlivého infiltrátu vytváří kyslíkové radikály, které přispívají k místnímu poškození tkáně.

U primárních glomerulopatií, jako jsou například membranózní glomerulopatie, fokálně segmentální glomeruloskleróza nebo mesangioproliferativní glomerulonefritida, se postupně s délkou trvání a tíží postižení glomerulů rozvíjí zánětlivý infiltrát v intersticiu s následnou intersticiální fibrózou. V progresi primárně glomerulárního postižení na tubulointersticium hrají důležitou roli postglomerulární peritubulární kapiláry a

tubulární epitel. Do glomerulárního ultrafiltrátu se při poškození glomerulů dostávají růstové faktory, prozánětlivé mediátory a albumin, které stimulují cestou NF-κB sekreci prozánětlivých a profibrotických cytokinů a chemokinů tubulárním epitelem (Abbate et al 2006, Segerer et al 2000b, Anders et al 2003). Navíc se mediátory zánětu secernované v glomerulárním kompartmentu dostávají do peritubulární postglomerulární cirkulace, kde aktivují endotel a tubulární epitel (Abbate et al 1998, Anders et al 2003). Dysbalance vazoaktivních substancí doprovázená aktivací renin-angiotensinového systému a uvolňováním angiotensinu II a následná vasokonstrikce eferentních arteriol spolu s glomerulárním poškozením snižují krevní zásobení v peritubulárním postglomerulárním kapilárním řečišti. Angiotensin II kromě vazoaktivního účinku působí ischemizaci indukcí oxidativního stresu (stimulací NADPH oxidázy), což zhoršuje využití kyslíku tubulárním epitelem. Navíc zvýšené metabolické nároky na tubulární buňky vedou k relativní hypoxii (Nangaku 2006, Nangaku et al 2008).

1.2.3 Fáze progrese

Trvalá stimulace renálních buněk při proteinurii, infiltrujícími leukocyty a uvolňovanými cytokiny vede k pokračující syntéze extracelulární matrix a k ireverzibilnímu poškození ledvinné struktury. Makrofágy, které infiltrují glomeruly, stimulují mesangiální buňky k syntéze a sekreci kolagenu IV, fibronektinu a lamininu, což přispívá k rozvoji glomerulosklerózy. Expanze mesangia působí zúžení a obliteraci glomerulárních kapilár (Kriz et al 1996). Akumulace makrofágů a T buněk v intersticiu ledviny a kontinuální sekrece prozánětlivých a profibrotických cytokinů vede k proliferaci fibroblastů, k epiteliální - mezenchymální přeměně epitelu tubulů a k produkci extracelulární matrix těmito buňkami. Aktivovaný tubulární epitel zde slouží jako další zdroj cytokinů a chemokinů (Strutz et al 2002). Pokročilé poškození tubulointersticiálního kompartmentu je spojeno s poškozením (obliterací) a ztrátou peritubulárních kapilár (Choi et al 2000, Ohashi et al 2000). Fibróza intersticia zhoršuje difúzi kyslíku, tím dochází k zhoršenému zásobení buněk intersticia a tubulů. Ischemie tubulárních buněk vede jednak k apoptóze a dále opět k epiteliální - mezenchymální přeměně, zhoršující fibrózu a následně i hypoxii tkáně, vzniká circulus vitiosus, končící

morfologickým obrazem end stage kidney s chronickým renálním selháním (Nangaku 2006).

1.2.4 Terminální fáze

Následkem difúzního jizvení tkáně ledviny a jejího nedostatečného krevního zásobení jsou těžká atrofie tubulů a glomeruloskleróza. Postupně dochází k rozvoji end-stage renálního onemocnění s klinickými příznaky urémie. Renální fibroblasty, stimulovány hypoxií a autokrinní sekrecí cytokinů, pokračují v produkci extracelulární matrix, myofibroblasty přispívají k obrazu kontrakcí fibrózní tkáně, rozvíjí se obraz svraštělé ledviny konečného stadia.

1.3 Poškození podocytů

Podocyty, epitelové buňky viscerálního listu Bowmanova pouzdra, jsou terminálně diferencované buňky pokrývající zevní povrch glomerulární bazální membrány. Jejich celulární výběžky, pedikly, jsou navzájem bazolaterálně propojeny glomerulární filtrační membránou, komplexem proteinů, který představuje hlavní selektivní filtrační bariéru ledvin; poškození podocytů je tedy zpravidla spojeno s rozvojem proteinurie. Bazolaterální oblast pediklů obsahuje tři hlavní membránové domény podílející se na základních funkcích podocytů: apikální membránovou doménu, komplex filtrační membrány a bazální membránovou doménu asociovanou s GBM. Všechny tři domény jsou strukturálně i funkčně propojeny s aktinovým cytoskeletem podocytů (Faul et al 2007).

K poškození podocytů dochází z různých příčin (Hostetter et al 2001, Pollak 2002, Kanwar et al 2008) u celé řady chorob glomerulů, jako jsou například nemoc minimálních změn, idiopatická FSGS, diabetická glomeruloskleróza nebo lupusová nefritida. Bez ohledu na vyvolávající příčinu je časné poškození podocytů charakterizováno změnami ve složení filtrační membrány, bez patrných morfologických změn, anebo častěji splýváním podocytárních výběžků - fúzí pedicel. Základní čtyři příčiny fúze pedicel a následné proteinurie jsou (Mundel et al 2002):

a. interference s komplexem filtrační membrány a spřaženými membránovými lipidovými rafty,

b. interference s GBM nebo vazbou GBM a podocytu,

c. interference s aktinovým cytoskeletem a s ním asociovaných proteinů (jako je α-aktinin-4),

d. interference s apikální membránovou doménou podocytů (např. ztráta negativního povrchového náboje).

Fúze pedicel je reverzibilní proces a jako takový je potenciálně terapeuticky ovlivnitelný, jinak může vést k progresi poškození glomerulu s deplecí podocytů a jizvením, a postupně k poškození celého nefronu a k rozvoji chronického renálního selhání. Proces fúze pedicel, případně jeho následná regenerace, obsahuje reorganizaci aktinového cytoskeletu, změny ve struktuře a funkci filtrační membrány a alteraci adheze podocytu ke GBM. Všechny tyto kroky jsou regulovány několika enzymatickými drahami, zahrnujícími signalizaci pomocí fosforylace a defosforylace proteinů, a dále proteolytickou regulační kaskádou krátké cytosolické varianty katepsinu L (Kistler et al 2010). Dosud byly popsány dva substráty cytosolické varianty katepsinu L, dynamin a synaptopodin. Dynamin v podocytech stabilizuje strukturální uspořádání aktinového cytoskeletu, synaptopodin reguluje funkci α-aktininu 4, proteinu vážícího aktin do paralelních svazků. Zabránění degradace synaptopodinu katepsinem L je recentně popsaným mechanismem antiproteinurického účinku imunosupresiva cyklosporinu A, používaného např. k léčbě primární FSGS (Faul et al 2008).

Z řady experimentálních prací (Shirato et al 1996, Kim et al 2001, Steffes et al 2001) vyplývá, že kritickým determinantem pro rozvoj glomerulosklerózy je počet podocytů, tedy že pokles podocytů vede k progresivnímu selhání ledvin. Počet podocytů v glomerulu je konečný. Za normálních okolností každý podocyt pokrývá svoji specifickou oblast GBM kapilární kličky; dojde-li k poklesu počtu podocytů, může být jejich počet nedostačující k pokrytí plochy GBM. Sekvence změn v rozvoji glomerulosklerózy je následující: poškození a ztráta podocytu vede k obnažení GBM. Následkem hydrostatického kapilárního tlaku dojde k vyklenutí kapilární kličky; to je dáno tím, že jednou z funkcí podocytů je i tlaková podpora kapilární stěny proti hydrostatickému tlaku působícímu její distenzi (Kriz et al 1994). Vyklenující se kapilární klička s obnaženou GBM přilne k Bowmanovu pouzdru, vytvoří

se adheze a následně dochází k rozvoji hyalinózy a jizvení kapilární kličky (Schwartz et al 1985).

Příčiny poklesu počtu podocytů jsou: neschopnost podocytů proliferovat, zánik podocytů mechanismem apoptózy a ztráta adheze podocytu ke GBM.

Apoptóza podocytů byla demonstrována na modelu TGF-β1 transgenních myší (Schiffer 2001), kde pokles podocytů vedl k rozvoji glomerulosklerózy. V kultuře potkaních podocytů lze apoptózu indukovat podáním angiotensinu II (Ding et al 2002), blokáda angiotensinových receptorů apoptóze zcela zabrání. V současné době jsou detailně popsány dvě signální dráhy vedoucí k apoptóze podocytů: aktivace p38 mitogenem aktivované proteinkinázy (MAPK) a působení TGF-β1. Aktivace fosfoinositid-3-kinázy a AKT (proteinkinázy B) naopak působí antiapoptoticky (Chuang a He 2009).

Dalším mechanismem, který vede ke snížení počtu podocytů, je ztráta jejich adheze ke GBM. Existuje řada studií, které v moči pacientů s různými glomerulárními chorobami prokázaly imunohistochemicky přítomnost podocytů (Hara et al 2001, Nakamura et al 2001). I v modelu membranózní glomerulopatie - pasivní Haymannovy nefritidy - a streptozotocinem indukované diabetické nefropatie se pomocí imunohistochemického průkazu podocinu a nefrinu podařilo prokázat v moči experimentálních zvířat podocyty (Mundel et al 2002).

Zajímavým pohledem na adaptivní změny podocytárního fenotypu jako odpovědi na poškození je hypotéza epiteliální - mezenchymální přeměny (EMT) podocytů. V závislosti na tíži poškození a jeho trvání dochází dle navrženého modelu nejprve k hypertrofii podocytů, následně k podocytární EMT, popřípadě až k odloučení transformovaných podocytů či k jejich apoptóze. V tkáňové kultuře podocyty inkubované s TGF-β1 ztrácí P-kadherin, ZO-1 a nefrin, tedy proteiny asociované s filtrační membránou, a zároveň nově exprimují desmin a produkují MMP-9, fibronektin a kolagen I. Změny podocytárního fenotypu byly studovány i in vivo na modelu streptozotocinem indukované diabetické nefropatie a demonstrovány i v biopsiích ledvin pacientů s diagnózou FSGS a diabetické nefropatie (Li et al 2008).

1.4 Vztah proteinurie a progrese renálního onemocnění

Chronické glomerulopatie spojené s proteinurií mají, bez ohledu na vyvolávající příčinu poškození glomerulu, za následek ztrátu normální funkce glomerulární bariéry selektivně filtrovat proteiny. Ukazuje se, že proteinurie není jen známkou poškození glomerulárních funkcí, ale i významným rizikovým a predikčním faktorem progrese renálního onemocnění (Remuzzi et al 2006, Hallan et al 2009) a kardiovaskulární mortality u řady chronických onemocnění ledvin (Peterson et al 1996, studie GISEN 1997, Brantsma et al 2008). Řada klinických studií postupně prokázala, že redukce proteinurie má významný renoprotektivní efekt (Brenner et al 2001, Jafar et al 2001, de Zeeuw et al 2004, Ruggenenti et al 2008).

V in vitro podmínkách stimulace buněk plazmatickými proteiny, jako jsou albumin, transferin nebo IgG, indukovala v buněčné kultuře epitelu proximálních tubulů syntézu endotelinu ET-1, chemokinů CCL2 (MCP-1), CCL5 (RANTES) a CXCL8 (IL-8) (Zoja et al 1995, 1998, Wang et al 1997). K produkci těchto proteinů dochází zejména směrem k bazolaterálnímu kompartmentu buňky. Nadměrná stimulace proteiny plazmy vede dále za účasti proteinkinázy C k rychlé syntéze H_2O_2, s následnou aktivací klíčového prozánětlivého transkripčního proteinu NF-κB (Morigi et al 2002).

Dosud není zcela jasné, které proteiny obsažené v primární moči hrají klíčovou roli v aktivaci tubulů. Albumin sám o sobě nejspíše není toxický pro tubulární epitel (Hirschberg et Wang 2005). Podle některých studií se zdá, že toxicky působí látky na albumin a ostatní proteiny navázané, jako jsou volné mastné kyseliny (Arici et al 2002, Kamijo et al 2002), léky, řada haptenů a toxických substancí (kadmium).

In vivo byla v celé řadě studií prokázána souvislost mezi proteinurií, aktivací transkripčních faktorů, expresí chemokinů a následnou akumulací mononukleárního zánětlivého infiltrátu. Například u potkanů s protein-overload proteinurií dochází v tubulárních epiteliích k up-regulaci syntézy CCL2 (MCP-1), spojené se zánětlivou reakcí v intersticiu a se zvýšenou aktivitou NF-κB (Eddy et al 1995). Podobné výsledky byly získány u modelů neimunitního (5/6 nefrektomie) i imunitního (Heymannova nefritis) glomerulárního poškození (Abbate et al 1998, Donadelli et al 2000).

Produkce chemokinu CCL2 (MCP-1) stimulovaná proteinurií se zdá být velmi významným faktorem progrese poškození ledvin u lidí. V prospektivní studii u pacientů s chronickým renálním onemocněním byla nalezena těsná korelace mezi albuminurií, hladinami CCL2 (MCP-1) v moči a intenzitou infiltrace intersticia makrofágy (Eardley et al 2006). Aktivace NF-κB v tubulárních buňkách signifikantně koreluje s tíží proteinurie (Mezzano et al 2001), to bylo potvrzeno i u diabetických pacientů (Mezzano et al 2004). Blokáda aktivace NF-κB pyrolidindithiokarbamátem u potkanů s adriamycinovou nefropatií vede k omezení tíže postižení tubulointersticia (Rangan et al 1999). Blokáda chemokinů či jejich receptorů vede k redukci akumulace leukocytů v intersticiu ledviny a k redukci následné fibrózy (Anders et al 2006).

Makrofágy infiltrující do intersticia na podkladě signálů z aktivovaných tubulárních buněk přispívají k rozvoji intersticiální fibrózy produkcí růstových faktorů, jako jsou TGF-β1, PDGF, ET-1 a PAI-1. TGF-β1 stimuluje transformaci intersticiálních buněk v myofibroblasty a je také hlavním stimulem procesu epiteliální-mezenchymální přeměny tubulárních epiteliálních buněk (Abbate et al 2002). In vitro stimuluje albumin produkci TGF-β1 v buněčné kultuře epitelu proximálních tubulů a působí zvýšení transkripce, syntézy a exprese TGF-β1 receptoru II (Yard 2001). Po navázání albuminu na povrchový receptor nazývaný megalin se aktivace sekrece TGF-β1 děje cestou signální dráhy mitogen-aktivované protein kinázy, a je nezávislá na endocytóze albuminu, jejíž inhibice simvastatinem nevede k blokádě albuminem indukované TGF-β1 produkce (Diwakar et al 2007). Ústřední roli megalinu při reabsorpci proteinů různé velikosti v proximálním tubulu a vliv reabsorpce proteinů při neselektivní proteinurii na rozvoj poškození tubulů potvrdila i další studie (Motoyoshi et al 2008).

Kromě albuminu, IgG, IgA a transferinu se do ultrafiltrátu primární moči při proteinurii dostávají také přímo růstové faktory jako IGF-1, HGF, TGF-β1 (Hirschberg et Wang 2005). Tyto faktory reagují s příslušnými receptory epitelu tubulů a stimulují produkci kolagenu I a IV, CCL2 (MCP-1) a CCL5 (RANTES). Řada cytokinů je produkována při zánětlivém poškození glomerulů, dostává se do lumen tubulů a přispívá k poškození intersticia.

Proteinurie je jednou z příčin apoptózy tubulárního epitelu. Přidání albuminu do buněčné kultury epitelu proximálních tubulů indukuje v závislosti na dávce

a délce expozice apoptózu aktivací dráhy Fas-FADD-kaspáza 8 (Erkan et al 2001). V ledvinách potkanů v modelu albumin overload proteinurie byla nalezena zvýšená apoptóza průkazem TUNEL metodou, a to jak v tubulointersticiálním kompartmentu, tak v glomerulech (Tejera et al 2004). V biopsiích pacientů s diagnózou primární FSGS byly detekovány apoptotické buňky v proximálních i distálních tubulech (Erkan et al 2005). U těchto pacientů byla prokázána těsná vazba mezi rozsahem apoptózy tubulárního epitelu a proteinurií, navíc se rozsah apoptózy ukázal jako silný faktor predikce dalšího vývoje onemocnění (Erkan et al 2005).

1.5 Renin-angiotenzin-aldosteronový systém (RAAS) a progrese chronického renálního poškození

RAAS je tradičně popisován jako endokrinní systém, na jehož počátku je angiotensinogen syntetizovaný v játrech, který je štěpen reninem uvolňovaným z buněk juxtaglomerulárního aparátu ledvin. Štěpením angiotensinogenu dochází ke vzniku angiotensinu I, který je štěpen angiotenzin konvertujícím enzymem (ACE) v plicích na aktivní angiotensin II. Angiotensin II se váže na receptory buněk kůry nadledvin, čímž dochází k uvolnění aldosteronu. Základní funkcí RAAS v tomto klasickém pojetí je kontrola krevního tlaku vazokonstrikčním působením angiotensinu II a aldosteronem regulovanou retencí sodíkových iontů v distálním tubulu ledvin. V současné době se tento systém jeví poněkud komplexnější (Rüster et al 2006).

Nejznámějším enzymem schopným tvorby angiotensinu II je ACE, dalšími enzymy jsou serinová proteáza chymáza, zodpovídající za zhruba 80% produkce angiotensinu II v srdci a asi 60% v cévách. Up-regulace chymázy v ledvinných tubulech byla pozorována u pacientů s diabetickou nefropatií; za chorobných stavů tedy dochází ke zvýšené aktivaci chymázy a tím ke zvýšené tvorbě angiotensinu II, která není blokovatelná ACE inhibicí (Huang et al 2003).

Na štěpení angiotensinu I se podílí i enzym ACE2, exprimovaný zejména endotelem (včetně cév ledvin), štěpící angiotensin I na nonapeptid

angiotensin 1-9, mající vazomodulační účinky. Angiotensin 1-9 může být ACE štěpen na angiotensin 1-7, který má vazodilatační účinky, podílí se na apoptóze a zástavě růstu endoteliálních buněk a buněk hladké svaloviny cév, působí antifibroticky a protizánětlivě (Ferrario et al 2005). Lokální exprese ACE2 úzce koreluje s koncentrací angiotensinu 1-7 a má antagonické účinky vůči angiotensinu II. Přesný význam těchto dat u onemocnění ledvin není dosud zcela objasněn (Danilczyk et al 2006). Angiotensin II může být dále štěpen aminopeptidázou A na angiotensin III a aminopeptidáza N štěpí angiotensin III na angiotensin IV (Velez 2009).

Kromě systémového RAAS byl popsán lokální nezávisle fungující RAAS. Epitel proximálních kanálků secernuje intaktní angiotensinogen do lumen tubulů a aktivně produkuje i angiotensin II, který uvolňuje do intersticia a do lumen tubulů. Epitelové buňky distálních tubulů konvertují angiotensinogen na angiotensin II, což vede k indukci sodíkových kanálů nezávislých na aldosteronu (Nishiyama et al 2002, Beutler et al 2003). Lokální syntéza angiotensinu II je cestou produkce kyslíkových radikálů stimulována hyperglykémií a proteinurií (Wolf et al 2003).

Působení angiotensinu II je zprostředkováno několika různými receptory. Hlavní dva receptory, AT1 a AT2, jsou různým způsobem exprimovány v ledvinách (Wolf et al 2003). Oba mají strukturu receptoru spřaženého s G-proteinem se sedmi transmembránovými doménami a mají jen asi 30% homologii v sekvenci aminokyselin. Exprese AT1 je zvyšována různými stimuly, jako jsou hypercholesterolémie a změny osmolality, je suprimována vysokou hladinou angiotensinu II (Wolf et al 2003). Receptor má schopnost tvořit homodimery i heterodimery s dalšími receptory, např. bradykininovým, což vede k významné akceleraci přenosu signálu po stimulaci angiotensinem II. AT2 receptor naopak není suprimován angiotensinem II; je up-regulován během zánětu a při tkáňovém poškození. Stimulace AT1 receptoru má za následek vazokonstrikci, uvolnění aldosteronu, stimulaci tubulárního transportu a prozánětlivé a profibrotické účinky. Role AT2 je méně jasná. Cestou uvolnění NO vede k poklesu krevního tlaku, inhibuje růst, indukuje diferenciaci a podílí se na apoptóze (Carey 2005). Vazba angiotensinu II na AT2 receptor antagonizuje antinatriuretický a vazopresorický efekt AT1. Intratubulární přeměna angiotensinu II na angiotensin III aminopeptidázou A

má zásadní význam v zesílení natriuretického účinku AT2, vzhledem k tomu, že angiotensin III je hlavní ligand AT2 receptoru (Velez 2009). Oba uvedené receptory (AT1 a AT2) se účastní aktivace NF-κB, významného prozánětlivého transkripčního faktoru.

RAAS hraje důležitou roli v řadě patofyziologických procesů spřažených s proteinurií. Angiotensin II buď přímo (eferentní vazokonstrikcí) nebo i nepřímo (cestou poškození autoregulace arteriol indukcí produkce TGF-β1) působí zvýšení kapilárního filtračního tlaku, zejména při systémové hypertenzi (Wolf 1998, Sharma et al 2005). Angiotensin II snižuje syntézu negativně nabitých proteoglykanů, inhibuje transkripci nefrinu, čímž má přímý efekt na integritu filtrační membrány. Následkem suprese nefrinu je indukce apoptózy podocytů, vzhledem k tomu, že signalizace nefrin-nefrin je důležitá pro jejich přežívání (Wolf 2003).

Kromě aktivace prozánětlivého transkripčního faktoru NF-κB stimuluje angiotensin II transkripční faktor Ets-1, podílející se na regulaci remodelace cévní stěny a regulaci chemotaxe T lymfocytů a makrofágů do cévní stěny (Wolf et al 2002, Zhan et al 2005). Angiotensin II zvyšuje expresi Toll-like receptoru 4 na mesangiálních buňkách (receptor pro LPS), což také vede ke zvýšené aktivaci NF-κB (Wolf et al 2006). Angiotensin II navíc stimuluje expresi adhezivních molekul VCAM-1, ICAM-1 a integrinů, což umožní zánětlivým buňkám adherovat k endotelu kapilár. Sekrece chemokinů zprostředkovaná NF-κB je zodpovědná za atrahování infiltrujících leukocytů do tubulointersticiální tkáně. Angiotensin II stimuluje proliferaci lymfocytů a lymfocyty samy jsou zdrojem angiotensinu II, což dále amplifikuje zánětlivý proces (Ruiz-Ortega et al 2006, Jankowski et al 2005).

Angiotensin II stimuluje proliferaci fibroblastů, mesangiálních a endotelových buněk v glomerulu, což může vést ke strukturálním změnám ledviny a její fibróze. Angiotensin II za určitých podmínek indukuje apoptózu (Wolf et al 2004).

Cílená overexprese reninu a angiotensinogenu v potkaních glomerulech vede při zachovalé normotenzi k expanzi mesangiální extracelulární matrix. V kultuře mesangiálních buněk indukuje angiotensin II syntézu mRNA prokolagenu I a fibronektinu, v epitelu proximálního tubulu stimuluje syntézu kolagenu IV (Wolf 1998). Angiotensin II stimuluje proliferaci renálních

fibroblastů a syntézu mRNA TGF-β1, fibronektinu a kolagenu I. Angiotensin II stimulací AT1 receptoru indukuje inhibitor aktivátoru plasminogenu 1 (PAI-1) a tkáňový inhibitor matrix metaloproteináz 1 (TIMP-1), které inhibují metaloproteinázy, čímž podporují rozvoj fibrózy (Abrahamsen et al 2002). Angiotensin II stimuluje produkci TGF-β1 a přispívá touto cestou k fibrotizaci intersticia i potencováním procesu epiteliální-mesenchymální přeměny; navíc inhibuje syntézu HGF, antagonisty TGF-β1 (Matsumoto et al 2003).

1.6 Intrarenální endotelinový systém, jeho role v rozvoji hypertenze a chronického renálního selhání

Endoteliny jsou skupinou vazokonstrikčních peptidů produkovaných endoteliemi cév, působící jako autokrinní/parakrinní regulátory (Abassi et al 2001). Jsou známy tři isoformy endotelinů, všechny mají délku 21 aminokyselin (Levin 1995). Endotelin 1 (ET-1), nejdůležitější z této skupiny peptidů, je nejúčinnější savčí přirozený vazokonstriktor. ET-1 působí prostřednictvím vazby na dva typy receptorů, ET_A a ET_B. ET_A váže převážně ET-1, ET_B má stejnou afinitu ke všem ET. ET_B je exprimován zejména endoteliálními buňkami, ET_A byl prokázán ve velkém množství v aortě, srdci a v ledvinách. Aktivace ET_A receptorů hladkosvalových buněk cév působí zvýšení intracelulární koncentrace kalcia a prolongovanou vazokonstrikci a proliferaci těchto buněk, aktivace ET_B receptorů endoteliálních buněk vede k uvolnění NO a prostaglandinů a tím k vazodilataci. Aktivace ET_B exprimovaných na hladkosvalových buňkách cév může navodit také vazokonstrikci (Haynes et al 1998, Levin 1995). Výsledný efekt působení endotelinu je tedy tkáňově specifický, daný rozdílnou expresí a denzitou receptorů a tkáňovou koncentrací endotelinů. ET_B má kromě převažující vazodilatační funkce ještě funkci clearance receptoru pro cirkulující ET-1. Po vazbě ET-1 na ET_B receptor dojde k internalizaci komplexu a následné intracelulární degradaci, zejména v plicní cirkulaci. Redukce nebo blokáda ET_B může redukovat clearance ET-1, čímž dojde ke zvýšení jeho koncentrace v plazmě.

ET-1 je produkován převážně endotelem, nicméně nezanedbatelné množství peptidu je syntetizováno i v ledvinách, srdci, mozku a buňkách hladké svaloviny cév (Haynes et al 1998, Levin 1995).

V ledvinách je ET-1 produkován glomerulárními epiteliálními a mesangiálními buňkami, tubulárním epitelem a epitelem sběracích kanálků. Dřeň ledviny je důležitým místem produkce ET-1, obsahuje nejvyšší koncentrace imunoreaktivního ET-1 ze všech orgánů (Kohan 1997). ET-1 ovlivňuje jednak hemodynamiku ledviny, dále výměnu elektrolytů a vody tubulárními buňkami, proliferaci a mitotickou aktivitu zejména buněk hladké svaloviny cév a mesangiálních buněk. ET_A je v ledvinách lokalizován na buňkách hladké svaloviny cév, ET_B receptory jsou dvakrát častější, vyskytují se zejména na buňkách sběracích kanálků.

ET-1 je mnohonásobně (30-50x) účinnější vazokonstriktor renálních cév, než jsou noradrenalin a angiotensin II, působí na interlobulárních arteriích, aa. arcuatae a na aferentních a eferentních arteriolách. Krátkodobá infuze ET-1 do renální arterie vede v pokusu ke snížení průtoku krve ledvinou, ke snížení glomerulární filtrace a diurézy (Kohan 1997). Systémová infúze vysokých dávek ET-1 má antidiuretický a antinatriuretický efekt, způsobený poklesem průtoku krve ledvinou a poklesem glomerulární filtrace. Nízké dávky ET-1 mají naopak diuretický a natriuretický efekt. Předpokládá se, že za tento efekt nízkých dávek ET-1 na exkreční funkce ledviny je zodpovědný tubulárním epitelem lokálně produkovaný ET-1, který působí autokrinně/parakrinně, a který zejména inhibuje Na^+/K^+-ATPázovou aktivitu a blokuje vliv vazopresinu na reabsorpci vody ve sběracích kanálcích. Efekt je zprostředkován přes ET_B receptory, jejich selektivní blokáda v experimentu vede k jeho odstranění.

Nezávisle na přímém působení na cévy, kde reguluje vaskulární tonus a krevní tlak, může tedy endotelinový systém ovlivňovat krevní tlak nepřímo, modulací renální hemodynamiky a exkrečních funkcí ledviny. Stejně jako na periferní cévy, působí ET-1 vazokonstrikčně prostřednictvím ET_A receptorů i na cévy v ledvinách, čímž snižuje průtok krve ledvinami. Efekt ET_B na regulaci cévního tonu je dán působením lokálně produkovaného ET-1 na vylučování sodíku a vody cestou stimulace receptorů (natriuretické a

diuretické působení ET-1 snižuje krevní tlak). Toto působení nicméně nebylo dodnes demonstrováno u člověka (Dhaun et al 2008).

Exprese ET-1 v cévní stěně je zvýšená u některých zvířecích modelů experimentální hypertenze: např. u Dahl sůl-senzitivních potkanů, „stroke-prone“ spontánně hypertenzních potkanů, DOCA sůl-senzitivních potkanů. Zvýšená produkce endotelinu cévami je u těchto modelů spojena s hypertrofií arteriální stěny (Li et al 1994). Podání neselektivních $ET_{A/B}$ a selektivních ET_A blokátorů vede u těchto modelů zvýšeně exprimujících ET-1 ke snížení krevního tlaku a k regresi hypertrofie cévní stěny (Schiffrin 1999). U lidí byla prokázána zvýšená koncentrace ET-1 v buňkách hladké svaloviny cév u pacientů s těžkou hypertenzí. Plazmatická hladina ET-1 nicméně u těchto pacientů nemusí být zvýšená (Schiffrin 2001). Blokátory ET_A systémově podané hypertenzním pacientům vedly ke snížení krevního tlaku asi o 10 mmHg, neselektivní blokáda byla účinná méně. Redukce tlaku byla v obou případech výrazně větší u nemocných osob než u zdravých kontrol, což podporuje domněnku, že u hypertenze je up-regulován ET-1 systém (Goddard et al 2004).

Význam role endothelinového systému v patofyziologii chronického onemocnění ledvin byl demonstrován v řadě studií (Barton 2008, Neuhofer et Pittrow 2009). S poklesem renálních funkcí stoupá hladina ET-1 v plazmě (Zoccali et al 1995), dochází k up-regulaci renálního ET-1 (Orisio et al 1993), a navíc jsou ledvinné cévy více senzitivní vůči vazokonstrikčnímu efektu ET-1, jak lze usuzovat ze zvířecího modelu (Kanai et al 1993); také exkrece ET-1 močí je u pacientů s chronickým onemocněním ledvin několikanásobně vyšší v porovnání se zdravými kontrolami. U pacientů s diabetickou nefropatií plazmatické hladiny ET-1 přímo korelují se sérovými hladinami kreatininu a stupněm albuminurie (Zanatta et al 2008), obdobně u pacientů s hypertenzí korelují plazmatické hladiny ET-1 s poklesem renálních funkcí (Cottone et al 2009). U pacientů s nefrotickým syndromem při fokálně segmentální glomeruloskleróze je koncentrace ET-1 v plazmě a v moči signifikantně vyšší než u zdravých kontrol (Chen et al 2001); imunosupresivní terapie vedoucí k redukci proteinurie způsobí i ke snížení exkrece ET-1 močí (Vlachojannis et al 1997).

Zvýšená produkce ET-1 v ledvinách je u chronického onemocnění ledvin stimulována cytokiny, chemokiny, vazoaktivními faktory, řadou růstových faktorů, hormony, reaktivními kyslíkovými radikály a dalšími látkami (Kohan 2010). U chronických nefropatií s proteinurií je produkce ET-1 v ledvinách výrazně zvýšená zejména v glomerulech, a koreluje s tíží proteinurie; sekrece ET-1 endotelem se naopak významně nemění. Hlavním zdrojem ET-1 za těchto patologických stavů jsou mesangiální buňky. Produkovaný ET-1 autokrinně/parakrinně prostřednictvím ET_A receptoru stimuluje proliferaci mesangiálních buněk a produkci extracelulární (mesangiální) matrix těmito buňkami. Tento mechanismus je považován za významný v rozvoji glomerulárního poškození u diabetu, hypertenze i chronických glomerulonefritid (Neuhofer et Pittrow 2009). Nespecifická blokáda $ET_{A/B}$ bosentanem zabrání deposici mesangiální matrix a ztluštění glomerulární bazální membrány u diabetických potkanů (Chen et al 2002). Při chronické selektivní ET_A blokádě dochází k redukci makroalbuminurie u diabetických pacientů (Wenzel et al 2009). Tento antiproteinurický efekt byl v dané studii pravděpodobně nezávislý na účinku použitého blokátoru na krevní tlak.
Stimulace ET_A receptoru mesangiálních buněk vede k syntéze CCL2 (MCP-1), tento chemokin je zodpovědný za infiltraci monocytů do poškozeného glomerulu, i tímto mechanismem se endotelinový systém podílí na rozvoji glomerulonefritid (Ishizawa et al 2004).
Rozvoj proteinurie u chronických glomerulopatií je spojen se splýváním výběžků podocytů. Expozice vysokým hladinám proteinu v primární moči vede ke strukturálním změnám podocytů se zvýšením produkce ET-1, který zřejmě autokrinně/parakrinně nadále potencuje strukturální podocytární změny (alterace cytoskeletu), což vede k poškození glomerulární filtrační bariéry (Morigi et al 2005). ET-1 se podílí na poškození filtrační membrány přímo odstraněním nefrinu, proteinu exprimovaného mezi výběžky podocytů a zodpovědného za udržování její integrity a glomerulární filtrační bariéry. Jak bylo demonstrováno na kulturách podocytů vystavených působení séra preeklamptických pacientek, prostřednictvím ET_A receptoru dojde k odštěpení extracelulární domény nefrinu proteázami a k následné redistribuci cytoskeletu podocytů (Collino et al 2008).

Intraglomerulárně produkovaný ET-1 přispívá k zvýšené filtraci proteinů do primární moče ještě dalším mechanismem – způsobuje vazokonstrikci eferentních arteriol, čímž zvyšuje intraglomerulární hydrostatický tlak a tím i glomerulární permeabilitu.

Reabsorpce filtrovaných proteinů epitelem proximálních kanálků stimuluje sekreci ET-1 (Zoja et al 1995). ET-1 je secernován abluminálně, vede k proliferaci intersticiálních fibroblastů a stimuluje produkci extracelulární matrix, čímž přímo přispívá k rozvoji fibrotizace intersticia. Navíc má ET-1 chemotaktické účinky na monocyty (Achmad 1992). Zvýšená produkce ET-1 v proximálních tubulech byla demonstrována v renálních biopsiích pacientů s IgA nefropatií, exprese prepro-ET-1 mRNA stanovená RT-PCR korelovala s tíží proteinurie (Lehrke et al 2001).

1.7 Role renálních fibroblastů a myofibroblastů u chronických onemocnění ledvin

Klíčovou efektorovou buňkou zodpovědnou za fibrogenezi v ledvinném parenchymu jsou fibroblasty a myofibroblasty. Fibróza ledvinného intersticia je hlavním rysem progresivního renálního onemocnění. Rozsah tubulointersticiálního poškození koreluje s postupným zhoršováním ledvinných funkcí lépe, než glomerulární změny. Tubulointersticiální fibróza je charakterizována akumulací komponent extracelulární matrix, jako jsou kolagen I a III, fibronektin a proteoglykany (Strutz et al 2006b).

V současném pojetí lze intersticiální fibroblast nejlépe negativně definovat jako „necévní“, neepiteliální, nezánětlivou buňku intersticia (Kaluri et al 2006). Fibroblasty syntetizují kolagen I, III, V a fibronektin, jsou hlavním zdrojem proteáz degradujících extracelulární matrix, jako jsou matrix metaloproteinázy. Fibroblasty jsou klíčové v udržování homeostázy extracelulární matrix. Jde o mezenchymální buňky vřetenitého tvaru, vzhledem k nedostatku specifických markerů je však problém odlišit tyto buňky od ostatních buněk mezenchymálního původu, jako jsou pericyty, mezenchymální kmenové buňky a buňky hladké svaloviny cév (Strutz et al 2006a). Jako myofibroblasty jsou označovány aktin pozitivní fibroblastické

elementy se schopností kontrakce; původně byly myofibroblasty popsány jako buňky zodpovědné za svrašťování ran (Tomasek et al 2002). V ledvinách jsou za stavů fibrózy myofibroblasty považovány za aktivovaný fenotyp fibroblastů zodpovědný za zvýšenou produkci extracelulární matrix. V elektronové mikroskopii jsou charakterizovány svazky mikrofilament, nápadně vyvinutým granulárním endoplazmatickým retikulem a hemidesmosomy. Imunohistochemicky jsou identifikovány pomocí exprese hladkosvalového aktinu α-SMA (ten je však přítomen i v buňkách hladké svaloviny cév a v pericytech).

Fibroblasty jsou aktivovány řadou stimulů spojených s poškozením tkáně. Infiltrující zánětlivé buňky aktivují fibroblasty produkcí TGF-β1, PDGF, FGF-2 (Alvarez et al 1992). Mezi další mechanismy aktivace patří přímý mezibuněčný kontakt, interakce integrin-extracelulární matrix, hypoxie a hyperglykémie (Qi et al 2006). Otázkou zůstává, jakým mechanismem jsou aktivované fibroblasty odstraňovány během rezoluce fibrotického procesu. Původ fibroblastů je dosud zčásti nejasný. Rezidentní „dospělé“ fibroblasty se nejspíše, jak bylo ukázáno v jiných orgánech, vyvíjí lokálně dělením a diferenciací mezenchymálních kmenových buněk. Předpokládá se, že část fibroblastů vzniká z kmenových buněk kostní dřeně, z periadventiciálních buněk a z epiteliálních buněk tubulů. Studie používající geneticky značené fibroblasty prokázaly, že zhruba 10% fibroblastů normální myší ledviny má původ v kostní dřeni. Podíl těchto buněk zůstal v modelu chronické fibrózy intersticia nezměněn, nicméně bylo prokázáno, že více než 30% fibroblastů v tomto modelu má původ v epiteliální - mezenchymální přeměně (transdiferenciaci) (Iwano et al 2006).

Termín epiteliální - mezenchymální přeměna (transdiferenciace, transformace, EMT) je používán k popisu přeměny terminálně diferencované epitelové buňky v buňku s mezenchymálním fenotypem. U renální fibrogeneze byla poprvé popsána Strutzem na myším modelu anti-TBM nefropatie za pomoci protilátky proti fibroblastickému specifickému proteinu (FSP-1) (Strutz et al 1995). V průběhu postižení se exprese tohoto proteinu objevuje nejen v intersticiu, ale i v tubulárních epitelových buňkách, které postupně získávají další mezenchymální markery (α-SMA, vimentin) a ztrácejí epiteliální adhezní molekuly, jako je E-kadherin a ZO-1. Navíc tyto

buňky migrují. Byla publikována práce používající expresi FSP-1 v renálních biopsiích jako prognostický marker u pacientů s IgA nefropatií (Nishitani et al 2005). Existence EMT v ledvinách není překvapující. Epitel tubulů (vyjma výstelky sběracích kanálků) má původ v metanefrickém mezenchymu, na proces EMT zde lze pohlížet jako na opakování vývojových programů ledvinných buněk.

EMT je regulovaný komplexní proces zahrnující čtyři klíčové kroky: ztrátu adheze epitelových buněk, de novo expresi α-SMA a reorganizaci aktinu, porušení TBM a migraci buněk do intersticia. Nejdůležitější faktory indukující EMT v ledvině jsou TGF-β1, IL-1, FGF-2, angiotensin II, MMP-2, tkáňový plazminogen aktivátor a hypoxie. Nejvíce prostudované faktory inhibující EMT jsou HGF a BMP7. Tři hlavní signálové dráhy podílející se v ledvině na procesu EMT jsou TGF-β/Smad, integrin/ILK a Wht/β-katenin (Liu 2010). Tyto tři dráhy jsou navzájem propojeny a konvergují v aktivaci β-kateninu, který vede k aktivaci transkripčních programů EMT.

Zeisbergová et al. na základě experimentálních studií u myší na modelu jednostranné obstrukce ureteru, modelu streptozotocinem indukovaného diabetické nefropatie a modelu Alportova syndromu odhadli, že 30-50% intersticiálních fibroblastů během rozvoje fibrózy intersticia ledviny vzniká procesem endoteliální - mezenchymální přeměny. Tyto fibroblasty exprimovaly jak markery endoteliální, jako je CD31, tak specifické markery fibroblastů či myofibroblastů, jako je FSP-1 a α-SMA. Přítomnost fibroblastů vznikajících procesem endoteliální - mezenchymální přeměny potvrdila na modelu jednostranné obstrukce ureteru použitím transgenních myší s fluorescenčně značenými endoteliálními buňkami (Zeisberg et al 2008).

1.8 Chemokiny a nenádorová onemocnění ledvin

1.8.1 Chemokiny

Chemokiny jsou velkou rodinou malých (8-15 kDa) **chemo**taktických cyto**kinů**, které mají podobnou sekundární strukturu a jsou syntetizovány většinou buněk v lidském těle. Účastní se zejména regulace migrace leukocytů a dále modulace funkcí řady imunitních i neimunitních buněk. Za

fyziologických stavů se chemokiny podílejí dále např. na mobilizaci CD34+ kmenových buněk (Flomenberg et al 2005), vaskularizaci orgánů během embryonálního vývoje (Tachibana et al 1998), neuronální komunikaci (Meucci et al 1998) a na vývoji T a B buněk (Forster et al 1994). Navíc se chemokiny účastní řady patologických procesů; známá je funkce chemokinových receptorů CCR5 a CXCR4 jako koreceptorů CD4, účastnících se vstupu viru HIV-1 do buňky (Feng et al 1996, Alkhatib et al 1996, Dragic et al 1996), chemokiny rovněž hrají významnou roli v procesu metastazování zhoubných nádorů (Ben-Baruch 2008).

Chemokiny imobilizované na luminálním endoteliálním povrchu spolu s adhezivními molekulami endotelu a leukocytů (selektiny, integriny a ICAM) jsou odpovědné za specificitu adheze leukocytů k endotelu a specificitu následného průniku leukocytů do poškozené tkáně. Chemokiny prezentované na endoteliálním povrchu interagují s příslušnými receptory rolujících leukocytů; to vede k rychlé aktivaci leukocytárních integrinů, čímž dojde k zastavení a pevné adhezi leukocytů na daném místě. Kromě indukce této adheze chemokiny napomáhají dosud neobjasněným mechanizmem transendoteliální migraci leukocytů.

V současné době je u člověka známo 42 chemokinů a 18 chemokinových receptorů (Colobran et al 2007a). Chemokiny vykazují navzájem 20-95% homologii v sekvenci aminokyselin. Všechny chemokiny jsou si navzájem podobné svou trojrozměrnou strukturou, mají relativně různou N-terminální oblast ukotvenou cysteinovými můstky k jádru molekuly tvořenému třemi antiparalelními úseky β-struktury a dále C-terminální α-helix.

Současná nomenklatura (Murphy et al 2000) dělí chemokiny do čtyř skupin CL, CCL, CXCL, CX3CL na základě strukturálního motivu čtyř cysteinů v blízkosti N-terminálního konce. CCL chemokiny mají na N-terminálním konci těsně vedle sebe dva cysteiny. U CXCL chemokinů, dva cysteiny nejblíže N-terminálnímu konci jsou odděleny jednou (variabilní) aminokyselinou. CX3CL chemokin (fraktalkin) je integrální membránový protein, který má na N-konci typickou chemokinovou strukturu se třemi aminokyselinami mezi prvními dvěma cysteiny. Tato doména je napojena na dlouhý řetězec aminokyselin substituovaných hlenovitými polysacharidy, navazuje transmembránový úsek a krátká intracytoplazmatická doména

(Bazan et al 1997). CL chemokiny nemají ve své molekule první a třetí cystein typické chemokinové struktury.

CXCL chemokiny jsou dále rozděleny na ELR+ a ELR- chemokiny na základě přítomnosti či nepřítomnosti tripeptidového motivu glutamová kyselina-leucin-arginin na N-terminálním konci za prvním cysteinem. ELR-CXC chemokiny - např. CXCL8 (IL-8) - působí jako chemoatraktanty neutrofilů a promotory angiogeneze (Kobayashi 2006). Non-ELR-CXC chemokiny mají různou funkci.

Další možné hledisko klasifikace chemokinů je dělení podle místa působení a jejich funkce a exprese na inducibilní (zánětlivé) – např. CXCL10, CCL2 a CCL5, které se účastní procesu migrace leukocytů do místa poškození tkáně a konstitutivní (homeostatické) např. CCL19, CCL20, produkované za normálních podmínek a účastnící se homingu leukocytů do lymfatické tkáně a organizace funkčního mikroprostředí v lymfatické tkáni za normálního stavu i během jejího vývoje (Murphy et al 2000). V poslední době se ukazuje, že řada „homeostatických“ chemokinů je produkována v zánětlivých lézích, kde se významně podílí na patogenezi zánětlivého procesu, navíc v řadě orgánů a tkáňových kompartmentů jsou i za normálních okolností produkovány „zánětlivé“ chemokiny, byť zde jejich funkce není dosud zcela objasněna.

Geny pro chemokiny se nacházejí ve dvou seskupeních (clusterech) na chromosomu 4q12-21 (CXC) a 17q11.2 (CC). V těchto seskupeních jsou uspořádány zejména základní zánětlivé chemokiny. CXC chemokiny uspořádané na 4. chromozomu atrahují převážně neutrofilní granulocyty, zatímco CC chemokiny na 17. chromozomu typicky atrahují lymfocyty a makrofágy (Colobran et al 2007a).

Uvnitř jedné skupiny (C, CC, CXC, CX3C) existuje několik chemokinů, které se váží jen na jeden receptor, který je pro ně specifický (jde zejména o homeostatické chemokiny), u většiny vazebných interakcí ligand – receptor (chemokin – chemokinový receptor) se vazebné specificity mezi jednotlivými členy skupiny různě překrývají, v rámci jedné chemokinové skupiny řada chemokinových receptorů váže více než jeden ligand, a některé chemokiny se mohou vázat na více než jeden chemokinový receptor.

Poměrně výrazná komplexnost až „promiskuita“ interakcí chemokin – receptor a jistá zdánlivá nadbytečnost jednotlivých chemokinů či buněčných

Tabulka 1. Chemokiny a jejich receptory

CC Chemokiny				
Název	**Chromozom**	**Ligand (člověk)**	**Ligand (myš)**	**Receptor**
CCL1	17q11.2	I-309	TCA-3/P500	CCR8
CCL2	17q11.2	MCP-1/MCAF	JE?	CCR2
CCL3	17q12	MIP-1α/LD78	MIP-1α	CCR1, CCR5
CCL3L1	17q12	LD78-ß	Neznámý	CCR1, CCR5
CCL4	17q12	MIP-1ß	MIP-1ß	CCR5
CCL5	17q12	RANTES	RANTES	CCR1,CCR3,CCR5
(CCL6)		Neznámý	C10/MRP-1	Neznámý
CCL7	17q11.2	MCP-3	MARC?	CCR1,CCR2,CCR3
CCL8	17q11.2	MCP-2	MCP-2?	CCR3, CCR5
(CCL9/10)		Neznámý	MRP-2/MIP-1χ	CCR1
CCL11	17q11.2	Eotaxin	Eotaxin	CCR3
(CCL12)		Neznámý	MCP-5	CCR2
CCL13	17q11.2	MCP-4	Neznámý	CCR2, CCR3
CCL14	17q12	HCC-1	Neznámý	CCR1, CCR5
CCL15	17q12	HCC-2/Lkn-1	Neznámý	CCR1, CCR3
CCL16	17q12	HCC-4/LEC/LCC-1	Neznámý	CCR1, CCR2
CCL17	16q13	TARC	TARC/ABCD-2	CCR4
CCL18	17q12	DC-CK1/PARC	Neznámý	Neznámý
CCL19	9p13.3	MIP-3ß /ELC/exodus-3	MIP-3ß /ELC/exodus3	CCR7/CD197
CCL20	2q36.3	MIP-3/ LARC/exodus-1	MIP-3 /LARC/exodus1	CCR6
CCL21	9p13.3	6Ckine /SLC/exodus-2	6Ckine /SLC/exodus2	CCR7/CD197
CCL22	16q13	MDC/STCP-1	ABCD-1	CCR4
CCL23	17q12	MPIF-1 /CKß8/CKß8-1	Neznámý	CCR1
CCL24	7q11.23	Eotaxin-2 /MPIF-2	MPIF-2	CCR3
CCL25	19p13.3	TECK	TECK	CCR9

CCL26	7q11.23	Eotaxin-3	Neznámý	CCR3
CCL27	9p13.3	CTACK/ILC	ALP/CTACK/ILC	CCR10
CCL28	5p12	MEC	Neznámý	CCR3/CCR10

CXC Chemokiny				
Název	**Chromozom**	**Ligand (člověk)**	**Ligand (myš)**	**Receptor**
CXCL1	4q21.1	GRO-α/MGSA-α	GRO/MIP-2/KC?	CXCR2 > CXCR1
CXCL2	4q21.1	GRO-ß/MGSA-ß	GRO/MIP-2/KC?	CXCR2
CXCL3	4q21.1	GRO-χ/MGSA-χ	GRO/MIP-2/KC?	CXCR2
CXCL4	4q21.1	PF4	PF4	Neznámý
CXCL5	4q21.1	ENA-78	GCP-2/LIX?	CXCR2
CXCL6	4q21.1	GCP-2	GCP-2/LIX?	CXCR1, CXCR2
CXCL7	4q21.1	NAP-2	Neznámý	CXCR2
CXCL8	4q21.1	IL-8	Neznámý	CXCR1, CXCR2
CXCL9	4q21.1	Mig	Mig	CXCR3/CD138
CXCL10	4q21.1	IP-10	IP-10/CRG-2	CXCR3/CD138
CXCL11	4q21.1	I-TAC	I-TAC	CXCR3/CD138
CXCL12	10q11.21	SDF-1α/ß	SDF-1/PBSF	CXCR4/CD184
CXCL13	4q21.1	BCA-1	BLC	CXCR5
CXCL14	5q31.1	BRAK/bolekin	BRAK	Neznámý
(CXCL15)		Neznámý	Lungkin/WECHE	Neznámý
CXCL16	17p13			CXCR6
C Chemokiny				
XCL1	1q24.2	Lymphotactin	Lymphotactin	XCR1
XCL2	1q24.2	SCM-1ß	Neznámý	XCR1
CX3C Chemokiny				
CX3CL1	16q13	Fractalkin	Neurotactin	CX3CR1

receptorů (žádný z chemokinů není pro danou populaci leukocytů ve své funkci zcela jedinečný a daná leukocytární subpopulace má na svém povrchu receptory pro různé chemokiny) je nezbytná pro zachování životně důležitých

funkcí, na jejichž řízení se chemokiny podílejí. Chemokinová síť je poměrně značně odolná vůči poškození svých jednotlivých komponent. Pokud je jeden receptor nebo ligand defektní, obvykle jiné chemokiny zachovávají funkční základní biologické procesy. Výjimkou je vazebný pár CXCL12/CXCR4 – knockout jak chemokinu, tak i receptoru je u myší letální již v embryonálním věku (Colobran et al 2007a).

Jeden chemokin může působit na různé buněčné populace a naopak, daná populace buněk je schopna odpovídat na stimulaci různými chemokiny. K dosažení požadované funkce, ať již v homeostatických procesech nebo během zánětu, je třeba velice přesné a jemné regulace. Během daného procesu dochází vždy k sekreci jen určitého specifického setu chemokinů, jejich produkce je časově a místně kontrolovaná, stejně jako je kontrolovaná exprese chemokinových receptorů u dané skupiny leukocytů (Colobran et al 2007a). Navíc existuje řada dalších mechanismů, které značně omezují a zjemňují nadbytečnost a jistou nespecificitu interakcí v daném systému. Každý receptor váže jen specifickou malou skupinu chemokinů, navíc, dva ligandy jednoho receptoru mají často různou vazebnou afinitu. Dva různé chemokiny se výrazně liší v tom, jakou funkci řídí (např. adheze, migrace, desenzitizace receptoru), s jakou efektivitou a jakým způsobem (agonista/antagonista) (Loetscher et al 2001). Množství chemokinů produkovaných určitou buněčnou populací či různými populacemi na podkladě stejného stimulu se může značně lišit (Schutyser et al 2003). Navíc daný stimulus indukuje produkci specifické sady chemokinů, tyto sady se pro jednotlivé populace buněk liší. Chemokiny produkované simultánně v jednom místě (mikroprostředí) mohou navzájem interagovat a modifikovat navzájem výsledný efekt působení, ať už ve smyslu deaktivace dané funkce nebo naopak vzájemného potencování efektu (Paoletti et al 2005). Různé subpopulace leukocytů mají na svém povrchu unikátní sadu chemokinových receptorů, často definující i jejich funkci.

Biologická aktivita daného chemokinu in vivo je dále ovlivňována citlivostí ke specifické degradaci enzymy (dipeptidylpeptidáza, metaloproteinázy), schopností vázat glykosaminoglykany a interakcí s nesignálními receptory, tzv. decoy receptory či interceptory (**inter**nalizing re**ceptors**), mezi něž patří zejména DARC (Duffy antigen receptor for chemokines), D6, CCX-CKR

(Colobran et al 2007a). Jde o povrchové receptory, které váží s vysokou afinitou a specificitou chemokiny, ale neindukují další přenos signálu v buňce. DARC receptor váže řadu CC a CXC chemokinů a kromě povrchu erytrocytů je exprimován endotelem postkapilárních venul a drobných vén. Zde se účastní přenosu (transcytózy) ve tkáni produkovaných chemokinů od abluminálního k luminálnímu povrchu buňky. Tím může dojít ke kontaktu chemokinů s cirkulujícími leukocyty a k indukci chemokiny zprostředkované migrace leukocytů do tkáně (Pruenster et al 2009). D6 váže CC chemokiny a je exprimován endotelem lymfatik. Po navázání na D6 je chemokin internalizován a cílen do lyzosomálního systému, kde dochází k jeho degradaci. Exprese D6 reguluje přísun leukocytů do místa zánětu a chrání tkáň před excesivním poškozením zánětlivými buňkami (Graham 2009).

Chemokiny se váží na solubilní glykosaminoglykany a glykosaminoglykany vázané na povrchu buněk a v extracelulární matrix (heparin, heparansulfát, chondroitinsulfát a dermatansulfát). Touto vazbou dochází k jejich imobilizaci, usnadní se vytváření haptotaktického chemokinového gradientu důležitého pro směrování migrace leukocytů a zvýší se koncentrace chemokinů v místě produkce. Tak dochází k omezení rozsahu působení chemokinů pouze na ložisko poškození tkáně. Chemokiny jsou transportovány endotelem z extracelulární matrix mechanismem transcytózy na luminální povrch, zde se naváží na glykosaminoglykany a jsou prezentovány chemokinovým receptorům na povrchu leukocytů. Glykosaminoglykany indukují oligomerizaci chemokinů, čímž se zvyšuje lokální koncentrace chemokinů a podporuje interakce s chemokinovými receptory (Hoogewerf et al 1997). Po interakci chemokinu s receptorem dojde k aktivaci integrinů, k pevné adhezi leukocytu k endotelu a následně k extravazaci. Vazba chemokinů k různým typům glykosaminoglykanů je selektivní. Důležitost vazby chemokinů s glykosaminoglykany ukázalo několik prací. Mutované chemokinové molekuly CCL2, CCL4, a CCL5 s porušenou vazbou k glykosaminoglykanům zachovávají v pokusu in vitro svoji chemotaktickou aktivitu, v pokusu na myši ale po intraperitoneálním podání nedokáží indukovat migraci leukocytů. Navíc tyto mutantní monomery chemokinů, ačkoliv jsou plně aktivní in vitro, neúčinkují in vivo, což ukazuje, že oligomerizace chemokinů zprostředkovaná vazbou na glykosaminoglykany je pro aktivaci některých

chemokinových receptorů zcela nezbytná (Proudfoot et al 2003). Varianta molekuly chemokinu CCL5 (RANTES), označovaná jako [44AANA47]-RANTES, neschopná vázat glykosaminoglykany, je in vivo nefunkční kvůli neschopnosti vytvářet oligomery nezbytné pro biologickou aktivitu chemokinu. Navíc tato molekula vytváří nefunkční heterodimery s endogenní molekulou CCL5 (RANTES). Při zachované plné schopnosti vázat chemokinový receptor je poškození vazebného místa pro glykosaminoglykan důvodem ztráty původní biologické funkce chemokinu (Johnson et al 2004).

Za určitých okolností mohou chemokiny pravděpodobně vytvářet specifické heterodimery se speciální funkcí. Komplex chemokinů CCL5-CXCL4 se účastní infiltrace monocytů do ateromových plátů, pokud je v experimentu cíleným farmakologickým zásahem zabráněno tvorbě tohoto heterodimeru, dochází ke snížení počtu infiltrujících monocytů a k redukci rozvoje aterosklerózy (Koenen et al 2009).

Produkce chemokinů buňkami je regulována na úrovni transkripce, posttranskripčních úprav, translace a na posttranslační úrovni. Sekrece homeostatických chemokinů je konstituční. Sekrece řady zánětlivých chemokinů je indukována cytokiny IL-1β, INF-γ a TNF-α. Na úrovni transkripce jsou zánětlivé chemokiny regulovány např. transkripčním nukleárním faktorem NFκB. Aktivace transkripčních faktorů vyžaduje komplexní kaskádu zahrnující fosforylaci kináz a fosfatáz a degradaci inhibitorů transkripce. Pro různé stimuly mohou být tyto signální dráhy různé a v jejich průběhu může docházet k jejich vzájemnému ovlivňování, to dovoluje integraci různých signálů a jemné vyladění komplexní biologické odpovědi, která je specifická pro danou tkáň a signál (Segerer et al. 2000b).

1.8.2 Chemokinové receptory

Chemokinové receptory jako jediné z cytokinových receptorů mají strukturu receptorů spřažených s G-proteinem tvořenou sedmi transmembránovými α-helixy (Rossi et al 2000). Receptory se podobně jako jejich ligandy dělí do čtyř tříd (CR, CCR, CXCR, CX3CR). Geny pro jednotlivé receptory jsou, podobně jako geny kódující jejich ligandy, sdruženy do seskupení (clusterů) na chromosomu 2 (CXCR) a chromosomu 3 (CCR). Lidské chemokiny působí na chemokinových receptorech převážně jako agonisté. V poslední

době se ukazuje, že některé z chemokinů mohou působit i jako antagonisté chemokinových receptorů. Například chemokin CCL7 (MCP-3) působí jako antagonista CCR5 receptoru (Blanpain et al 1999), chemokiny CXCL9, CXCL10 a CXCL11, účastnící se imunitní odpovědi řízené Th1 buňkami, jsou antagonisty CCR3 receptoru, který je považován za receptor Th2 imunitní odpovědi (Loetscher et al 2001).

Po navázání ligandu dochází u chemokinových receptorů ke změně konformace, umožňující navázání heterotrimeru Gi proteinu, vedoucí k spuštění intracelulární signální kaskády vedoucí ke změnám v chování buňky. Na receptor se naváže Gα-podjednotka, dojde k disociaci heterotrimeru na βγ-podjednotku a GTP-vážící α-podjednotku. Zpětnou vazbou dochází navíc ke kovalentním modifikacím receptoru. Tyto modifikace mění jeho strukturu a funkci – po fosforylaci dojde například k navázání β-arestinů. To má zásadní význam v regulaci chemokinových receptorů – vazba arestinu inhibuje vazbu G-proteinu, směruje receptor k internalizaci do klathrinem obalených jamek, a řídí následný intracelulární pohyb receptoru. Jinou modifikací je ubikvitinizace receptoru po navázání ligandu, směřující receptor do lyzosomálního kompartmentu k degradaci.

Signalizace zprostředkovaná G proteinem zahrnuje aktivaci fosfolipázy C, vedoucí k formaci inositoltrifosfátu a diacylglycerolu, zodpovědných za influx vápníkových iontů a aktivaci proteinkinázy C. Dále dochází k indukci aktivace fosfolipázy A2 a uvolnění arachidonové kyseliny, účastnící se chemotaktické odpovědi, a ke spuštění fosfolipázy D, účastnící se transformace buňky a vezikulárního transportu. Chemotaxe řízená chemokiny je podmíněna vysoce komplexními buněčnými procesy, které zahrnují změnu tvaru buňky, polymerizaci/depolymerizaci aktinu a buněčnou adhezi. Modulaci těchto procesů zajišťují guaninové nukleotidy a zahrnují regulaci GTP-vážícími proteiny, jako je Rho, Rac, a Cdc42. Tyto malé GTP-vážící proteiny se podílejí na regulaci buněčné adheze a tvorby filopodií a lamelipodií.

Na základě novějších studií se v současné době předpokládá, že chemokinové receptory jsou na povrchu buněk exprimovány jako dimery či oligomery, ve shlucích přirovnávaných ke svazkům doutníků. Dynamika těchto receptorových komplexů, vliv navázání ligandu na jejich tvorbu ani jejich biologický význam nejsou dosud blíže prozkoumány (Thelen et al 2010).

Některé chemokinové receptory (např. CXCR1, CXCR2, CXCR4) mohou tvořit se stejnou afinitou homodimery i heterodimery. Společná internalizace (mechanismus desenzitizace chemokinových receptorů) těchto různých receptorů po navázání ligandu nebyla pozorována, což svědčí proti přítomnosti stabilních heterodimerů.

V recentně publikované studii popisují autoři tvorbu heterooligomerů chemokinových receptorů CCR2-CCR5-CXCR4 v experimentálních buněčných modelech i na primárních buňkách. V experimentálním modelu in vivo ukázali, že specifický ligand jednoho z receptorů, který indukuje konformační změny receptoru, může ovlivnit vazebnou afinitu a funkční odpověď sousedních receptorů. Protilátky blokující funkci receptoru, které neindukovaly konformační změny, neovlivňovaly okolní receptory (Sohy et al 2009).

1.8.3 Chemokiny a ledviny

Vývoj a průběh renálního onemocnění úzce koreluje svou intenzitou, ale i časově a místně s expresí chemokinů a chemokinových receptorů.

Všechny typy renálních buněk dokáží za určitých podmínek produkovat prozánětlivé chemokiny (Segerer et al 2000b). Syntézu a sekreci chemokinů dokáže stimulovat řada prozánětlivých látek, oxidační stres, vazoaktivní látky a růstové faktory. Experimentálně byla prokázána produkce chemokinů buňkami proximálních tubulů indukovaná lipopolysacharidem (Tsuboi et al 2002), vysokými hladinami albuminu (Wang et al 1999), kalcium oxalátovými, kalciumfosfátovými a urátovými krystaly (Umekawa et al 2003). Hyaluronan, produkt degradace glykosaminoglykanů extracelulární matrix, jehož akumulace je prokázána v intersticiu ledvin během onemocnění, dokáže indukovat sekreci CCL2 (MCP-1) (Beck-Schimmer et al. 1998). Interakce CD40 s ligandem CD154, spolu s IL-4 a IL-3, vedou v buněčné kultuře epitelií proximálních tubulů k produkci CCL2 (MCP-1), CCL5 (RANTES) a CXCL8 (IL-8) (Deckers et al 1998). Mezangiální buňky v buněčné kultuře vytvářejí chemokiny po stimulaci IgA a IgG imunokomplexy, popřípadě aktivovanými komponentami komplementu (Hora et al 1992). Glomerulární endotel může produkovat chemokin CCL5 (RANTES) na podkladě stimulace AT2 receptoru

angiotenzinem II (Wolf et al 1997), infúze angiotenzinu II potkanům vede k blokovatelnému přílivu makrofágů do glomerulu.

Chemokiny produkované renálními buňkami působí prozánětlivě na několika úrovních. Jednak atrahují cirkulující leukocyty do místa poškození, jednak zřejmě působí místně na samotné renální buňky exprimující chemokinové receptory. Experimentálně bylo například na modelu proliferativní glomerulonefritidy prokázáno, že chemokin CXCL10 (IP-10) indukuje proliferaci mezangiálních buněk (Romagnani et al 1999). V buněčné kultuře podání CCL2 (MCP-1) vedlo u fibroblastů ke zvýšené expresi mRNA pro kolagen. Význam těchto poznatků in vivo dosud není jasný.

Kromě samotných buněk ledvin jsou hlavním zdrojem chemokinů v místě poškození infiltrující leukocyty, což má za následek další cílenou infiltraci tkáně zánětlivými buňkami.

Produkce chemokinů v zánětlivém ložisku je časově omezená. Hladina rychle indukovaných chemokinů, jako je např. CCL2 (MCP-1), se vrací k normě během jednoho dne. Naproti tomu exprese CCL5 (RANTES) nastupuje pomaleji, ale hladiny zůstávají zvýšené několik dní (Schwarz et al 1997).

1.8.3.1 Experimentální studie na zvířecích modelech

Expresí chemokinů a jejich receptorů v ledvinách u zvířecích modelů renálních onemocnění se zabývala řada experimentálních prací. Z výsledků těchto prací je zřejmé, že na podkladě poškození jak glomerulů, tak tubulointersticia ledviny dochází k nadprodukci chemokinů, což bylo prokázáno na úrovni zvýšené exprese mRNA i proteinu (Anders et al 2001, Perez de Lema et al 2001, Vielhauer et al 2001). Naproti tomu ve zdravé tkáni ledviny nebyla produkce chemokinů prokázána. Výsledky in situ-hybridizace potvrdily data získaná in vitro průkazem exprese chemokinů nejen v infiltrujících zánětlivých buňkách, ale i v buňkách samotného parenchymu poškozené ledviny.

U klasického modelu **nefrotoxické sérové nefritidy** je poškození glomerulů způsobeno injekcí protilátek proti GBM. U myší s akcelerovanou nefrotoxickou sérovou nefritidou je zvýšená exprese mRNA pro CCL5, CCL2, CXCL10 (Lloyd et al 1997). Dochází taktéž k indukci CCR1, CCR2 a CCR5.

Působení jednotlivých chemokinů bylo u tohoto modelu studováno pomocí specifických antagonistů a neutralizujících protilátek. Použití Met-RANTES, antagonisty CCL5 (RANTES), redukovalo proteinurii a infiltraci ledviny T lymfocyty a monocyty. Tvorba srpků naproti tomu nebyla ovlivněna. Podání protilátky neutralizující CCL2 (MCP-1) vedlo k výrazné redukci tvorby srpků, snížení produkce kolagenu, snížení počtu infiltrujících makrofágů a redukci proteinurie. Zdá se tedy, že na stimulaci tvorby srpků se podílí CCL2, ale nikoli CCL5 (Lloyd et al 1997a). V průběhu onemocnění lze vysledovat korelaci mezi expresí neutrofily-atrahujících chemokinů a následnou infiltrací tkáně neutrofily a pozdější nástup infiltrace makrofágy poté, co dojde k expresi makrofágy- atrahujících chemokinů (Tang et al 1995). V průběhu onemocnění se také mění hlavní zdroj secernovaných chemokinů. Po třech hodinách lze prokázat expresi mRNA pro CCL2 v glomerulárních buňkách. Po 24 hodinách jsou převažujícím zdrojem CCL2 infiltrující makrofágy (Tang et al 1996).

Modelem **systémového lupus erythematodes** a zároveň i lupusové nefritidy je myš NZB/W, novozélandská černá myš křížená s bílou. U F1 generace se objevují cirkulující autoprotilátky proti nukleovým kyselinám, imunokomplexy v ledvinách a proteinurie. Během prvních šesti měsíců onemocnění je prokazována zvýšená exprese mRNA pro CCL2 (MCP-1). Pomocí in situ hybridizace byla mRNA pro CCL2 lokalizována v glomerulárních buňkách, infiltrujících mononukleárech a v tubulárních epiteliích. V pozdějších stádiích onemocnění, exprese CCL2 v tubulech korespondovala s intenzitou intersticiálního leukocytárního infiltrátu (Zoja et al 1997). Podávání bindaritu vede u myší k redukci exprese CCL2, k delšímu přežívání, snížení proteinurie, zmenšení rozsahu poškození glomerulů a tubulointersticiálních změn (Zoja et al 1998). Dalším modelem lupusové nefritidy je myší kmen MRL-lpr. Tento kmen má mutovaný Fas gen (lpr-gen), což vede k tvorbě funkčně defektní povrchové Fas molekuly, s poruchou Fas-zprostředkované apoptózy. Vzhledem k důležitosti Fas pro odstraňování aktivovaných lymfocytů se tento defekt projeví zejména zvýšenou kontinuální proliferací lymfocytů, produkcí autoprotilátek proti řadě antigenů, včetně jaderných proteinů a DNA, vede k imunokomplexové vaskulitidě a rozvoji

glomerulonefritidy. Asi 50% myší umírá okolo 24 týdne věku. Průběh exprese chemokinů a chemokinových receptorů v ledvinné tkáni těchto myší během počátečních stadií lupusové nefritidy popisuje Perez de Lema se spolupracovníky (Perez de Lema et al 2001). Zvýšené hladiny chemokinů CCL2 (MCP-1), CCL5 (RANTES), CCL4 (MIP-1β) a CXCL10 (IP-10) se objevily ve tkáni ledvin osmý týden, kdy již byly zvýšeny hladiny imunokomplexů v krvi, a bylo patrné ukládání imunokomplexů v glomerulech. V tuto dobu myši neměly proteinurii a ve světelné mikroskopii nebyly známky poškození ledvin. Imunohistochemicky a in situ hybridizací byla exprese chemokinů lokalizována do glomerulů a intersticia. Mononukleární infiltráty se objevily v histologickém obrazu 10-12 týden. Dvanáctý týden byla detekována i zvýšená exprese chemokinových receptorů CCR1, CCR2, CCR5, u myši se objevila proteinurie a histologické známky renálního poškození. Od 12-14 týdne byla detekovatelná zvýšená produkce proinflamatorních cytokinů (IL-1β, TNFα, INFγ). Uvedené výsledky svědčí pro to, že chemokiny jsou v ledvinách produkovány před rozvojem proteinurie, morfologicky patrného poškození parenchymu a před nástupem leukocytárního infiltrátu. K produkci chemokinů dochází v místech, do kterých později míří mononukleární infiltrace. Chemokinové receptory jsou v ledvinné tkáni prokazatelné až v době, kdy dochází k infiltraci tkáně leukocyty a paralelně s infiltrujícími leukocyty je detekována i zvýšená produkce prozánětlivých cytokinů; v tuto dobu dochází také k rozvoji proteinurie.

Modelem tubulointersticiálního poškození a následné progresivní fibrotizace ledvinného parenchymu provázené intersticiálním zánětlivým infiltrátem je **jednostranná obstrukce ureteru**. Na tomto modelu lze studovat změny probíhající v tubulointersticiálním kompartmentu ledviny, ke kterým dochází při progresi renálního poškození a rozvoji end-stage kidney nezávisle na primárním poškození parenchymu (ať jde o poškození glomerulů, tubulů či cév). V podvázané ledvině se vyvíjí hydronefróza, dochází k poškození tubulárních buněk, k rozvoji zánětu v intersticiu a následně k fibróze intersticia. Exprese mRNA CC chemokinů, zejména CCL2, CCL5 a jejich receptorů CCR1, CCR2, CCR5 stoupá rychle mezi 2-10 dnem. Během této doby nastává i masivní příliv makrofágů a lymfocytů do ledviny (Vielhauer et

al 2001). Morfologicky je patrné poškození tubulů s jejich dilatací, s atrofií tubulární výstelky spojenou s oploštěním a nekrózou buněk. Dochází k rozšíření intersticia způsobenému zvýšenou tvorbou a ukládáním extracelulární matrix, tj. k rozvoji fibrózy, s imunohistochemicky prokazatelnou zvýšenou akumulací aktivovaných fibroblastů v intersticiu. Za pomoci in situ hybridizace byla v histologických řezech prokázána exprese mRNA pro CCL2 (MCP-1) a CCL5 (RANTES) a jejich receptorů CCR2 a CCR5 v místech mononukleárního zánětlivého infiltrátu. Na povrchu infiltrujících makrofágů a lymfocytů izolovaných z poškozené ledviny byla pomocí průtokové cytometrie prokázána exprese CCR2 a CCR5. Data získaná Vielhauerovou studií (Vielhauer et al 2001) ukazují korelaci poškození tubulointersticiální tkáně a rozvoje fibrózy spojené se zvýšenou expresí mRNA některých CCL chemokinů s intersticiální akumulací lymfocytů a makrofágů exprimujících na svém povrchu příslušné chemokinové receptory.

1.8.3.2 Chemokiny u lidských onemocnění ledvin

Relevance dat získaných experimentálními studiemi na zvířecích modelech pro lidská onemocnění ledvin byla potvrzena řadou morfologických studií v biopsiích ledvin (např. přehled Segerer et al. 2000b).

Expresi chemokinů a chemokinových receptorů u různých typů glomerulárních onemocnění prokázala řada prací. U IgA nefropatie byla zjištěna zvýšená hladina CXCL8 (IL-8) v moči, která korelovala se stupněm hematurie u pacientů s akutní formou onemocnění. V ledvinné tkáni byla exprese CXCL8 lokalizována v glomerulech se známkami endokapilární proliferace. Naproti tomu hladiny CCL2 (MCP-1) v moči byly signifikantně vyšší u chronického onemocnění, korelovaly s morfologickými známkami progrese, jako jsou mezangiální proliferace a intersticiální infiltrace makrofágy (Yokoyama et al 1998). CCL2 (MCP-1) byl imunohistochemicky detekován v endoteliích cév, v tubulárních epiteliích a infiltrujících mononukleárních buňkách v intersticiu. U neproliferativních glomerulopatií, jako jsou membranózní nefropatie, nemoc minimálních změn, diabetická nefropatie a nefropatie tenkých bazálních membrán, se nepodařilo v glomerulech prokázat CCL2 (Rovin et al 1994), naproti tomu v tubulech

proteinurických pacientů byla v biopsii patrná exprese CCL5 (RANTES) a CCL2 (MCP-1) spojená s intersticiálním zánětlivým infiltrátem a fibrózou (Prodjosudjadi et al 1995). Imunohistochemicky byla prokázána exprese CCL2 v mezangiu glomerulů u rychle progredující glomerulonefritidy (RPGN) se srpky, Wegenerovy granulomatózy a lupusové nefritidy (Rovin et al 1994). In situ hybridizací byla prokázána mRNA pro CCL2 (MCP-1) v infiltrujících mononukleárech v intersticiu, v kortikálních tubulech a v endotelu u pacientů s lupusovou nefritidou, u glomerulonefritidy se srpky navíc i v glomerulárním trsu a v srpcích (Segerer 2000a).

Pomocí PCR byly v biopsiích u pacientů s IgA nefropatií detekovány CCR1, CCR2a, CCR2b (Yamauchi et al 1996). Furuichi et al. (2000) se zabývali expresí CCR1 a CCR5 u různých chorob ledvin v korelaci s hladinami jejich ligandů, CCL3 (MIP-1α), CCL4 (MIP-1β) a CCL5 (RANTES) v moči. Buňky exprimující CCR1 a CCR5 a zároveň CD3 nebo CD68 (T lymfocyty a makrofágy) nalezli v glomerulech a v intersticiu. Počet CCR1-pozitivních buněk v glomerulech koreloval s hladinami CCL3 (MIP-1α) v moči, počet CCR1-pozitivních buněk v intersticiu koreloval s močovými hladinami jak CCL3 (MIP-1α), tak CCL5 (RANTES). Většina CCR1-pozitivních buněk v intersticiu byly makrofágy, jejich množství korelovalo s intenzitou intersticiální fibrózy a tubulární atrofie. Počet CCR5-pozitivních buněk v glomerulech koreloval s extrakapilárními lézemi a CCL3 (MIP-1α) hladinami v moči, zatímco počet CCR5-pozitivních buněk v intersticiu,většinou CD3 pozitivních T buněk, koreloval s intersticiálním poškozením a hladinami CCL5 (RANTES) v moči. U renálních allograftů v tubulointersticialním kompartmentu a v glomerulech exprese CCL2 (MCP-1), CCL5 (RANTES), a jejich receptorů CCR1, CCR2 a CCR5 korelovala s monocytárním infiltrátem (Rüster et al 2004). Biopsie u pacientů s chronickou transplantační nefropatií vykazovaly nižší expresi CCL2 (MCP-1), CCL5 (RANTES), CCR1, CCR2 and CCR5 na buňkách v tubulointersticiálním kompartmentu, a signifikantně nižší infiltraci monocyty než biopsie s akutní rejekcí (Pozn.: autor si je vědom faktu, že současná modifikace Banffské klasifikace k hodnocení změn v ledvinných štěpech výše uvedené termíny nepoužívá). CCR2, receptor pro CCL2 (MCP-1), byl prokázán in situ hybridizací u biopsií s obrazem RPGN

v glomerulárním trsu, v srpcích a v zánětlivém infiltrátu v tubulointersticiu na CD3 a v menší míře na CD68 pozitivních buňkách (Segerer et al. 2000a). Segerer et al. (1999) sledovali imunohistochemicky přítomnost CCR5 receptoru v biopsiích pacientů s různými onemocněními ledvin. Nezávisle na typu onemocnění nalezli tento receptor pouze na infiltrujících mononukleárech; pozitivita barvení převážně korelovala s pozitivitou CD3, a to jak v intersticiu, tak i v menší míře v glomerulech, zejména u membranoproliferativní glomerulonefritidy. Exprese CCR5 v ledvinné tkáni byla nově studována za použití CCL5 binding assay u pacientů s akutní transplantační glomerulitidou, tedy s patrnou infiltrací glomerulu T lymfocyty. CCR5 zde byl prokázán na infiltrujících zánětlivých buňkách v mezangiu, v glomerulárních endoteliích, v extracelulární matrix mezangia a v intersticiu na mononukleárním zánětlivém infiltrátu. Exprese CCR5 na bazolaterálním povrchu epitelu tubulů a na tubulární bazální membráně byla obdobná jako u kontrolních nefrektomií (Segerer et al 2007).

1.8.4 Možnosti terapie lidských onemocnění ovlivněním chemokinové sítě

Receptory spřažené s G-proteinem jsou v současné době jedním z nejvýznamnějších cílů farmakologické terapeutické intervence. Na trhu je celá řada nízkomolekulárních látek používaných k léčbě onemocnění, na jejichž etiopatogenezi se uvedené receptory podílejí, zejména léků ovlivňujících adrenergní, dopaminergní, cholinergní, histaminové a serotoninové receptory.

Chemokinové receptory jsou významným cílem farmakologického výzkumu, vzhledem k jejich důležité roli u akutních a chronických zánětlivých onemocnění, v angiogenezi, metastazování nádorů a jako koreceptorů vstupu viru HIV do buňky (Onuffer et al 2002).

Terapeutické strategie cílené na chemokinovou síť lze schematicky rozdělit do tří skupin:

a. Blokáda leukocytární adheze k endotelu cév a blokáda transendoteliální migrace – zde lze využít antagonisty chemokinových receptorů, analoga chemokinů, popřípadě neutralizující protilátky

b. Inhibice lokální produkce chemokinů – blokádou leukocytární infiltrace do místa zánětu mechanizmy popsanými v předchozím bodě, popřípadě inhibice receptorů, jejichž aktivace vede k produkci chemokinů (jako jsou například Toll-like receptory)

c. Selektivní eliminace cílových buněk, na něž chemokiny působí (identifikovatelné jako buňky exprimující daný receptor) – pomocí protilátek s dvojí specificitou, nebo protilátek proti chemokinovým receptorům s navázaným exotoxinem.

Blokáda lymfocytárního a monocytárního zánětlivého infiltrátu u chronických onemocnění ledvin pomocí blokády chemokinových receptorů, které tyto buňky exprimují, se jeví do budoucna jako použitelná terapeutická strategie. Blokáda leukocytárního zánětlivého infiltrátu pomocí polyklonální protilátky proti CCR1 redukovala v experimentu na myši rozvoj bleomycinem indukované plicní fibrózy (Tokuda et al 2000). Na modelu kolagenem indukované artritidy u myši bylo demonstrováno, že blokáda CCR1 může být efektivním přístupem k terapii zánětlivých chorob, kde poškození tkáně působí či spolupůsobí leukocytární zánětlivý infiltrát (Plater-Zyberk et al 1997).

Zablokování chemokinového receptoru CCR1 pomocí nízkomolekulárního antagonisty BX471 vedlo v experimentu ke snížení počtu makrofágů a lymfocytů infiltrujících intersticium ledviny o 50-60%. Zároveň došlo ke snížení počtu aktivovaných fibroblastů a k redukci exprese kolagenu I v intersticiu ledviny (Anders et al 2002). Přehledně je výčet zvířecích modelů renálních onemocnění, u kterých byl v experimentu podán BX471, uveden v práci Ninichuk et al. (2005).

V experimentu byly použity i chemokiny s modifikací na N-konci, u kterých je molekula chemokinu upravena delecí aminokyselin nebo přidáním funkční chemické skupiny. Takto upravené molekuly působí jako antagonisté na příslušných receptorech. Modifikací CCL5 (RANTES) byly vyvinuty AOP-RANTES a Met-RANTES. Jejich působení bylo studováno například na modelu anti-GBM glomerulonefritidy a Thy-1 nefritidy, kde došlo k redukci leukocytárního infiltrátu a zároveň i proteinurie (Lloyd et al 1997) a snížené depozici kolagenu IV v mezangiu (Panzer et al 1999). U modelu transplantace ledviny vedlo podávání Met-RANTES k redukci rozvoje rejekce

(Gröne et al 1999). Furuichi et al. (2003) použili vektorový konstrukt produkující 7ND - CCL2 protein s delecí na -NH2 konci, blokující CCR2 aktivaci. Po přenesení tohoto vektoru do femorálního svalu BALB/c myši došlo k výraznému poklesu tkáňového poškození u ischemického-reperfúzního poškození ledviny, spolu se značným snížením počtu infiltrujících makrofágů. Obdobně byl 7ND použit na modelu jednostranné obstrukce ureteru, kde došlo k významné redukci rozvoje fibrózy intersticia ledviny (Wada et al 2004).

Aptamery jsou třídimenzionální oligonukleotidy schopné se specificky vázat na cílové molekuly (zhruba podobně jako protilátky). U nově vyvinuté skupiny aptamerů zvané spiegelmery díky konfiguraci (L-enantiomery) nedochází k degradaci nukleázami. Chemokin CCL2 (MCP-1) blokující spiegelmer, pojmenovaný mNOX-E36, u MRL lpr/lpr myší vede ke zlepšení lupusové nefritidy a kožních a plicních lézí (Kulkarni et al 2007, 2009).

Pokud již došlo k infiltraci leukocytů do tkáně, lze jako přídavnou terapeutickou strategii použít cílenou eliminaci zánětlivých buněk identifikovaných specifickými chemokinovými receptory, které exprimují na svém povrchu. K depleci CCR5 pozitivních monocytů a T buněk lze například použít jednořetězcové protilátky s dvojí specificitou, které simultánně váží CCR5 na cílových buňkách a CD3 na T buňkách. Vazbou dochází k aktivaci T buněk, které posléze zničí navázané CCR5 pozitivní buňky. Jinou možností, jak eliminovat CCR5 pozitivní buňky, je použití fúzního proteinu, složeného z CCL5, ligandu CCR5, a větvené verze pseudomonádového exotoxinu A, který specificky destruuje cílové buňky po svém navázání na receptor. Eliminace jen určitých definovaných skupin leukocytů na základě exprese chemokinových receptorů může být v budoucnu strategií selektivní imunosuprese, která nevede k závažným vedlejším účinkům „klasické“ terapie, jako je leukopenie nebo těžká imunosuprese (Brühl et al 2001).

V nedávné době byla publikována řada výsledků klinických studií zkoumajících možnosti využití antagonistů chemokinových receptorů, jednak jako antiretrovirové terapie, a jednak jako terapie autoimunitních onemocnění (např. roztroušené sklerózy, SLE a revmatoidní artritidy). Zatímco možnosti léčby HIV antagonisty chemokinových receptorů se jeví jako slibné, studie

využití těchto antagonistů k léčbě autoimunitních onemocnění byly dosud převážně neúspěšné (Horuk 2009).

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2 CÍLE PRÁCE

V první skupině prací byla zkoumána účast vybraných chemokinových receptorů v průběhu chronického renálního onemocnění. Cílem první práce bylo zjistit, zda chemokinové receptory CCR1 a CCR5 hrají roli při infiltraci makrofágů a T lymfocytů do intersticia ledviny a při následném rozvoji renální fibrózy v experimentálním modelu jednostranné obstrukce ureteru u myši, reprezentujícím protrahované poškození tubulointersticia, ke kterému dochází v průběhu chronického renálního onemocnění. V práci byly použity CCR1 a CCR5 deficientní (knock-out) myši.

V další práci byl studován efekt terapeutické blokády chemokinového receptoru CCR1 na průběh progresivního renálního poškození na modelu lupus-like nefritidy u myšího kmene MRLlpr/lpr, autoimunitního onemocnění, které vede k imunokomplexové vaskulitidě a glomerulonefritidě s následným tubulointersticiálním poškozením a s rozvojem chronického renálního selhání. K blokádě receptoru byl použit nízkomolekulární antagonista BX471.

Cílem třetí práce bylo určit efekt farmakologické blokády na průběh progresivního renálního poškození spojeného s těžkou proteinurií na myším modelu adriamycinem indukované fokální segmentální glomerulosklerózy s nefrotickým syndromem a fibrózou renálního parenchymu.

Druhá skupina prací byla zaměřena na roli ET-1 při rozvoji hypertenze a orgánového poškození u homozygotních a heterozygotních TGR (mRen-2)27 transgenních potkanů, modelu monogeneticky definované hypertenze. Hlavním cílem bylo charakterizovat rozdíl vlivu selektivní ET_A a neselektivní a $ET_{A/B}$ blokády u mladých jedinců ihned po odstavení, s cílem zjistit, zda časně zahájená antihypertenzivní terapie dokáže zabránit rozvoji hypertenze či alespoň zmírnit její průběh a tím i orgánové poškození. Vzhledem k tomu, že jde o sůl senzitivní model experimentální hypertenze, u kterého je produkce ET-1 zvýšena, byly pokusy prováděny v normoslaném i vysokoslaném dietním režimu a byl zkoumán i vliv vysokého příjmu soli na průběh hypertenze, orgánového poškození a přežití pokusných zvířat.

Dále byla řešena otázka, zda blokáda ET systému (selektivní či neselektivní) může mít protektivní účinky na orgánové poškození a vliv na krevní tlak a přežití, pokud je zahájena u dospělých jedinců s již stabilizovanou hypertenzí, a to jednak na homozygotních potkanech, kde průběh onemocnění odpovídá těžké maligní hypertenzi, tak na potkanech heterozygotních, kde postupný rozvoj hypertenze s pozvolnějším rozvojem orgánových změn dovoluje dlouhodobější studie morfologie renálního poškození lépe modelující postupný rozvoj sekundární FSGS jako u lidí.

3 VÝSLEDKY

3.1 Chemokine receptor CCR1 but not CCR5 mediates leukocyte recruitment and subsequent renal fibrosis after unilateral ureteral obstruction

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Chemokine Receptor CCR1 But Not CCR5 Mediates Leukocyte Recruitment and Subsequent Renal Fibrosis after Unilateral Ureteral Obstruction

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Abstract. As chemokine receptor CCR1 and CCR5 expression on circulating leukocytes is thought to contribute to leukocyte recruitment during renal fibrosis, the authors examined the effects of unilateral ureteral obstruction (UUO) in mice deficient for CCR1 or CCR5. Analysis of UUO kidneys from CCR1-deficient mice revealed a reduction of interstitial macrophages and lymphocytes (35% and 55%, respectively) compared with wild-type controls. CCR1-deficient mice had reduced CCR5 mRNA levels in UUO kidneys, which correlated with a reduction of CCR5+ T cell infiltrate as determined by flow cytometry. Interstitial fibroblasts, renal TGF-β1 mRNA expression, interstitial volume, and collagen I deposits were all significantly reduced in CCR1-deficient mice. In contrast, renal leukocytes and fibrosis were unaffected in CCR5-deficient mice with UUO. However, if treated with the CCR1 antagonist BX471, CCR5-deficient mice showed a similar reduction of renal leukocytes and fibrosis as CCR1-deficient mice. To determine the underlying mechanism labeled macrophages and T cells isolated from either wild-type, CCR1-deficient, or CCR5-deficient mice were injected into wild-type mice with UUO. Three hours later, renal cell recruitment was reduced for CCR1-deficient cells or cells pretreated with BX471 compared with CCR5-deficient or wild-type cells. Thus, CCR1 but not CCR5 is required for leukocyte recruitment and fibrosis after UUO in mice. Therefore, CCR1 is a promising target for therapeutic intervention in leukocyte-mediated fibrotic tissue injury, *e.g.* progressive renal fibrosis.

Chronic inflammation and tissue fibrosis are common causes of progressive organ dysfunction. In the kidney, the extent of leukocyte infiltration and tubulointerstitial fibrosis are strong prognostic factors for the degree of renal insufficiency and the progression to end-stage renal disease (1). Interstitial fibrosis is characterized by the accumulation of interstitial T cells, macrophages, and fibroblasts that contribute to extracellular matrix production and tubular atrophy (2). Strategies that specifically block the infiltration of leukocytes and thereby reduce interstitial inflammation may provide a potential option to reduce progressive renal fibrosis and to prevent end-stage renal disease (3).

Infiltration of circulating leukocytes is triggered by locally secreted chemokines (4), and chemokine-mediated leukocyte infiltration is thought to contribute to the initiation and progression of renal disease (5). Studies on progressive renal fibrosis using the model of unilateral ureteral ligation (UUO) in the mouse have demonstrated that increasing amounts of the chemokine receptors CCR1 (ligands CCL3 and CCL4) and CCR5 (ligands CCL5 and CCL4) were expressed on infiltrating macrophages and T cells in parallel to the development of renal fibrosis (6). CCR1 and CCR5 are thought to mediate the migration of T cells and macrophages into inflamed tissues (4,7). Evidence for the role of CCR1 in renal disease comes from a study that showed that the CCR1 antagonist BX471 was similar to cyclosporin in its ability to prevent renal allograft rejection and to improve survival in rabbits (8). Furthermore, we have recently shown that BX471 reduced leukocyte accumulation and renal fibrosis after UUO in mice (9). However,

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when CCR1-deficient mice were injected with nephrotoxic serum they showed enhanced glomerular T cell infiltration, crescent formation, and proteinuria in association with an enhanced Th1-response, indicating CCR1 is also involved in the regulation of systemic immune responses (10). Conflicting results have also been found with Met-RANTES or AOP-RANTES, two antagonists that can block CCR5. AOP-RANTES reduced macrophage infiltration in Thy1.1 antibody-induced glomerulonephritis in rats (11), and Met-RANTES improved rat renal allograft rejection (12). However, both antagonists aggravated immune complex glomerulonephritis in mice, which was associated with antagonist-induced modulation of macrophage function toward a proinflammatory phenotype (13).

Although the multiple roles of CCR1 and CCR5 activation on leukocytes may show different outcomes in systemic immune responses, their specific roles for localized leukocyte recruitment *in vivo* at sites of restricted inflammation or tissue injury remain do be determined. *In vitro* studies have suggested different roles of CCR1 and CCR5 for leukocyte recruitment. CCR1 but not CCR5 was found to be required for the initial adhesion of human monocytes and T cells to activated endothelium, whereas CCR5 seemed to be involved in the subsequent transendothelial cell migration (14).

We therefore hypothesized that both CCR1 and CCR5 might be involved in macrophage and T cell infiltration and the development of renal fibrosis after UUO in mice. Using a multipronged approach involving combinations of CCR1-deficient and CCR5-deficient mice, transfer of leukocytes from these animals into wild-type mice with UUO, and a specific CCR1 antagonist, we could clearly demonstrate that CCR1 but not CCR5 is required for leukocyte recruitment in this model. We conclude that CCR1 but not CCR5 may be a valuable target for therapeutic intervention for chronic nephropathies accompanied by leukocyte-mediated progressive interstitial fibrosis.

Materials and Methods

Animal Studies

CCR1-deficient mice were generated as described (15). CCR1-deficient mice were backcrossed into the C57BL/6 background for eight generations under specific pathogen-free housing conditions. Details about the generation of CCR5-deficient mice will be described elsewhere (Luckow *et al.*, manuscript in preparation). In brief, almost the entire coding region of the murine CCR5 gene was deleted by homologous recombination in E14–1 ES cells using standard gene-targeting methods. ES cells with a targeted deletion of the CCR5 gene were identified by Southern blot analyses and subsequently used to generate chimeric mice by morula aggregation. After germ line transmission, the resulting heterozygous CCR5 knockout mice were backcrossed for five generations into the inbred strain C57BL/6 (Charles River, Germany) and then intercrossed to obtain homozygous CCR5 knockout mice. Deletion of CCR1 or CCR5 was confirmed by repeated genotyping in all individual mice by PCR analyses using genomic DNA prepared from tail snips as described (15). After UUO was performed, all mice were housed in groups of 7 to 9 mice in filter top cages with a 12-h dark/light cycle and unlimited access to food and water. Cages, bedding, nestlets, food, and water were sterilized by autoclaving before use. For all experiments, 8- to 12-wk-old animals were used. One group of CCR5-deficient mice were treated with the CCR1 antagonist BX471 (50 mg/kg body wt, thrice daily, subcutaneously) from day 0 to day 10 as described (9). Sex-matched and age-matched controls were obtained from Charles River, Sulzfeld, Germany. Tissue was obtained at day 10. Contralateral kidneys served as intraindividual control. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Morphologic Evaluation

Tissue sections from the obstructed and the contralateral kidneys of each mouse were fixed in 4% formalin in PBS and embedded in paraffin. Two-micrometer sections were stained with periodic acid-Schiff reagent and silver following the instructions of the supplier (Bio-Optica, Milano, Italy). To count interstitial cells, 15 high power fields (hpf, ×400) were analyzed by a blinded observer. Positive cells were counted per hpf omitting positive cells in glomerular fields. Quantitation of the interstitial volume (I_{Vol}), interstitial collagen deposition (I_{col}), and tubular dilatation (I_{Tdil}) was performed as described previously (9).

Immunohistochemistry

All immunohistologic studies were performed on paraffin-embedded sections as described (6). The following rat and rabbit antibodies were used as primary antibodies: rat anti-F4/80 (1:50; Serotec, Oxford, UK), rat anti-CD3 (1:50; Serotec), rabbit anti-human TGF-β crossreacting with mouse TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-FSP1 (1:500; kindly provided by Dr. E. G. Neilson, Vanderbilt University, Nashville, TE).

In Situ Hybridization

In situ hybridization for murine TGF-β1 was performed as described previously (16). The TGF-β1 probe was a gift from H.L. Moses (Department of Cell Biology, Vanderbilt University, Nashville, TN) as described. Negative controls included hybridization performed on replicate tissue sections using the sense riboprobe.

Isolation of Renal Cells for FACS Analysis

A preparation of isolated renal cells including infiltrating leukocytes was obtained as described previously (6) from obstructed and contralateral kidneys. For flow cytometry, the resulting cell suspensions were incubated with 5 μg/ml monoclonal antibodies against murine CCR5 or the isotype control rat IgG_{2b} (Pharmingen, Hamburg, Germany), as described (17). To identify CD8, T cells samples were incubated with a Cy-chrome-labeled anti-mCD8 antibody (Pharmingen).

In Vivo Assay of Renal Leukocyte Infiltration

F4/80 macrophages and CD8 T cells were prepared from spleens of CCR1 −/−, CCR5 −/−, and wild-type mice by a previously described isolation and labeling method (18). In brief, spleen T cells and F4/80 macrophages were isolated by immunomagnetic selection using the following antibodies: CD8a (Ly-2) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), FITC rat anti-mouse F4/80 (Serotec, Düsseldorf, Germany), and Anti-FITC MicroBeads (Miltenyi). Purity of isolated cells was verified by flow cytometry. Separated cells were labeled with PKH26 (Red Fluorescence Cell Linker Kit, Sigma-Aldrich Chemicals, Steinheim, Germany), and labeling efficacy was assessed by flow cytometry to be >97%. Viability assessed by trypan

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blue exclusion was >90%. Wild-type mice underwent surgery for UUO and were injected with either 7.5×10^5 F4/80 positive cells or CD8 positive T cells in 200 μl of isotonic saline into a tail vein 10 d after surgery. One group of mice was injected with CCR5-deficient cells that were preincubated with 600 μM CCR1 antagonist BX471 for 30 min (9). Mice of this group received a single subcutaneous injection of BX471 (50 mg/kg) at the time of injection. Renal tissue was obtained after 3 h, snap frozen, and prepared for microscopy. Interstitial cells from UUO and contralateral kidneys were analyzed as above.

Isolation of Cells for Real-Time RT-PCR

To assess CCR1 and CCR5 mRNA expression in renal fibroblasts and tubular epithelial cells, renal tissue samples were obtained 10 d after UUO. Tubular segments were microdissected from RNase inhibitor treated tissue in ice-cold PBS, as described previously for human renal biopsies (19). For isolation of primary renal fibroblasts, small pieces of renal tissue were incubated in DMEM (Invitrogen Corporation, Karlsruhe, Germany) supplemented with 10% FCS (Invitrogen), penicillin, and streptomycin for 21 d. Adherent cells were lifted with 1.5 mM EDTA (Calbiochem-Novabiochem, San Diego, CA) and were depleted for leukocytes by immunomagnetic selection using FITC anti-mCD45 (Pharmingen) and anti-FITC MicroBeads as described (Miltenyi). F4/80-positive macrophages and CD8-positive T cells were obtained from wild-type mice as described above. mRNA of isolated cells was prepared by standard methods as described (9)

Real-Time Quantitative RT-PCR

Pieces of kidney from each animal were snap frozen in liquid nitrogen and stored at −80°C. RNA preparation and real-time RT-PCR on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) were performed as described (9). Controls consisting of ddH_2O were negative for target genes and the housekeeper gene GAPDH. The following oligonucleotide primers (300 nM) and probes (100 nM) were used. Murine CCR1: forward 5'-TTAGCTTCCATGCCTGCCTTATA-3', reverse 5'-TCCACT-GCTTCAGGCTCTTGT-3', internal fluorescence labeled probe (FAM) 5'-ACTCACCGTACCTGTAGCCCTCATTTCCC-3', the probe is located in the deleted region of the CCR1 gene in CCR1-deficient mice; murine CCR5: forward 5'-CAAGACAATCCT-GATCGTGCAA-3', reverse 5'-TCCTACTCCCAAGC-TGCATA-GAA-3'; FAM 5'-TCTATACCCGATCCACAGGAG-AACATG-AAGTTT-3', the probe is located in the deleted region of the CCR5 gene in CCR5-deficient mice, CCR5 specificity of primers and probe were tested on CCR plasmids; murine TGF-β1: forward 5'-CACAG-TACAGCAAGGTCCTTGC-3', reverse 5'-AGTAGACGATGGG-CAGTGGCT-3', FAM 5'-GCTTCGGCGTCACCGTGCT-3'; murine GAPDH: forward 5'-CATGGCCTTCCGTGTTCCTA-3', reverse 5'-ATGCCTGCTTCACCACCTTCT-3', internal fluorescence labeled probe (VIC) 5'-CCCAATGTGTCCGTCGTGGATCTGA-3'. All primers and probes were obtained from PE Biosystems. For the expression, CCR levels in isolated cells values are expressed as ratio of respective CCR mRNA to GAPDH mRNA expression. Expression levels of CCR in the UUO model were analyzed accordingly, showing the aforementioned ratio in each animal separately for UUO and contralateral kidneys (CLK). For comparison of mRNA expression, levels between different groups the ratio of UUO to CLK is shown.

RNase Protection Assay

Total spleen RNA was isolated from three C57BL/6 wild-type mice, five CCR1−/− mice, and four CCR5−/− mice. Multiprobe RNase protection assay was performed with the RiboQuant multi-probe template set for murine CC chemokine receptors (mCR-5) obtained from Pharmingen using 10 μg of total spleen RNA per lane as described (6). Unfortunately, the CCR1-specific RPA probe from the multiprobe template set is located outside of the deleted region of the CCR1 gene in CCR1-deficient mice and therefore not suited to differentiate between wild-type and knockout transcripts.

Statistical Analyses

Data are presented as mean ± SD. Comparison of groups was performed using univariant ANOVA and post-hoc Bonferroni correction was used for multiple comparisons. Paired *t* test was used for the comparison of single groups (FACS data). A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Chemokine Receptor Expression in Renal Cells

As we intended to study the role of CCR1 and CCR5 in the fibrotic kidney, we first determined the expression of CCR1 and CCR5 by real-time RT-PCR using total renal RNA. There was a marked increase of mRNA of both chemokine receptors in obstructed compared with unobstructed kidneys (Figure 1A). To characterize whether intrinsic renal cells contribute to renal CCR1 and CCR5 expression, we performed real-time RT-PCR for both receptors on renal fibroblasts and tubular epithelial cells. Neither cell type expressed mRNA for CCR1 or CCR5 (Figure 1B). CCR1 and CCR5 were examined on naïve macrophages and T cells, as these receptors may contribute to recruitment of these cells to the kidney after UUO. Both F4/80 macrophages and T cells expressed CCR1 and CCR5 mRNA. In macrophages, the expression of CCR1 mRNA was much higher than that of CCR5 mRNA. In contrast T cells expressed CCR5 mRNA to a greater extent than CCR1 mRNA (Figure 1B). To further characterize the expression of CCR5 protein on infiltrating T cells, FACS analysis on renal cell isolates was performed. The ratio of CD4/CD8-positive among the CD3-positive lymphocytes infiltrating the kidney was 1:1; 93 ± 3% of CD8 T cells infiltrating the obstructed kidney were positive for CCR5. Flow cytometry of F4/80 macrophages for CCR5 was not feasible due to unspecific binding of isotype antibodies. Deficiency of CCR1 was not compensated by higher expression levels for CCR2 and CCR5 and *vice versa* as analyzed by RNase protection assays with spleen cell mRNA from all mouse strains (Figure 1C), confirming data reported by others (10). These data indicate that both CCR are expressed by infiltrating leukocytes but not by intrinsic interstitial cells such as tubular epithelial cells or fibroblasts in the UUO kidneys. Furthermore, lack of either CCR1 or CCR5 is not followed by upregulation of the other CCR on immune cells and *vice versa*.

CCR1 But Not CCR5 Is Required for Leukocyte Infiltration after UUO

At day 10 after UUO, a prominent cell infiltrate of CD3-positive lymphocytes and F4/80-positive macrophages was localized to the peritubular interstitium in wild-type mice by immunostaining (Figure 2A). CCR1-deficient but not CCR5-

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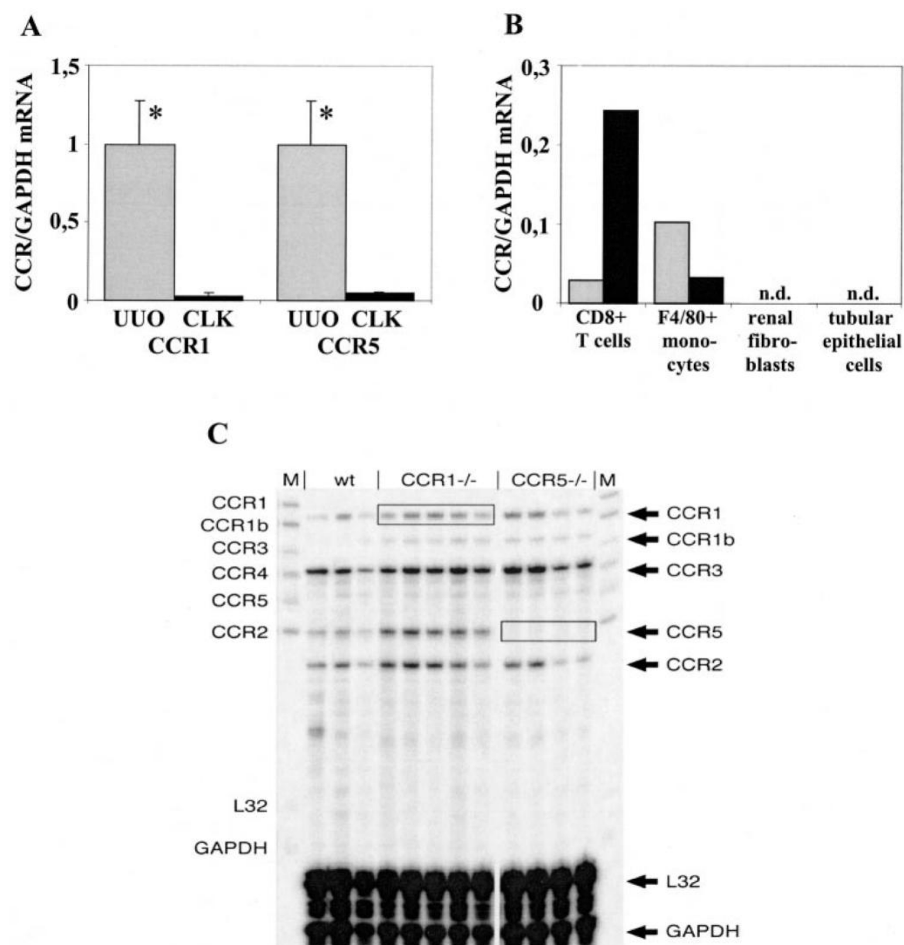

Figure 1. Chemokine receptor mRNA expression. (A) Renal expression of CCR1 and CCR5 mRNA was determined by real-time RT-PCR from total renal isolates 10 d after unilateral ureteral obstruction (UUO). CCR mRNA expression of obstructed kidneys (UUO) and contralateral unobstructed kidneys (CLK) is expressed as ratio to the respective GAPDH mRNA expression ($n = 5$). CCR mRNA expression in UUO kidneys is set as 1. * $P < 0.05$. (B) The expression of CCR1 (gray) and CCR5 (black) mRNA in different cell types was determined in duplicate by real-time RT-PCR and expression levels are expressed as ratio to the respective GAPDH mRNA expression. (C) Expression of CC chemokine receptor mRNA in splenocytes determined by RNase protection assay. The two lanes designated M show the undigested templates, the arrows indicate the positions of the RNase digested templates. The lower box shows the absence of the corresponding message in CCR5 −/− mice. A CCR1 signal was obtained from the CCR1 −/− mice (upper box) because the probe used for CCR1 RPA was located outside of the region that has been deleted in the CCR1 −/− mice; therefore, a positive signal was obtained. It is obvious from the gel, that deletion of either CCR1 or CCR5 has no major effect on the expression of the other closely related chemokine receptors. Similar results have been obtained for the ligands of CCR5 using RPA. WT, wild-type; CCR −/−, mice negative for respective chemokine receptor expression; n.d., not detected.

deficient mice showed a reduction of interstitial CD3-positive lymphocytes and of interstitial F4/80-positive macrophages in obstructed kidneys 10 d after UUO compared with UUO kidneys of wild-type control mice (Figure 2B). However, if treated with BX471, CCR5-deficient mice revealed a reduction of interstitial CD3-positive lymphocytes and of interstitial F4/80-positive macrophages in obstructed kidneys similar to what was observed in CCR1-deficient mice (Figure 2B).

FACS analysis of isolated renal cells revealed that this decrease was accompanied by a significant reduction of CCR5/CD8-positive T cells in UUO kidneys of CCR1-deficient mice compared with wild-type controls (93 ± 3% *versus* 50 ± 17%; $P = 0.036$; Figure 3A). When CCR5-positive CD4 cells were evaluated in one experiment, they were also reduced by 33%. Due to the lack of appropriate antibodies against mCCR1 that work in FACS or immunostaining, we were unable to demonstrate the amount of CCR1-positive cells in UUO kidneys. We therefore used real-time RT-PCR to determine the amount of CCR1 and CCR5 mRNA expression in UUO kidneys of all groups. Corresponding to reduced renal leukocyte counts, CCR1-deficient mice revealed a significant reduction of renal CCR5 mRNA expression (Figure 3B). In line with their geno-

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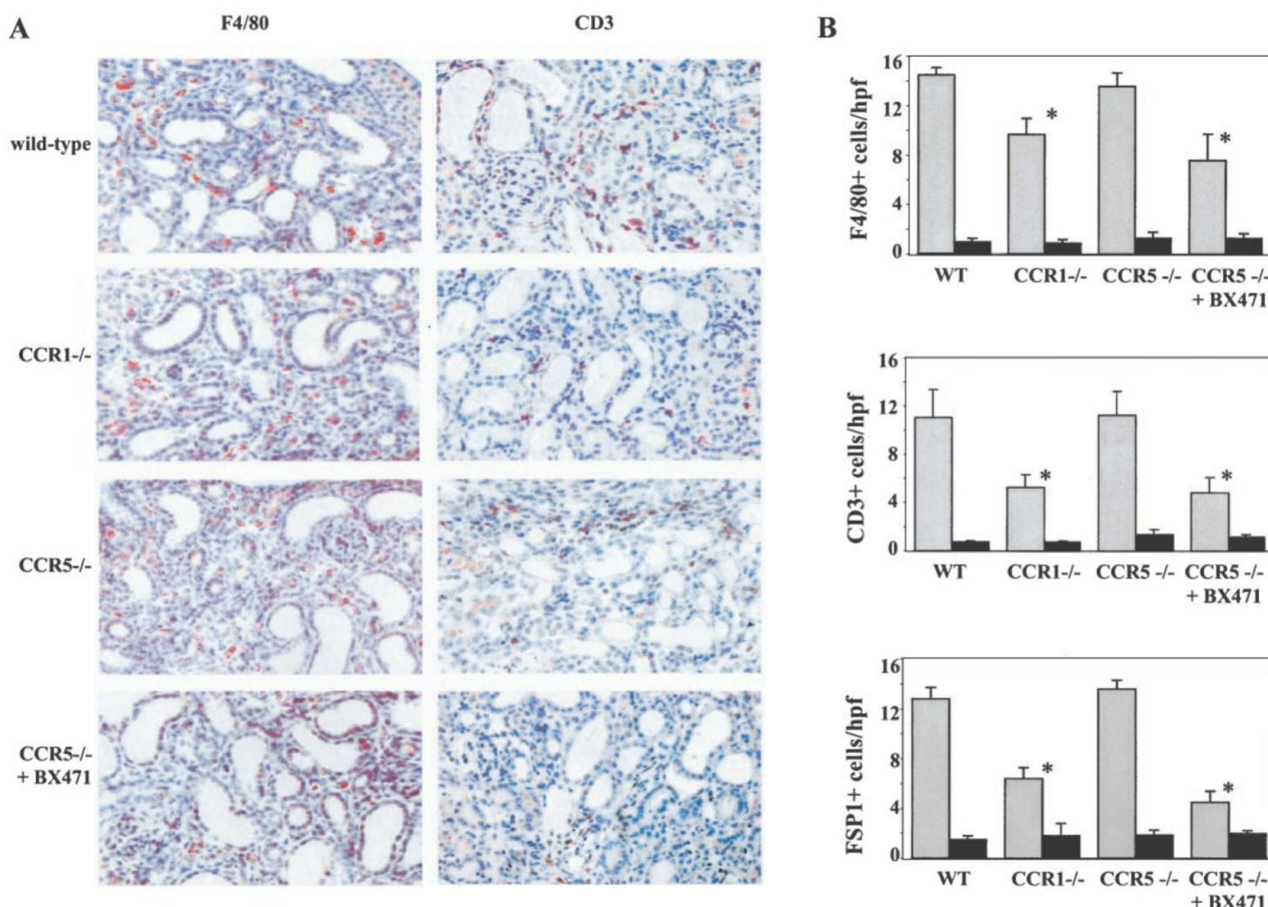

Figure 2. Accumulation of leukocytes after UUO. (A) Immunohistochemistry. Cortical renal sections were stained for F4/80-positive macrophages (left) and CD3-positive lymphocytes (right). Images illustrate representative sections of UUO kidneys from the respective groups 10 d after UUO (original magnification, ×400). (B) Quantitative analysis of F4/80-positive macrophages, CD3-positive T cells, and FSP1-positive fibroblasts 10 d after UUO in obstructed (gray bars) and contralateral unobstructed kidneys (black bars). Values are means ± SD of cell counts per 10 high power fields (hpf) per kidney from 5 to 7 mice in each group. WT, wild type; CCR −/−, mice negative for respective chemokine receptor expression. BX471 was administered in CCR5-dificient mice as described in methods. * $P < 0.05$.

type, CCR5 mRNA expression was not detectable in CCR5-deficient mice. However, in analyzing renal CCR1 mRNA expression, we found that UUO kidney CCR1 mRNA levels of CCR5-deficient mice were comparable to those of wild-type control mice. In contrast, when treated with the CCR1 antagonist BX471, CCR1 mRNA expression levels of UUO kidneys from CCR5-deficient mice were markedly reduced compared with those of wild-type mice (Figure 3B). In line with their genotype CCR1 mRNA expression was not detectable in UUO kidneys of CCR1-deficient mice.

CCR1 But Not CCR5 Mediates Renal Infiltration of Macrophages and T Cells In Vivo

On the basis of previous *in vitro* studies, we investigated the role of CCR1 and CCR5 for renal T cell and macrophage recruitment *in vivo* and used the approach of injecting purified and fluorescence-labeled leukocytes from the various knockout strains intravenously into wild-type mice at day 10 after UUO. The tissue was analyzed 3 h after injection. CD8-positive T cells or F4/80-positive macrophages purified from wild-type mice both accumulated in the interstitium of UUO kidneys (Figure 4A). When the same cells were isolated from CCR1-deficient mice, there was a 66% reduction of interstitial F4/80 positive macrophages and a 35% reduction of interstitial CD8 T cells in the UUO kidney after injection compared with wild-type controls (Figure 4B). In contrast, macrophages and T cells isolated from CCR5-deficient mice accumulated to the same extent in UUO kidneys as cells isolated from wild-type animals. When cells were isolated from CCR5-deficient mice and both the cells and the recipient mice were pretreated with BX471 before injection of the labeled cells, there was a 35% reduction of labeled interstitial F4/80-positive macrophages and a reduction of 35% of interstitial CD8-positive T cells in UUO kidneys compared with injection of untreated CCR5-negative cells or cells from wild-type controls (Figure 4B). No cells were detected in unobstructed contralateral kidneys from

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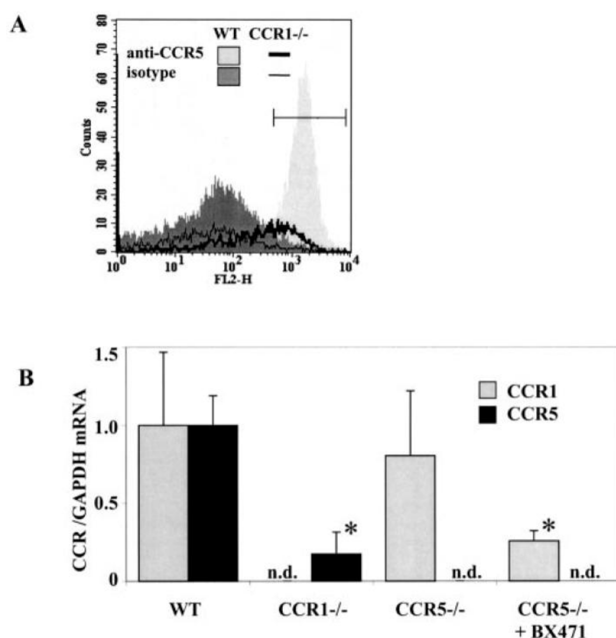

Figure 3. Renal chemokine receptor expression after UUO. (A) Flow cytometry analysis of CCR5 expression on CD8-positive T cells isolated from UUO kidneys of CCR1-deficient and wild-type mice 10 d after UUO. Staining with the CCR5 antibody is illustrated as ░ (CCR1+/+) or thick black line (CCR1−/−) and IgG2b isotype control as ▓ (CCR1+/+) and thin black line (CCR1−/−), respectively. Data shown are from obstructed kidneys of one animal of each group and are representative of four independent samples. (B) Real-time RT-PCR was performed using total renal RNA of UUO kidneys of 4 to 5 mice 10 d after UUO. Levels of mRNA expression for CCR1 (gray) and CCR5 (black) in UUO kidneys are expressed in relation to renal GAPDH mRNA expression and to the respective ratio of the unobstructed contralateral kidney. The expression of control mice is set as 1. Primers for CCR1 were located in the deleted region of the CCR1 gene. Compared with kidneys of wild-type (WT) mice, CCR1-deficient mice revealed a significant reduction of CCR5 mRNA expression ($P = 0.03$). As to be expected, CCR1 mRNA expression was not detectable in CCR1-deficient mice. Renal CCR1 mRNA expression in CCR5-deficient mice was not reduced compared with wild-type controls. CCR5-deficient mice treated with BX471 revealed a significant reduction of CCR1 mRNA expression (* $P < 0.04$). BX471 was administered in CCR5-deficient mice as described in Materials and Methods. As to be expected, CCR5 mRNA was not detectable in CCR5-deficient mice. WT, wild-type; CCR −/−, mice negative for respective chemokine receptor expression; n.d., not detected; * $P < 0.05$.

all groups, indicating that the injected cells only localize to the UUO kidneys (not shown). These data indicate that, in the mouse, CCR1 but not CCR5 is involved in the infiltration of circulating macrophages and T cells into the kidney after UUO.

Lack of CCR1 But Not of CCR5 Is Associated with a Reduction of Interstitial Fibrosis after UUO

As a cellular marker of fibrosis, the amount of FSP1-positive fibroblasts was assessed by immunohistochemistry. Fibroblasts accumulated in the peritubular interstitium in areas of marked tubular dilatation in the obstructed kidney 10 d after UUO compared with contralateral unobstructed kidneys (Figure 5). In CCR1-deficient but not in CCR5-deficient mice, the amount of interstitial FSP1-positive fibroblasts in UUO kidneys was significantly reduced compared with wild-type controls. In contrast, when treated with BX471, CCR5-deficient mice revealed a reduction of interstitial FSP1-positive fibroblasts in UUO kidneys by 65%, similar to that of CCR1-deficient mice (Figure 2B).

Morphometric analysis of silver-stained renal sections revealed a marked increase of tubular dilation, interstitial volume, and collagen deposition in obstructed kidneys compared with the respective unobstructed contralateral kidneys (Figure 5A). CCR1-deficient but not CCR5-deficient mice revealed a reduction of interstitial volume and collagen deposition, respectively (Figure 5B). In contrast, when treated with BX471, interstitial volume and collagen were also reduced in UUO kidneys of CCR5-deficient mice similar to that of CCR1-deficient mice (Figure 5B). The extent of tubular dilation in UUO kidneys was comparable in all groups as expected after persistent UUO (Figure 5B). No changes in interstitial cell counts or morphometric parameters were observed in contralateral kidneys.

Reduced Interstitial Leukocyte Infiltration in CCR1-Deficient Mice Is Associated with a Decrease of Renal TGF-β1 mRNA Expression

We were intrigued by the finding that the extent of renal fibrosis after UUO in CCR1-deficient and wild-type mice directly paralleled the amount of interstitial leukocytes. As renal fibroblasts were negative for CCR1, the lack or blockade of CCR1 is unlikely to directly affect fibroblast activation. In contrast, the observed reduction of renal fibrosis could be secondary to reduced secretion of profibrotic cytokines. We therefore determined the expression of TGF-β1 mRNA in total renal RNA by real-time RT-PCR. UUO kidneys from mice that lack CCR1 had a marked reduction of TGF-β1 mRNA expression compared with wild-type mice (Figure 6A). In contrast, lack of CCR5 did not affect renal TGF-β1 mRNA expression. However, if treated with BX471, CCR5-deficient mice revealed a significant reduction of renal TGF-β1 mRNA expression compared with untreated CCR5-deficient mice (Figure 6A). To determine the source of renal TGF-β1, we performed *in situ* hybridization and immunostaining for TGF-β. In unobstructed contralateral kidneys of wild-type mice, the *in situ* hybridization yielded only a weak diffuse deposition of silver grains not different from the sense controls (Figure 6B). The strongest signal for TGF-β mRNA was found in areas of tubulointerstitial infiltrates (Figure 6B). The resolution of the *in situ* hybridization did not allow assignment of the signal in the infiltrate to specific cells. In areas without prominent cell infiltration, only the background signal was present, similar to unobstructed control kidneys. No clear tubular expression of TGF-β mRNA was apparent. Immunostaining for TGF-β protein was also restricted to the interstitial cell infiltrates of UUO kidneys from wild-type mice (Figure 6B). In unobstructed

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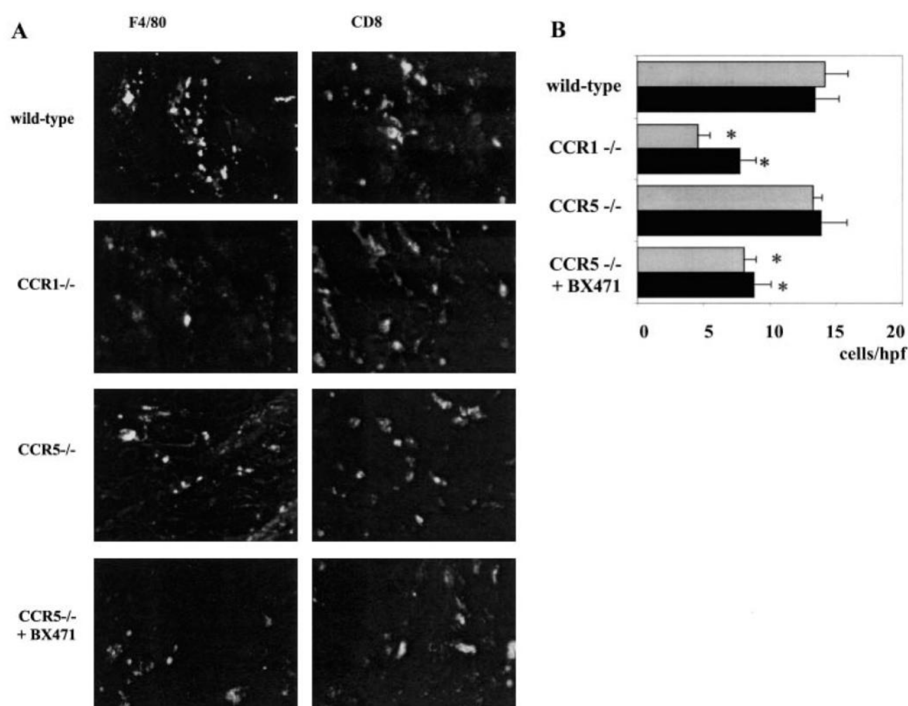

Figure 4. Renal infiltration of leukocyte subsets. (A) Wild-type mice were injected intravenously 10 d after UUO with PKH26-labeled F4/80$^+$ macrophages (left lane) or CD8$^+$ T cells (right lane) that were isolated from wild-type mice, CCR1-deficient mice, CCR5-deficient mice, and CCR5-deficient mice pretreated with BX471. Kidneys were obtained 3 h after injection and underwent fluorescence microscopy (original magnification, ×400). (B) Cell counts for labeled F4/80$^+$ macrophages (gray bars) or labeled CD8$^+$ T cells (black bars) were determined by fluorescence microscopy from 10 hpf and are expressed as means ± SD. WT, wild type; CCR −/−, mice negative for respective chemokine receptor expression. BX471 was administered in CCR5-deficient mice as described in Materials and Methods. * $P < 0.05$.

contralateral kidneys, only a weak background immunostaining for TGF-β was detected. UUO kidneys of CCR1-deficient mice had fewer interstitial TGF-β positive cells as detected by immunostaining, which correlated with less tubulointerstitial deposition of silver grains by *in situ* hybridization for TGF-β mRNA. (Figure 6B) and with the lower degree of cell infiltrate in these mice (Figure 2). In contrast, lack of CCR5 did not affect tubulointerstitial expression of TGF-β mRNA and protein (Figure 6B).

These data indicate that the source of TGF-β is in the interstitial infiltrate and not the tubular cells. Furthermore, the reduction of interstitial leukocyte infiltration observed in CCR1-deficient mice is associated with a decrease of TGF-β1 mRNA and protein, a cytokine that can stimulate epithelial-mesenchymal transformation, apoptosis, and collagen secretion by renal fibroblasts.

Discussion

We used the UUO model to study the role of CCR1 and CCR5 for leukocyte infiltration and renal fibrosis. Using combinations of genetically generated CCR1-deficient and CCR5-deficient mice, their respective leukocytes for transfer, and the CCR1 antagonist BX471, we could unequivocally demonstrate that lack or blockade of CCR1 effectively reduced the infiltration of macrophages and T cells into the UUO kidney and subsequent renal fibrosis, whereas lack of CCR5 had no effect.

CCR1 Is Required for Leukocyte Infiltration after UUO

Here we show that CCR1 is involved in renal macrophage and T cell recruitment after UUO in mice. Other studies using CCR1-deficient mice have documented its role for neutrophil migration in the defense of certain infectious organisms such as *Toxoplasma gondii*, *Paramyxovirus*, and *Aspergillus fumigatus* (15,20,21). In the context of progressive fibrotic disease states, a recent study reported the effects of a neutralizing antibody against murine CCR1 in the bleomycin-induced pulmonary fibrosis model (22). Antibodies against CCR1 but not against CCR2 reduced pulmonary mononuclear cell infiltration 10 d after induction of disease. We have recently shown in the UUO model in mice that the small molecule CCR1 antagonist BX471 reduces the amount of interstitial leukocytes (9). Previous *in vitro* studies demonstrated that CCR1 mediates leukocyte adhesion to activated endothelium of human T cells and monocytes under conditions of shear stress and flow (8,17). In addition, our studies with labeled leukocyte subsets *in vivo* clearly confirmed a role of CCR1 for macrophage and T cell recruitment into the UUO kidney in mice. As the UUO model does not involve a systemic immune response, our data are not

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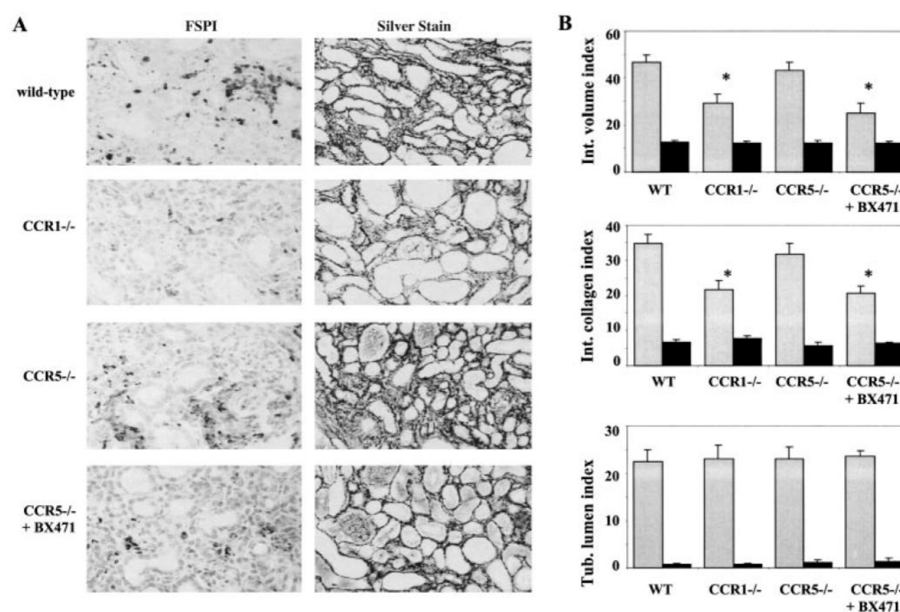

Figure 5. Morphometric analysis of tubulointerstitial injury and renal fibrosis after UUO. (A) Cortical renal sections were either stained with an FSP1-specific antibody (left) or underwent silver staining (right) as described in the Materials and Methods section. Images illustrate representative sections of UUO kidneys 10 d after UUO in mice of the respective group as indicated (original magnification, ×400). (B) The indices for interstitial volume, interstitial collagen deposition, and tubular dilatation were determined in obstructed kidneys (gray bars) and unobstructed contralateral kidneys (black bars) 10 d after UUO by quantitative morphometry as decribed in the methods section. Values represent means ± SD from 10 hight power fields of UUO (gray bars) and unobstructed contralateral kidneys (black bars). Five to seven mice were evaluated in each group. WT, wild-type; CCR −/−, mice negative for respective chemokine receptor expression. BX471 was administered in CCR5-deficient mice as described in Materials and Methods. * $P < 0.05$.

in conflict with a report that lack of CCR1 enhances Th1 responses and glomerular injury during nephrotoxic serum nephritis (10). The data on nephrotoxic nephritis would indicate that CCR1 is also involved in systemic immune responses. The fact that genetically generated CCR1 deficiency and intermittent CCR1 blockade with a specific antagonist had the same effect on renal inflammatory infiltrates and fibrosis after UUO provides powerful evidence for the role of CCR1 for macrophage and T cell recruitment after UUO and the concomitant fibrosis. Our results are consistent with the beneficial effects of CCR1 blockade on the functional outcome in renal and heart transplantation, pulmonary fibrosis, or experimental encephalomyelitis and argue for an important role of CCR1-mediated leukocyte recruitment in these models (8,23,24).

Lack of CCR5 Expression Does Not Affect Leukocyte Infiltration and Renal Fibrosis after UUO

CCR1 and CCR5 are coexpressed to different extents on macrophages and T cells. Although T cells expressed only low levels of CCR1 mRNA, the absence of CCR1 or its blockade resulted in significant reduction of renal CCR5-positive T cells in the UUO kidneys, arguing for a role of CCR1 in the recruitment of CCR5-positive cells. In contrast, despite expression of high CCR5 mRNA levels on T cells in wild-type mice, the lack of CCR5 itself did not influence renal leukocyte recruitment after UUO. Interestingly, CCR5 is involved in recruitment of leukocyte subsets in other disease models. For example, lack of CCR5 has been shown to attenuate disease activity in a model of *Aspergillus fumigatus conidia*–induced asthma, which was associated with a decrease of T cells but not of macrophages in bronchial lavage fluids (25). In a model of dextran sodium sulfate–mediated colitis, improvement of colitis in CCR5-deficient mice was also associated with impaired intestinal lymphocyte recruitment compared with wild-type controls, whereas macrophage recruitment did not depend on CCR5 expression (26). Furthermore, lack of CCR5 improved outcome after cardiac transplantation in fully MHC-mismatched mice, which was associated with reduced host T cell and macrophage recruitment to the rejected allograft (27). Interestingly, in a model of pulmonary *Cryptococcus neoformans*-infection, 12-wk survival was markedly reduced in CCR5-deficient mice, although no defects in lung leukocyte recruitment were observed (28). However, in this model, CCR5-deficient mice showed impaired leukocyte recruitment into the brain, leading to a reduction in cerebral elimination of cryptococcal polysaccharide (28). It was concluded that CCR5-mediated leukocyte trafficking is organ-specific during host defense against *C. neoformans*. In contrast, macrophage recruitment did not depend on CCR5 in experimental encephalomyelitis (29) and a model of peripheral nerve injury induced by sciatic nerve axotomy (30). The lack of an effect of CCR5 elimination on inflammatory infiltration after UUO cannot be

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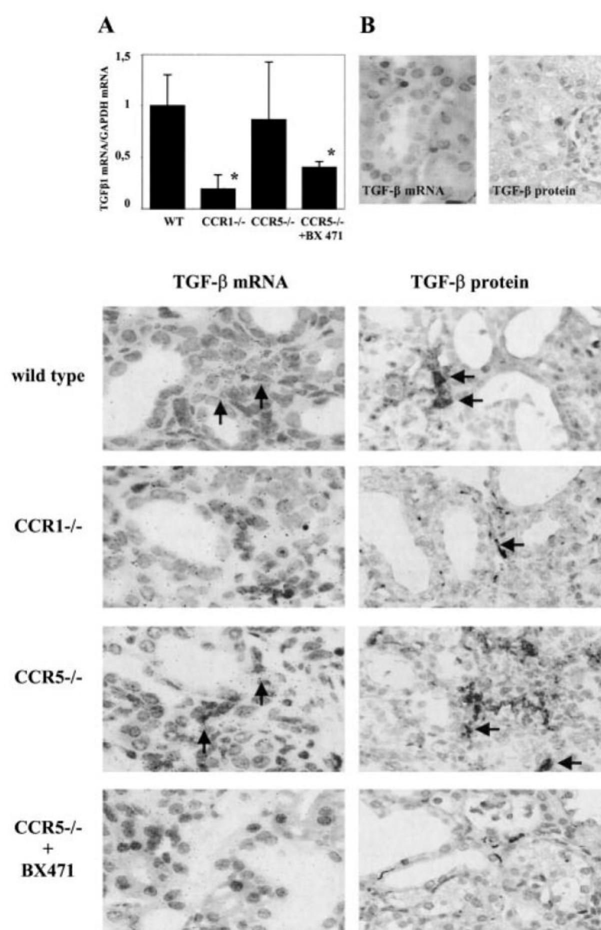

Figure 6. Renal TGF-β expression. (A) Total renal mRNA from UUO kidneys 10 d after UUO was analyzed for TGF-β1 mRNA expression by real-time RT-PCR. Levels of mRNA expression for TGF-β1 is expressed in relation to renal GAPDH mRNA expression as indicated under Materials and Methods. TGF-β1 mRNA expression of wild-type mice was set as 1 and values represent means ± SD from 5 mice per group. WT, wild-type; CCR −/−, mice negative for respective chemokine receptor expression. BX471 was administered in CCR5-dificient mice as described in Materials and Methods. * $P < 0.05$. (B) *In situ* hybridization for TGF-β mRNA (left column) and immunostaining for TGF-β protein (right column) in wild-type mice with UUO revealed marked TGF-β signals in interstitial cell infiltrates but only background signals in renal tubular cells of obstructed kidneys. In obstructed kidneys of CCR1-deficient mice, a reduction of TGF-β signals in interstitial infiltrates was noted. In contrast, obstructed kidneys of CCR5-deficient mice revealed similar TGF-β–positive interstitial cell infiltrates as wild-type controls. In contrast CCR1 blockade by BX471 in CCR5-deficient mice resulted in reduced inflammatory infiltrates and TGF-β signals. Immunostaining for TGF-β protein and with *in situ* hybridization using sense primers for TGF-β mRNA were negative in unobstructed contralateral kidneys (see panel upper right).

explained by lack of the chemokine ligand CCL5 in UUO kidneys. The chemokine ligand CCL5 for CCR5 is markedly expressed in UUO kidneys (6). Thus the interaction of CCL5-CCR5 may have functions other than cell recruitment in UUO. In contrast, our data clearly demonstrate the nonredundant role of CCR1 (ligands CCL3 and CCL4) for renal leukocyte recruitment after UUO in mice. The finding that CCR1 is important for renal leukocyte recruitment *in vivo* is consistent with *in vitro* data showing that CCR1 but not CCR5 is essential for the initial leukocyte attachment to endothelium (14). Initial attachment mediated by CCR1 is therefore critical for renal leukocyte recruitment. We therefore conclude that the role of CCR5 for leukocyte recruitment may vary in different disease models, which may relate differential chemokine expression patterns or to additional functions of CCR5 in these models.

Lack of CCR1 on Leukocytes Reduces Renal Fibrosis after UUO

How could the reduction in mononuclear leukocyte infiltration in CCR1-deficient mice or with CCR1 blockade relate to the concomitant reduction in interstitial fibroblasts and fibrosis? The infiltrating leukocytes could via secretion of, *e.g.* cytokines such as TGF-β, contribute to epithelial-mesenchymal transformation, fibroblast proliferation, and collagen production (2). In fact, CCR1-deficient mice showed a marked reduction of renal TGF-β1 mRNA expression, a key cytokine for the induction of fibroblast proliferation and the development of renal fibrosis (31). To localize the site of TGF-β production, we therefore performed both *in situ* hybridization and immunostaining for TGF-β. Both methods localized the TGF-β production to the interstitial cell infiltrate. Compared with real-time RT-PCR data, both *in situ* hybridization and immunostaining signals for TGF-β were reduced in UUO kidneys of CCR1-deficient mice and in mice treated with the CCR1 antagonist. The resolution of both methods did not allow assignment of the signal to specific cells in the infiltrate. Clearly, however, the TGF-β is not coming from the tubular epithelial cells or from the interstitium in areas without inflammatory cell infiltrate. It therefore appears reasonable to assign the TGF-β signals to inflammatory cells, *i.e.*, the infiltrating mononuclear leukocytes. This interpretation is also consistent with another report that localized TGF-β to infiltrating leukocytes in the same model using the same TGF-β antibody (32). It appears that the reduction in TGF-β mRNA and immunostaining in the UUO kidney from CCR1-deficient mice or with CCR1 receptor blockade relates directly to the reduced number of infiltrating cells observed under these conditions and is less likely a result of TGF-β production per infiltrating cell. In fact, *in vitro* studies with peritoneal macrophages from mice of all three strains expressed comparable amounts of TGF-β mRNA after stimulation with LPS (unpublished data).

In summary, CCR1 appears to play an important role in mediating the infiltration of mononuclear cells after UUO. Lack of CCR1 reduced the infiltration of macrophages and lymphocytes in the obstructed kidney and the resultant interstitial fibrosis. In contrast, lack of CCR5 had no effect on leukocyte infiltration in the UUO model. Previous studies have demonstrated a similar effect with a CCR1 antagonist; we therefore propose that CCR1 blockade may offer a new therapeutic strategy to reduce renal leukocyte infiltration and re-

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sulting fibrosis in chronic nephropathies leading to end-stage renal disease or even leukocyte-mediated progressive tissue fibrosis in general.

Acknowledgments

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3.2 Late onset of treatment with a chemokine receptor CCR1 antagonist prevents progression of lupus nephritis in MRL-Fas(lpr) mice.

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Late Onset of Treatment with a Chemokine Receptor CCR1 Antagonist Prevents Progression of Lupus Nephritis in MRL-Fas(lpr) Mice

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Abstract. Slowly progressive renal injury is the major cause for ESRD. The model of progressive immune complex glomerulonephritis in autoimmune $MRL^{lpr/lpr}$ mice was used to evaluate whether chemokine receptor CCR1 blockade late in the disease course can affect progression to renal failure. Mice were treated with subcutaneous injections of either vehicle or BX471, a nonpeptide CCR1 antagonist, three times a week from week 20 to 24 of age. BX471 improved blood urea nitrogen levels (BX471, 35.1 $\pm$ 5.3; vehicle, 73.1 $\pm$ 39.6 mg/dl; $P < 0.05$) and reduced the amount of ERHR-3 macrophages, CD3 lymphocytes, Ki-67 positive proliferating cells, and ssDNA positive apoptotic cells in the interstitium but not in glomeruli. Cell transfer studies with fluorescence-labeled T cells that were pretreated with either vehicle or BX471 showed that BX471 blocks macrophage and T cell recruitment to the renal interstitium of $MRL^{lpr/lpr}$ mice. This was associated with reduced renal expression of CC chemokines CCL2, CCL3, CCL4, and CCL5 and the chemokine receptors CCR1, CCR2, and CCR5. Furthermore, BX471 reduced the extent of interstitial fibrosis as evaluated by interstitial smooth muscle actin expression and collagen I deposits, as well as mRNA expression for collagen I and TGF-β. BX471 did not affect serum DNA autoantibodies, proteinuria, or markers of glomerular injury in $MRL^{lpr/lpr}$ mice. This is the first evidence that, in advanced chronic renal injury, blockade of CCR1 can halt disease progression and improve renal function by selective inhibition of interstitial leukocyte recruitment and fibrosis.

Progressive tubulointerstitial fibrosis is the main predictor for the progression to ESRD irrespective of the trigger mechanism (1). In patients with chronic renal failure, renal histology is characterized by a mixed tubulointerstitial inflammatory cell infiltrate and increased matrix deposition leading to tubular atrophy (2). During this process, infiltrating macrophages and lymphocytes are a major source of inflammatory mediators such as cytokines, nitric oxide, and growth factors. Inhibition of leukocyte infiltration may reduce the production of such mediators and therefore may be an option to prevent or to delay ESRD.

The leukocytic cell infiltrate is triggered by locally secreted chemokines (3). *In vitro* studies suggest a role for CCR1 in leukocyte adhesion and transendothelial migration (4), which may explain the beneficial effects of CCR1 antagonists in certain disease models, including pulmonary fibrosis (5) as well as in heart and renal transplant rejection (6,7). Using the model of unilateral ureteral obstruction in mice, we showed recently that blockade of CCR1 with the nonpeptide antagonist BX471 reduced leukocyte infiltration even when treatment was started at a time when renal fibrosis was already present (8). These data indicate that CCR1 blockade is a potential target for therapeutic intervention of progressive renal fibrosis. As chemokines are also involved in systemic immune responses (3), data from the unilateral ureteral obstruction model may not apply to renal manifestations of systemic autoimmunity, *e.g.*, lupus nephritis. In fact, lack of CCR1 has been reported to modulate the course of nephrotoxic serum nephritis in mice in association with a Th1-like immune response (9). We therefore studied the effects of therapeutic CCR1 blockade in progressive renal injury of lupus-like nephritis in $MRL^{lpr/lpr}$ mice, an autoimmune disease that leads to progressive immune complex glomerulonephritis with tubulointerstitial disease resulting in end-stage renal failure that resembles human lupus nephritis. We recently characterized the expression of chemokines and

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chemokine receptors during the course of this model and found that, among other chemokine receptors, CCR1 and its chemokine ligand CCL3 are expressed in kidneys of $MRL^{lpr/lpr}$ mice (10). We hypothesized that the CCR1 antagonist BX471 might improve renal outcome during the progressive phase of disease by inhibiting renal leukocyte recruitment. This hypothesis proved to be correct for the interstitial compartment but not for the glomerular leukocyte recruitment.

Materials and Methods

Animals and Experimental Protocol

Ten-week-old female $MRL^{lpr/lpr}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in groups of five mice in filter-top cages with a 12-h dark/light cycle and unlimited access to food and water. Cages, bedding, nestlets, food, and water were sterilized by autoclaving before use. All experimental procedures were performed according to the German animal care and ethics legislation and were approved by the local government authorities. At week 20 of age, mice were distributed into two groups ($n = 8$–10) that received subcutaneous injections three times a week until week 24 as follows: vehicle group, 50 μl of 40% cyclodextrin (#33260-7; Sigma-Aldrich, Deisenhofen, Germany) prepared as described previously (8); and BX471 group, 50 mg/kg BX471 in 50 μl of vehicle. BX471 is a nonpetide antagonists that is 10,000-fold more specific for CCR1 than for 32 other G protein–coupled receptors including the chemokine receptors CXCR3, CCR2, and CCR5 (8,10,11) (R. Horuk, personal communication). All mice were killed by cervical dislocation at the end of week 24 of age.

Evaluation of Glomerulonephritis

Blood samples were collected from each animal at the end of the study by bleeding from the retro-orbital venous plexus under general anesthesia with inhaled ether. After centrifugation, all serum samples were stored at −80°C until analysis. Spot urine samples were collected from each animal at the end of the study for determination of proteinuria. The following parameters were determined using standard analytical protocols as described previously (10): Bradford assay for urine protein concentration, urease/glutamate dehydrogenase method for blood urea nitrogen (BUN) measurements (Merck Diagnostika), IgG ELISA for analysis of DNA autoantibodies using the following antibodies for detection: IgG_1 (Pharmingen, Hamburg, Germany; 1:100) and IgG_{2a} (Dianova, Hamburg, Germany; 1:100). The left kidney from each mouse was fixed in 4% buffered formalin, processed, and embedded in paraffin. Sections for Silver and hematoxylin-eosin stains were prepared as described (8,12). The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis (13) and previously used for this murine model (10). All morphologic evaluations were performed by a renal pathologist who was unaware of the source of the tissue.

Immunohistology

Paraffin-embedded sections were prepared as described (12). As primary antibodies, a rat anti–ERHR-3 (1:50, monocytes/macrophages; DPC Biermann, Bad Nauheim, Germany), a rat anti-CD3 (1:100, T lymphocytes, clone CD3-12; Serotec, Raleigh, NC), a mouse anti–smooth muscle actin (SMA; 1:100, myofibroblasts, clone 1A4; Dako, Carpinteria, CA), an anti–collagen I (LF-67, 1:50; provided by Dr. L.W. Fischer, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD), an anti-CCL5 (1:50; Peprotech, Rocky Hill, NJ), a rabbit anti-CCL2 (1:20, rabbit antiserum, prepared as described (12)), anti–Ki-67 (1:25, cell proliferation; Dianova), anti-ssDNA (1:50, apoptotic cells; Chemicon, Hofheim, Germany) were applied. Staining for immunoglobulins was performed on acetone-fixed frozen section using anti-IgG_1 (rabbit, 1:50; Dianova) and anti-IgG_{2a} (rabbit, 1:100; Dianova) as detection antibodies. For quantitative analysis, glomerular cells were counted in 10 cortical glomeruli per section and interstitial cells in 10 high-power fields per section selected by uniform random sampling, from each animal. For the assessment of glomerular Ig and complement deposits, 15 cortical glomeruli were analyzed from each section. Glomerular signals were scored using a semiquantitative index as follows: 0 = no signal, 1 = low signal, 2 = moderate signal, and 3 = strong signal intensity. For the quantification of interstitial collagen I immunostaining, digital pictures of 10 random high-power fields were taken using a digital camera (DC 300F; Leica Microsystems, Cambridge, UK). The area of positive staining for collagen I was measured and expressed as percentage using image analysis software (Leica Imaging Solutions, Cambridge, UK).

In Situ Hybridization

In situ hybridization for murine TGF-β1 was performed as described previously (14). The TGF-β1 probe was a gift from H.L. Moses (Department of Cell Biology, Vanderbilt University, Nashville, TN). Negative controls included hybridization performed on replicate tissue sections using the sense riboprobe.

RNA Preparation and RNase Protection Assay

Renal tissue from each mouse was snap-frozen in liquid nitrogen and stored at −80°C. From each animal, total renal RNA was prepared as described (12). Multiprobe template sets (mouse CC chemokines; Pharmingen, San Diego, CA) and 20 μg of total kidney RNA were used to perform RNase protection assays as described (12). Efficacy of RNase digestion was ensured by a yeast t-RNA sample in each assay. Gels were dried and exposed on phosphor screens of a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Bands were quantified using the ImageQuant software (Molecular Dynamics).

Real-Time Quantitative (TaqMan) Reverse Transcription–PCR

Reverse transcription from total renal RNA was performed as described (12). Real time reverse transcription–PCR (RT-PCR) was performed on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) using a heat-activated *Taq*DNA polymerase (Amplitaq Gold; PE Biosystems) as described previously (11). Controls that were composed of ddH_2O were negative for target and housekeeper genes. Primers and probes were from PE Biosystems. Oligonucleotide primer (300 nM) and probes (100 nM) were used as described: murine GAPDH (8); murine collagen I (8); murine CCR1: forward 5'-TTAGCTTCCATGCCTGCCTTATA-3', reverse 5'-TCCACTGCTTCAGGCTCTTGT-3'; internal fluorescence labeled probe (FAM): 5'-ACTCACCGTACCTGTA-GCCCTCAT-TTCCC-3'; murine CCR2: forward 5'-CCTTGG-GAATGAGTAACTGTGTGA-3', reverse 5'-ACAAAGGCATAAATGACAGGATTAATG-3'; FAM: 5'-TGACAAGCACTTAGAC-CAGGCCATGCA-3'; CCR5: forward 5'-CAAGACAATCCTGATCGTGCAA-3', reverse 5'-TCCTACTC-CCAAGCTGCATAGAA-3'; FAM: 5'-TCTATACCCGATCCACAG-GAG-AACATGAAGTTT-3'; murine TGF-β1: forward 5'-CACAGT-ACAGCAAGGTCCTTGC-3', reverse 5'-AGTAGACGAT-GGGCA-GTGGCT-3'; fluorescence labeled probe (FAM): 5'-GCTTCGGCGT-

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CACCGTGCT-3'; murine GAPDH: forward 5'-CATGGCCTTCCGTGTTCCTA-3', reverse 5'-ATGCCTGCTTCACCACCTTCT-3'; internal fluorescence labeled probe (VIC): 5'-CCCAATGTGTCCGTCGTGGATCTGA-3'.

CCR1 Expression of Leukocytes and Intrinsic Renal Cells

For assessing CCR1 mRNA expression in macrophages, T cells or intrinsic renal cells were prepared from MRL$^{lpr/lpr}$ mice as follows: Macrophages and T cells were isolated from spleens by immunomagnetic selection as described (15). Tubular segments were microdissected from RNase inhibitor–treated tissue in ice-cold PBS, as described previously for human renal biopsies (16). For isolation of primary renal fibroblasts, small pieces of renal tissue were incubated in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Invitrogen), penicillin, and streptomycin for 16 d. Adherent cells were obtained by treatment with 1.5 mM EDTA (Calbiochem-Novabiochem, San Diego, CA) and were depleted of leukocytes by immunomagnetic selection using FITC anti-mCD45 (Pharmingen) and anti-FITC MicroBeads as described (Miltenyi Biotec, Bergisch Gladbach, Germany). mRNA of isolated cells was prepared by standard methods (8). Baseline CCR1 mRNA expression was determined by real-time RT-PCR as above.

In Vivo Assay of Renal T Cell Infiltration

ERHR-3–positive macrophages and CD8 T cells were prepared from spleens of MRL$^{lpr/lpr}$ mice by a previously described isolation and labeling method (15). In brief, spleen cells were isolated by immunomagnetic selection using anti-CD8 (Ly-2) and anti–ERHR-3 MicroBeads (Miltenyi Biotec). Purity of isolated cells was verified by flow cytometry. Separated cells were labeled with PKH26 (Red Fluorescence Cell Linker Kit; Sigma-Aldrich Chemicals, Steinheim, Germany), and labeling efficacy was assessed by flow cytometry. Viability as assessed by trypan blue exclusion was >90%. Twenty-week-old MRL$^{lpr/lpr}$ mice received an injection of either 3.5×10^5 CD8-positive T cells or ERHR-3–positive macrophages in 200 μl of isotonic saline through tail vein. Two groups of mice received an injection of either labeled T cells or macrophages that were preincubated with 600 μM of the CCR1 antagonist BX471 for 30 min. Mice in these groups received a single subcutaneous injection of BX471 (50 mg/kg). Renal tissue was obtained after 3 h, snap-frozen, and prepared for microscopy.

Statistical Analyses

Data were expressed as mean ± SEM. Comparison of groups was performed using unpaired *t* test. $P < 0.05$ was considered to indicate statistical significance.

Results

Renal Disease of Autoimmune MRL$^{lpr/lpr}$ Mice at 24 Weeks of Age

Renal Function. At 24 wk of age, MRL$^{lpr/lpr}$ mice had impaired renal function with serum BUN levels elevated to 73 mg/dl (Table 1). As a marker of glomerular damage, marked proteinuria was present (Table 1).

Glomerular Injury. At 24 wk, kidneys of vehicle-treated MRL$^{lpr/lpr}$ mice revealed diffuse mesangioproliferative glomerulonephritis with crescents and marked proteinuria (Figure 1). Glomeruli showed few ERHR-3–positive macrophages. A mixed periglomerular inflammatory cell infiltrate that consisted of ERHR-3–positive macrophages and CD3-positive lymphocytes was present around glomerular crescents (Figure 1).

Interstitial Injury. Kidneys showed diffuse tubulointerstitial disease with tubular atrophy, inflammatory cell infiltrates, and confluent areas of interstitial fibrosis (Figure 1).

Renal Expression of CCR1 in MRL$^{lpr/lpr}$ Mice

As appropriate antibodies that allow detection of CCR1 protein by cell fluorescence or immunostaining in mice were not available, we used real-time RT-PCR to determine the expression of CCR1 mRNA in kidneys of MRL$^{lpr/lpr}$ mice. Kidneys of 24-wk-old MRL$^{lpr/lpr}$ mice showed a marked induction of CCR1 mRNA compared with MRL wild-type mice of the same age (Figure 2), a finding that is consistent with our previously reported analysis using RNase protection assays from kidneys of MRL$^{lpr/lpr}$ mice (10). For determining the source of renal CCR1 expression, tubular segments were microdissected manually from kidneys of the same MRL$^{lpr/lpr}$ mice and primary renal fibroblasts were isolated from kidneys of MRL$^{lpr/lpr}$ mice as described in Materials and Methods. Furthermore, we isolated macrophages and T cells from spleens of MRL$^{lpr/lpr}$ mice by magnetic bead isolation. Real-time RT-PCR for CCR1 was performed with RNA isolates from all types of cells prepared. Both macrophages and CD8 T cells expressed CCR1 mRNA, but CCR1 mRNA transcripts were not detected in tubular epithelial cells or renal fibroblasts (Figure 2B). These data indicate that renal CCR1 expression does not originate from renal tubular cells or interstitial fibroblasts; in contrast, CCR1 positive macrophages and T cells may contribute to renal CCR1 expression after infiltrating the kidney.

CCR1 Blockade with BX471 Does Not Affect the Humoral Immune Response in MRL$^{lpr/lpr}$ Mice

Lack of CCR1 has been reported to modulate the course of nephrotoxic serum nephritis in mice associated with a Th1-shift of the immune response (9). We therefore examined parameters of the Th1/Th2 balance of systemic autoimmunity in MRL$^{lpr/lpr}$ mice. First, we studied serum titers of DNA autoantibodies of the IgG_1 and IgG_{2a} isotype, because an increase of IgG_{2a} autoantibodies would indicate a shift toward a Th1 response (16). No difference in serum titers for DNA autoantibodies of either IgG isotypes was found between BX471- and vehicle-treated MRL$^{lpr/lpr}$ mice (Table 1). Furthermore, the amount of mesangial IgG immune complex deposits evaluated by semiquantitative scoring of renal sections showed a comparable extent of mesangial IgG_1 and IgG_{2a} deposits in BX471- and vehicle-treated mice (Table 1). Taken together, these findings argue against a shift of the Th1/Th2 balance by CCR1 blockade with BX471.

CCR1 Blockade with BX471 Reduces Renal Damage in MRL$^{lpr/lpr}$ Mice

Renal Function. Daily treatment with BX471 from 20 to 24 weeks of age significantly improved renal function as illustrated by a reduction of serum BUN levels compared to

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Table 1. Serum, urinary, and histologic findings in MLR$^{lpr/lpr}$ mice[a]

	Vehicle (n = 10)	BX471 (n = 8)
Functional parameters		
BUN (mg/dl)	73.1 ± 39.6	35.1 ± 5.3[b]
proteinuria (μg/mg creatinine)	2179 ± 1459	1214 ± 1047
body weight (g)	37.0 ± 2.75	36.6 ± 3.4
Histologic scores		
activity index	8.0 ± 4.6	4.0 ± 1.9
chronicity index	2.9 ± 3.6	0.1 ± 0.1[b]
Cellular response (cells/glomerulus or hpf)		
glomerular		
EHR3+	1.2 ± 1.2	1.1 ± 0.3
CD3+	0.3 ± 0.1	0.4 ± 0.1
Ki-67+	5.8 ± 1.4	5.8 ± 1.1
interstitial		
EHR3+	15.3 ± 11.8	1.9 ± 0.4[b]
CD3+	26.3 ± 10.8	12.9 ± 3.8[b]
Ki-67+	7.2 ± 1.6	2.6 ± 0.9[b]
ssDNA+	1.5 ± 0.8	0.4 ± 0.2[b]
tubular		
Ki-67+	6.5 ± 1.0	3.3 ± 1.3[b]
ssDNA+	1.0 ± 0.6	0.3 ± 0.3[b]
Humoral response		
serum titers		
Anti-DNA IgG_1	6963 ± 4751	6162 ± 3611
Anti-DNA IgG_{2a}	5325 ± 2621	6349 ± 4014
IgG_{2a}/IgG_1 ratio	0.7 ± 0.6	1.0 ± 1.1
glomerular deposit score		
IgG_1	1.3 ± 0.4	1.2 ± 0.5
IgG_{2a}	0.8 ± 0.3	0.9 ± 0.4
IgG_{2a}/IgG_1 ratio	0.6 ± 0.7	0.7 ± 0.7

[a] BUN, blood urea nitrogen. Values are means ± SEM.
[b] $P < 0.05$ BX471 *versus* vehicle.

vehicle-treated MRL$^{lpr/lpr}$ mice (Table 1). In contrast, BX471-treatment did not affect proteinuria and body weight compared to vehicle-treated MRL$^{lpr/lpr}$ mice (Table 1).

Glomerular Injury. Daily treatment with BX471 did not significantly affect the extent of mesangioproliferative glomerulonephritis, the number of ERHR-3–positive glomerular macrophages, and the number of Ki-67–positive proliferating glomerular cells compared with vehicle-treated controls (Table 1). Apoptotic cells were rarely detected in glomeruli of both groups by immunostaining with an anti-ssDNA antibody (not shown). No significant difference in the activity index was found between the two groups. This index includes mostly histopathologic abnormalities of the glomerular compartment in lupus nephritis, such as proliferation of the mesangium, glomerular leukocyte infiltration, mesangial matrix, focal glomerular necrosis, and cellular crescents (13).

Interstitial Injury. In contrast to the lack of effect on glomerular inflammation and proteinuria and consistent with improved serum BUN levels, BX471 markedly reduced the extent of tubulointerstitial disease compared with vehicle-treated control mice. In BX471-treated mice, no tubular atrophy or confluent areas of interstitial fibrosis were observed. Furthermore, there was a marked reduction in periglomerular and interstitial accumulation of ERHR-3–positive macrophages and CD3 lymphocytes compared with vehicle-treated control mice (Figure 1). Immunostaining for KI-67–positive proliferating cells and ssDNA-positive apoptotic cells was performed as markers of cell turnover in the renal tubulointerstitium. BX471-treated mice showed a marked reduction of KI-67– and ssDNA-positive tubular cells as well as interstitial cells compared with vehicle-treated mice (Table 1). Immunostaining for SMA-positive myofibroblasts and interstitial collagen I deposits was performed as additional markers of interstitial fibrosis. BX471-treated mice showed a marked reduction of SMA-positive cells and collagen I deposits in the interstitium compared with those in vehicle-treated mice (Figure 3A). The latter finding was confirmed by real-time RT-PCR for renal collagen I mRNA expression, which showed a 10-fold reduction by treatment with BX471 (Figure 3B). Quantitative analysis of interstitial immunostaining for collagen I by auto-

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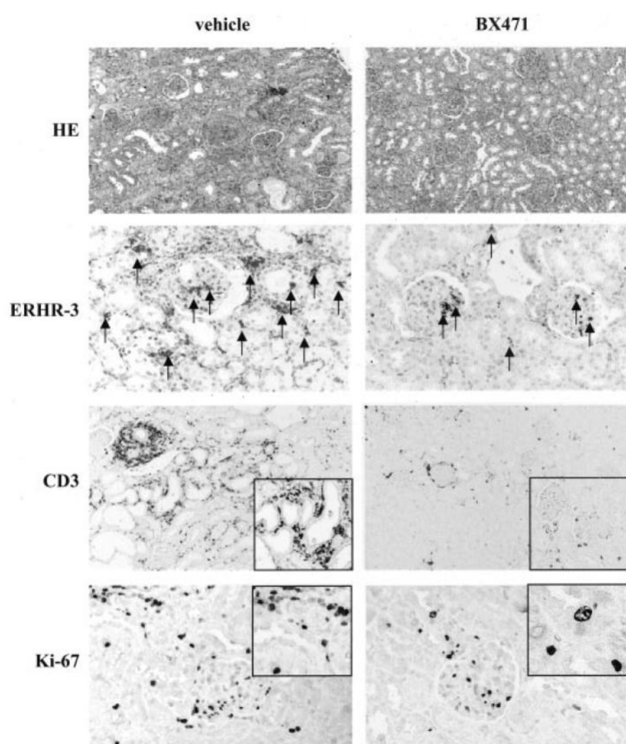

Figure 1. Renal histopathology in MRL$^{lpr/lpr}$ mice. At 24 wk of age vehicle-treated MRL$^{lpr/lpr}$ mice had proliferative glomerulonephritis (GN) and diffuse interstitial fibrosis and tubular atrophy. BX471-treated MRL$^{lpr/lpr}$ mice also had proliferative GN but no major tubulointerstitial abnormalities. HE, hematoxylin eosin staining. ERHR-3–positive macrophages and Ki-67–positive proliferating cells were present in glomeruli and the interstitium of both groups. CD3-positive cells were present only in the interstitial compartment but were not detected in glomeruli of either group. BX471 markedly reduced accumulation of ERHR-3–positive macrophages, KI-67–positive cells, and CD3-positive lymphocytes in the interstitial compartment compared with vehicle-treated mice. For quantification, see Table 1. Images illustrate representative sections of kidneys from 8 to 10 mice of respective groups at 24 wk of age. Magnifications: ×40; ×100 in inserts.

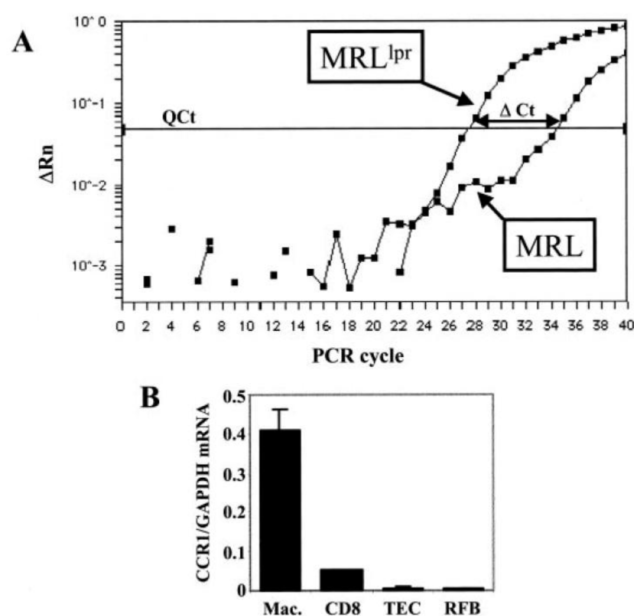

Figure 2. Renal CCR1 expression in MRL$^{lpr/lpr}$ mice. (A) Quantitative real-time reverse transcription–PCR (RT-PCR) analysis was performed on total cDNA derived from kidneys of 24-wk-old MRL$^{lpr/lpr}$ mice or MRL wild-type controls. The cDNA was amplified using primers specific for mCCR1 for 40 PCR cycles. CCR1 mRNA expression in kidneys of MRL$^{lpr/lpr}$ mice is indicated by a left shift of the amplification profile in real-time RT-PCR. The data shown are from a single mouse of each group and are representative of duplicate analysis of four mice of each group. (B) The expression of CCR1 mRNA was assessed by real-time PCR in macrophages, CD8-positive T cells, and renal cells isolated from MRL$^{lpr/lpr}$ mice as described in Materials and Methods. CCR1 mRNA levels are expressed in relation to the respective GAPDH mRNA expression. Macrophages and CD8-positive T cells expressed CCR1, in contrast to isolated renal fibroblasts and microdissected tubular segments of MRL$^{lpr/lpr}$ mice.

mated digital evaluation of the collagen I–positive area per high-power field demonstrated a 2.5-fold reduction in the BX471-treated group compared with vehicle-treated MRL$^{lpr/lpr}$ mice (Figure 3C). These findings are also illustrated by a significant reduction of the chronicity index between the two groups (Table 1). This index evaluates histopathologic abnormalities of the interstitial compartment in lupus nephritis, such as the extent of tubular atrophy and interstitial fibrosis (13).

CCR1 Blockade with BX471 Reduces Renal Expression of Chemokines and Chemokine Receptors in MRL $^{lpr/lpr}$ Mice

To study whether treatment with BX471 affects renal chemokine expression in MRL$^{lpr/lpr}$ mice, we performed RNase protection assays for CC chemokines from renal RNA isolates. At 24 wk, kidneys of vehicle-treated MRL$^{lpr/lpr}$ mice contained mRNA for various CC chemokines, such as CCL5, CCL2, CCL4, and the CCR1 ligand CCL3. In contrast, kidneys of healthy MRL wild-type mice contained no detectable mRNA levels for these chemokines (Figure 4A). Treatment with BX471 reduced renal expression of CCL2, CCL3, CCL4, and CCL5 compared with vehicle-treated MRL$^{lpr/lpr}$ mice (Figure 4, A and B). To localize further the source of renal CCL2 and CCL5 expression, we performed immunostaining for these chemokines (Figure 4B). At 24 wk, CCL2 immunostaining localized to glomerular cells and interstitial cell infiltrates but not to tubular epithelial cells. Treatment with BX471 markedly reduced immunostaining for CCL2 and CCL5 in the renal interstitium of MRL$^{lpr/lpr}$ mice (Figure 4C).

To study the effect of CCR1 blockade on renal chemokine receptor expression, we performed real-time RT-PCR for the chemokine receptors CCR1, CCR2, and CCR5. Previously, we found these receptors to be progressively upregulated in kidneys of MRL$^{lpr/lpr}$ mice during progression of renal disease (10). Treatment with BX471 from 20 to 24 weeks of age

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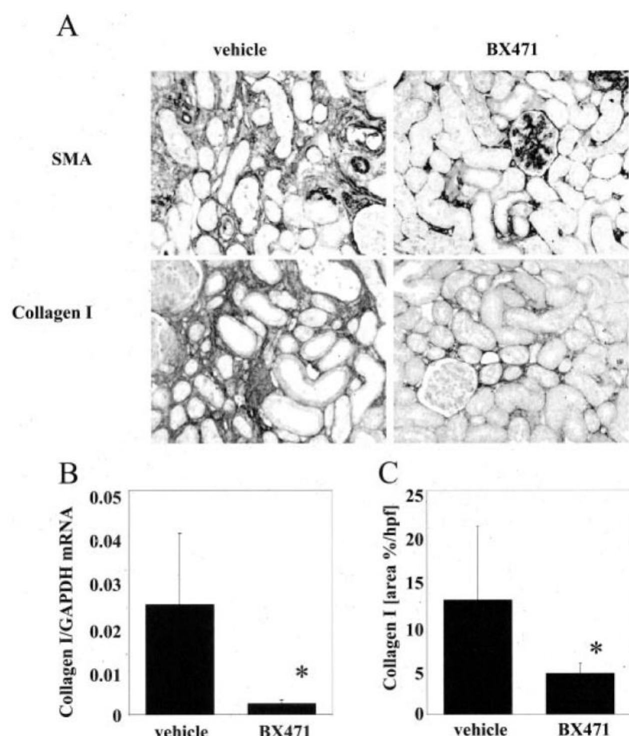

Figure 3. Renal fibrosis in MRL$^{lpr/lpr}$ mice. (A) At 24 wk of age, vehicle-treated MRL$^{lpr/lpr}$ mice had diffuse interstitial smooth muscle actin (SMA) and collagen I immunostaining in glomeruli and areas of interstitial fibrosis. In contrast, BX471-treated MRL$^{lpr/lpr}$ mice had only minimal interstitial SMA and collagen I protein expression. Images illustrate representative sections of kidneys from the respective groups at 24 wk of age. (B) The renal mRNA expression for collagen I was determined by real-time RT-PCR using total renal RNA of five to seven mice for each group. Collagen I mRNA levels for vehicle- and BX471-treated MRL$^{lpr/lpr}$ mice are expressed in relation to respective renal GAPDH mRNA; *$P < 0.02$. (C) Quantification of renal immunostaining for collagen I was performed by automated digital analysis as described in Materials and Methods. Values are expressed as collagen I–positive area per high-power field (×40) in percentage and are from seven to nine mice of each group; *$P < 0.02$. Magnification, ×40.

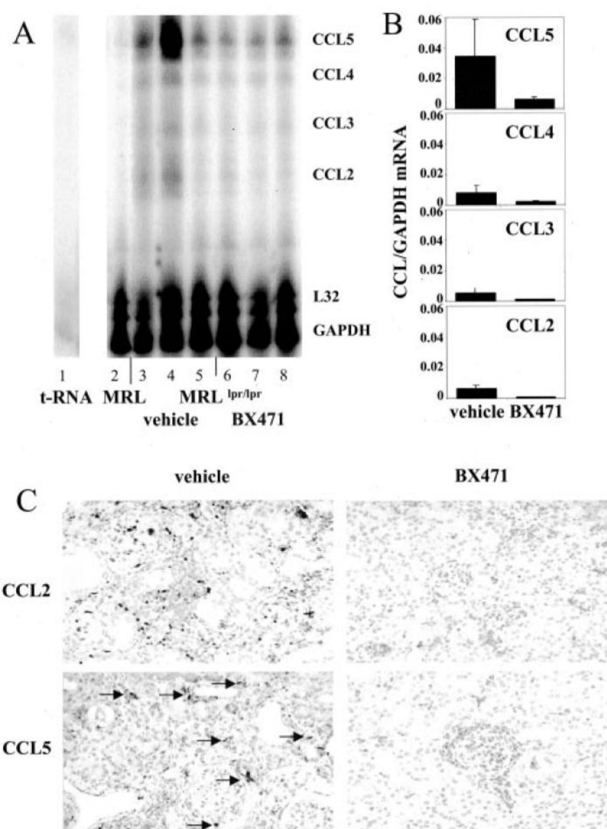

Figure 4. Renal chemokine expression in MRL$^{lpr/lpr}$ mice. (A) The renal chemokine mRNA expression was determined by RNase protection assay using total renal RNA from each group at the end of the study. The unprotected probe is shown on the left, and the protected fragments are indicated on the right. Healthy mice of the MRL strain did not reveal renal chemokine expression compared with diseased kidneys of MRL$^{lpr/lpr}$ mice. Treatment with BX471 reduced renal mRNA expression of CCL2, CCL3, CCL4, and CCL5. The illustration is representative of assays on tissue from three to four mice of each group. (B) Bands from RNase protection analysis were quantified using the ImageQuant software as described in Materials and Methods. Values are expressed as CCL per respective GAPDH mRNA expression for both groups as indicated. (C) Spatial chemokine expression in affected kidneys of MRL$^{lpr/lpr}$ mice was determined by immunostaining for CCL2 and CCL5 as described in Materials and Methods. Arrows indicate CCL5-positive cells. Images illustrate representative sections of kidneys from the respective groups at 24 wk of age. Magnification, ×40.

reduced renal expression of the chemokine receptors CCR1, CCR2, and CCR5 compared with vehicle-treated controls (Figure 5). Taken together, late onset of treatment with BX471 reduced renal expression of chemokines and chemokine receptors in kidneys of MRL$^{lpr/lpr}$ mice.

Pretreatment of Leukocytes with the CCR1 Antagonist Blocks Their Recruitment to the Kidney in MRL$^{lpr/lpr}$ Mice

CCR1 has been shown to mediate macrophage and T cell adhesion and subsequent transendothelial migration *in vitro* under conditions of shear stress and flow (4). Thus, BX471-induced reduction of interstitial leukocyte infiltration in kidneys of MRL$^{lpr/lpr}$ mice could be related to blockade of CCR1-dependent leukocyte recruitment. We therefore studied the effect of BX471 on recruitment of CCR1-positive renal macrophages and T cells in MRL$^{lpr/lpr}$ mice. ERHR-3–positive macrophages and CD8-positive T cells were isolated from spleens of MRL$^{lpr/lpr}$ mice, fluorescence labeled, and incubated with BX471 or vehicle for 1 h before intravenous injection into 20-wk-old MRL$^{lpr/lpr}$ mice. Three hours later, the number of labeled cells was determined by fluorescence microscopy of frozen sections of renal tissue. All labeled macrophages and T cells that were detected in kidneys of MRL$^{lpr/lpr}$ mice localized to the interstitial area, whereas glomeruli and perivascular

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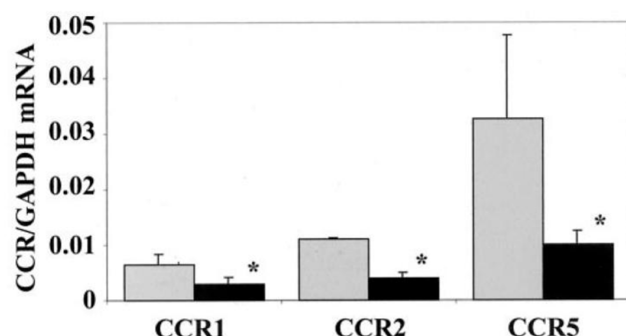

Figure 5. Renal chemokine receptor mRNA expression in kidneys of MRL$^{lpr/lpr}$ mice. (A) The chemokine mRNA expression was determined by real-time RT-PCR using total renal RNA of five to seven mice from each group. CCR mRNA levels for vehicle- (■) and BX471-treated (■) MRL$^{lpr/lpr}$ mice are expressed in relation to respective GAPDH mRNA expression of each kidney. *$P < 0.05$.

fields were negative for these cell types. Pretreatment with BX471 significantly reduced the amount of the injected labeled ERHR-3 macrophages and CD8 T cells that infiltrated into the interstitium of kidneys of MRL$^{lpr/lpr}$ mice (Figure 6). These data indicate that BX471 blocks CCR1-dependent macrophage and T cell recruitment into the interstitium of kidneys of MRL$^{lpr/lpr}$ mice.

Reduced Interstitial Leukocyte Infiltration in BX471-Treated MRL$^{lpr/lpr}$ Mice Is Associated with a Decrease of Renal TGF-β1 mRNA Expression

We were intrigued by the finding that the extent of renal fibrosis in BX471- and vehicle-treated MRL$^{lpr/lpr}$ mice directly paralleled the amount of interstitial leukocytes. As renal fibroblasts were negative for CCR1, the blockade of CCR1 could not directly affect fibroblast activation. In contrast, the observed reduction of renal fibrosis could be secondary to reduced secretion of profibrotic cytokines, *e.g.*, TGF-β. We therefore determined the expression of TGF-β mRNA in total renal RNA by real-time RT-PCR. Kidneys from BX471-treated mice had an 85% reduction of TGF-β mRNA expression compared with vehicle-treated MRL$^{lpr/lpr}$ mice (Figure 7A). These data show that at a late stage of nephritis in MRL$^{lpr/lpr}$ mice, BX471 reduces renal leukocyte infiltration and TGF-β mRNA expression, a cytokine that has been implicated in renal fibrosis. To determine the source of renal TGF-β, we performed *in situ* hybridization for TGF-β. In sense control kidneys of vehicle-treated MRL$^{lpr/lpr}$ mice, the *in situ* hybridization yielded only a weak diffuse deposition of silver grains (not shown). The strongest signal for TGF-β mRNA was found in areas of tubulointerstitial infiltrates (Figure 7B). The resolution of the *in situ* hybridization was not sufficient to assign the signal in the infiltrate to specific cells. In areas without prominent cell infiltration, only background signal was present, similar to incubation with sense controls. No clear tubular expression of TGF-β mRNA was apparent. These results suggest that the source of TGF-β is in the interstitial infiltrate and not the tubular cells. Furthermore, the reduction of interstitial leukocyte infiltration observed in BX471-treated MRL$^{lpr/lpr}$ mice is associated with a decrease in levels of TGF-β mRNA and protein, a cytokine that can stimulate epithelial-mesenchymal transformation, apoptosis, and collagen secretion by renal fibroblasts.

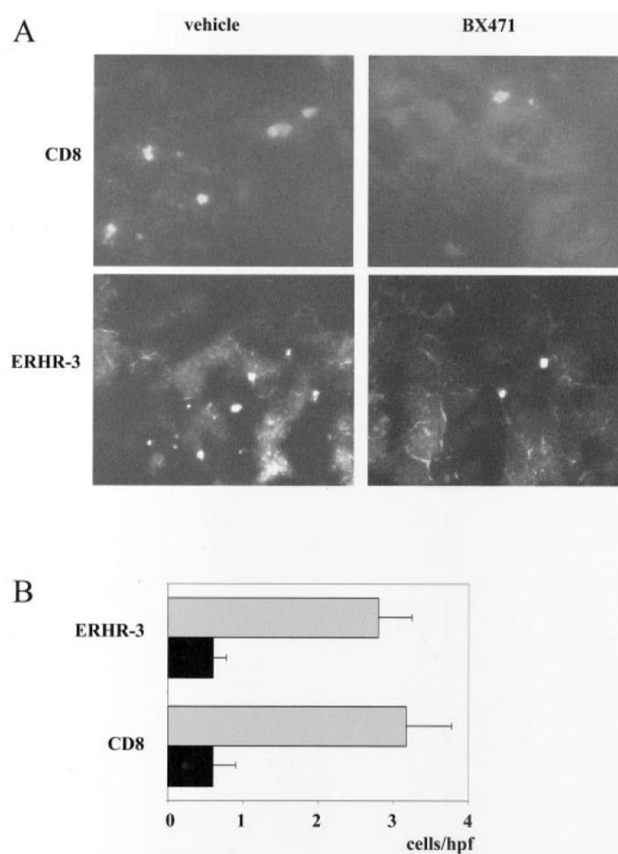

Figure 6. Renal infiltration of labeled leukocytes in kidneys of MRL$^{lpr/lpr}$ mice. (A) MRL$^{lpr/lpr}$ mice 20 wk of age received an intravenous injection of PKH26-labeled ERHR-3 macrophages or CD8 T cells. The cells were isolated from MRL$^{lpr/lpr}$ mice, labeled, and pretreated with either vehicle or BX471 as indicated. Recipient mice received subcutaneous injections of either vehicle or BX471 before injection of the respective cells and kidneys were obtained 3 h after injection of cells and examined by fluorescence microscopy. Single fluorescence-labeled cells locate to the renal interstitium. (B) Cell counts for interstitial fluorescence-labeled ERHR-3 macrophages and CD8 T cells were determined by fluorescence microscopy from 10 high-power fields and are expressed as means ± SEM. ■, vehicle-treated cells; ■, BX471-treated cells; *$P < 0.05$. Magnification, ×400.

Discussion

We hypothesized that late onset of CCR1 blockade with BX471 would improve renal disease in MRL$^{lpr/lpr}$ mice by inhibiting renal leukocyte recruitment. This hypothesis proved to be correct as demonstrated by histologic evaluation of leukocyte infiltrates of the interstitial compartment and further illustrated by transfer studies with labeled macrophages and T

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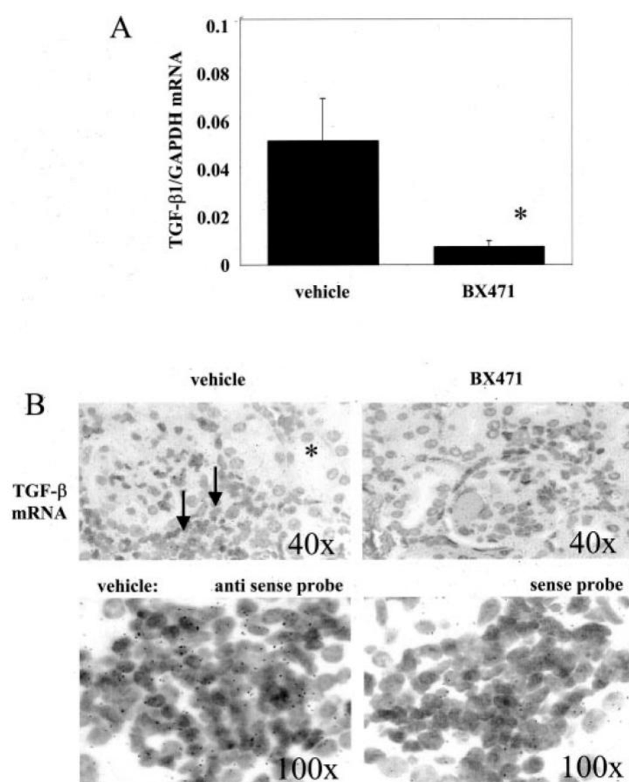

Figure 7. Renal TGF-β1 mRNA expression in MRL$^{lpr/lpr}$ mice. (A) The renal TGF-β1 mRNA expression was determined by real-time RT-PCR using total renal RNA of five to seven mice for each group of MRL$^{lpr/lpr}$ mice at the end of the study. TGF-β1 mRNA levels for vehicle- and BX471-treated MRL$^{lpr/lpr}$ mice are expressed in relation to respective GAPDH mRNA expression; $*P < 0.001$. (B) *In situ* hybridization for TGF-β mRNA in kidneys of vehicle-treated MRL$^{lpr/lpr}$ mice revealed TGF-β signals in interstitial cell infiltrates as compared with background signals in renal tubular cells or sections hybridized with sense probes, as indicated at higher magnification (×100). In kidneys from BX471-treated MRL$^{lpr/lpr}$ mice, signals for TGF-β in interstitial infiltrates were hardly detectable.

cells. The BX471-induced reduction of interstitial leukocyte infiltration markedly diminished tubulointerstitial fibrosis and improved renal function despite no change in systemic autoimmunity, proteinuria, and glomerular pathology. The last may relate to the unexpected finding that glomerular leukocyte recruitment was not affected by CCR1 blockade, indicating a different role for CCR1 in the recruitment of leukocytes into the interstitial and glomerular compartments of the kidney. This is the first evidence that blockade of CCR1 can halt disease progression and improve renal function by selective inhibition of interstitial leukocyte recruitment late in the course of chronic renal failure.

BX471 Reduces Renal Damage in MRL$^{lpr/lpr}$ Mice by Blocking CCR1-Mediated Leukocyte Recruitment to the Renal Interstitium

Leukocyte migration to sites of tissue injury involves concerted interaction of adhesion molecules and chemokines and their receptors. Our data demonstrate that CCR1 is involved in interstitial macrophage and T cell infiltration in MRL$^{lpr/lpr}$ mice. The evidence comes from the results of the histologic evaluation and from the transfer experiments using a technique of injecting labeled macrophages and T cells *in vivo*. These studies confirm that the reduction of renal leukocytes and chemokine receptor expression in BX471-treated MRL$^{lpr/lpr}$ mice relates to impaired leukocyte recruitment into the kidney. As we have recently shown that CCR1 antagonism reduces the amount of interstitial leukocytes in the mouse kidney after unilateral ureteral obstruction (8), these data indicate that interstitial leukocyte infiltration may be CCR1 dependent in other mouse models of renal disease as well. CCR1 blockade has also been shown to have beneficial effects on the outcome of other disease models and in other species. For example, BX471 improved survival and renal function after kidney transplantation in rabbits (7), delayed heart transplant rejection in rats (6), and improved functional performance of rats with experimental encephalomyelitis (18). Our data show that renal CCR1 is expressed only on the infiltrating leukocytes, and *in vitro* studies confirmed the role of CCR1 for leukocyte adhesion and transmigration through activated endothelium (4). Furthermore, these data suggest that proteinuria-induced activation of proximal tubular cells may not be sufficient to maintain progression of tubulointerstitial injury in the absence of interstitial inflammatory cell infiltrates (19). Apparently, additional signals from the infiltrating leukocytes such as proinflammatory and profibrotic cytokines are required for progression of interstitial injury. This hypothesis is supported by numerous experimental and human biopsy studies indicating roles for T cells and macrophages in local cytokine and chemokine expression and in progressive tubulointerstitial injury (20–22). Our study shows that even in chronic renal injury, preventing tubulointerstitial leukocyte infiltrate improves the disease despite unaltered glomerular damage and proteinuria. Although of interest, from this study it remains unclear whether CCR1 blockade halts or just slows disease progression in MRL$^{lpr/lpr}$ mice. However, these data indicate that CCR1-mediated leukocyte recruitment is important for interstitial inflammation in the kidney as well as in other model systems and that therapeutic blockade of CCR1 with a small molecule antagonist late in the course of immune complex glomerulonephritis can have beneficial effects on disease progression.

BX471 Reduces Renal Fibrosis in MRL$^{lpr/lpr}$ Mice

How could the reduction in mononuclear leukocyte infiltration in BX471-treated MRL$^{lpr/lpr}$ mice relate to the concomitant reduction in interstitial fibroblasts and fibrosis? The infiltrating leukocytes, via secretion of cytokines such as TGF-β, EGF, PDGF, or fibroblast growth factor, could contribute to epithelial-mesenchymal transformation, fibroblast proliferation, and collagen production (2). In fact, BX471-treated MRL$^{lpr/lpr}$ mice showed a marked reduction of renal TGF-β mRNA expression, a key cytokine for the induction of fibroblast proliferation and the development of renal fibrosis (23). To localize the site of TGF-β production, we performed *in situ* hybridization for TGF-β and localized the TGF-β production to the interstitial

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cell infiltrate. Similar to the real-time RT-PCR data, *in situ* hybridization signals for TGF-β were reduced in kidneys of BX471-treated MRL$^{lpr/lpr}$ mice. The resolution of the latter method did not allow assignment of the signal to specific cells in the infiltrate, but the reduction of renal TGF-β mRNA expression with BX471 treatment corresponds to the reduction of interstitial macrophages. It therefore seems reasonable to assign the TGF-β signals to inflammatory cells, *i.e.*, the infiltrating mononuclear leukocytes, although low-level TGF-β mRNA expression may be beyond the sensitivity of the *in situ* hybridization method as the reported data about tubular TGF-β expression in mice are conflicting (24,25). TGF-β can also mediate a suppressive effect on systemic immune responses (26). In our study, reduced renal TGF-β mRNA levels were not associated with a change of systemic autoimmunity in MRL$^{lpr/lpr}$ mice. It therefore seems that the reduction in TGF-β mRNA in BX471-treated MRL$^{lpr/lpr}$ mice relates directly to the reduced renal leukocyte recruitment observed under these conditions.

BX471 Does Not Affect DNA Autoantibodies, Glomerular Macrophage Recruitment, and Proteinuria in MRL$^{lpr/lpr}$ Mice

The unchanged serum anti-DNA IgG isotype titers or glomerular IgG isotype deposits in BX471-treated mice compared with vehicle-treated controls indicate that BX471 does not induce a major shift in the Th1/Th2 balance of the systemic immune response in our lupus model. Such a shift toward the Th1 response was observed by Topham *et al.* (9) when nephrotoxic serum glomerulonephritis was induced in CCR1-deficient mice. This discrepancy may relate to different pathomechanisms of the nephrotoxic serum nephritis *versus* the MRL$^{lpr/lpr}$ lupus model. In nephrotoxic serum nephritis, a specific immune response against the planted glomerular antigen is required, whereas the MRL$^{lpr/lpr}$ lupus mouse model shows a broad polyclonal unregulated antibody production. Given this difference in pathoimmunology of the two models, the discrepancy seems less surprising, especially as in our model, the CCR1 blockade occurs after the establishment of the immune complex disease rather then during the generation of the immune response. It is interesting that we found that BX471 does not affect glomerular macrophage or T cell recruitment compared with vehicle-treated control mice, despite reduced interstitial macrophage counts with BX471. Surprising is that after injection, labeled and vehicle-treated macrophages did not localize to glomeruli, although there are clearly macrophages in glomeruli of lupus mice. Whether this observation relates to a different temporal interaction of circulating macrophages with glomerular endothelium remains unclear. Nevertheless, these data indicate that CCR1 is involved in interstitial but not in glomerular leukocyte recruitment. These data add to the growing body of evidence indicating marked differences in the mechanisms of leukocyte recruitment to the glomerular and tubulointerstitial compartment. For example, Tesch *et al.* (27) reported that CCL2/MCP-1–deficient mice were protected from tubulointerstitial but not from glomerular injury after induction of nephrotoxic serum nephritis. Furthermore, we recently showed in a model of Apoferritin-induced immune complex glomerulonephritis in mice that Met-RANTES, a presumed CCR5 antagonist, reduced glomerular macrophage infiltration by ~50% (28). Conversely, Met-RANTES or CCR5-deficient mice showed no impairment of interstitial macrophage recruitment after unilateral ureteral ligation in mice (11). Together, these data support different roles for CCR1 and CCR5 in renal leukocyte recruitment with CCR5 involved in the glomerulus, while CCR1 is involved in the interstitial compartment. This phenomenon may relate to different adhesion molecules present on these different vascular beds, which modulate chemokine-mediated leukocyte adhesion and transmigration differentially (29,30). Further evidence for this hypothesis comes from the observation that CD3–positive lymphocytes are rarely found in the glomerulus in murine or human glomerulopathies, suggesting that glomerular endothelia may not support lymphocyte recruitment in this compartment, which again is demonstrated by the lack of recruitment of labeled CD8 T cells after intravenous injection in the present study. Together, CCR1 blockade with BX471 did not affect serum anti-DNA IgG isotype titers, glomerular immune complex deposition, glomerular macrophage recruitment, and proteinuria in MRL$^{lpr/lpr}$ mice. These data indicate that CCR1 blockade does not interfere with the humoral immune response in systemic autoimmunity of MRL$^{lpr/lpr}$ mice and that the recruitment and activation state of glomerular macrophages is not mediated by CCR1.

In summary, CCR1 mediates interstitial but not glomerular recruitment of mononuclear cells in the mouse kidney. When given late in the course of progressive renal injury in MRL$^{lpr/lpr}$ mice, BX471 improved renal function and diminished interstitial injury but did not affect glomerular damage, proteinuria, and systemic autoimmunity. These data signify the importance of interstitial injury for progressive renal dysfunction and provide the first evidence that blockade of CCR1—late in the course of chronic renal failure—can halt disease progression and improve renal function by selective inhibition of interstitial macrophage and T cell recruitment. Therefore, we propose that CCR1 blockade, currently evaluated for the treatment of multiple sclerosis in Phase II trials (31), may offer a new therapeutic strategy for lupus nephritis and perhaps for other chronic nephropathies that lead to end-stage renal failure.

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3.3 CCR1 blockade reduces interstitial inflammation and fibrosis in mice with glomerulosclerosis and nephrotic syndrome

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CCR1 blockade reduces interstitial inflammation and fibrosis in mice with glomerulosclerosis and nephrotic syndrome

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CCR1 blockade reduces interstitial inflammation and fibrosis in mice with glomerulosclerosis and nephrotic syndrome.

Background. CC chemokines mediate leukocyte infiltration into inflamed tissue. We have recently shown that blockade of the CC chemokine receptor CCR1 reduces interstitial inflammation and fibrosis in murine obstructive nephropathy. However, it is not known whether CCR 1 blockade is protective in progressive renal injury associated with severe proteinuria. We therefore studied the effect of the small-molecule CCR1 antagonist BX471 in a murine model of adriamycin-induced focal segmental glomerulosclerosis (FSGS) with nephrotic syndrome and progressive interstitial inflammation and fibrosis.

Methods. Adriamycin nephropathy with persistent proteinuria was induced in male BALB/c mice by two intravenous injections of adriamycin (13 mg/kg) at day 0 and 14. BX471 treatment was started at day 14 when proteinuria and interstitial inflammation had developed. At 6 weeks, renal histology was studied by morphometry and immunohistochemistry.

Results. At week 6, adriamycin-treated mice showed FSGS, associated with tubulointerstitial injury consisting of tubular dilation and atrophy, interstitial leukocyte infiltration, and fibrosis. The mRNA expression of CCR1 and CC chemokines, including the CCR1 ligands CCL3 (MIP-1α) and CCL5 (RANTES), was up-regulated in diseased kidneys, with a prominent interstitial expression of CCL5. Compared to vehicle-treated controls BX471 significantly reduced the amount of macrophages and T lymphocytes in interstitial lesions by 51% and 22%, respectively. Markers of renal fibrosis such as interstitial fibroblasts (48%) and interstitial volume (23%) were significantly reduced by BX471 treatment. In contrast, the extent of proteinuria and glomerular sclerosis was not affected by BX471 treatment.

Conclusion. Blockade of CCR1 substantially reduced interstitial leukocyte accumulation and the subsequent renal fibrosis in a murine model of nephrotic syndrome and FSGS. These findings support a role for CCR1 in interstitial leukocyte recruitment and suggest that CCR1 blockade might be a new therapeutic strategy in progressive nephropathies such as FSGS.

Key words: chemokines, receptor blockade, focal segmental glomerulosclerosis, progressive nephropathy, fibrosis, macrophages.

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Regardless of etiology, glomerulosclerosis and tubulointerstitial fibrosis are the final common pathways of progression seen in most chronic renal diseases [1]. Glomerulosclerosis is characterized by increased glomerular matrix accumulation and collapse of the capillary lumina. Initially, this process may affect only some glomeruli (focal), with only part of the glomerular tuft involved (segmental), leading to the characteristic histology seen in focal segmental glomerulosclerosis (FSGS) [2]. The structural alterations in the glomerulus are accompanied by sustained proteinuria, often of nephrotic range. Associated with progressive glomerulosclerosis are tubular atrophy and dilation, interstitial inflammatory infiltrates, accumulation of interstitial fibroblasts, and increased matrix deposition, which ultimately lead to interstitial fibrosis and loss of renal function [1, 2]. The interstitial recruitment of macrophages and lymphocytes as a major source of inflammatory and profibrotic mediators plays an important role in chronic interstitial inflammation and fibrosis [3, 4]. Independent of the primary glomerular lesion it is the severity of this tubulointerstitial damage and the degree of interstitial leukocyte infiltration that correlates best with the loss of renal function and the risk for progression to end-stage renal disease (ESRD) [4, 5]. Therapeutic strategies to prevent or delay loss of organ function in chronic renal disease must therefore target pathways instrumental in the fibrotic tissue remodeling.

Locally secreted chemokines trigger leukocyte adhesion to activated endothelium and subsequent infiltration into inflamed tissue [6, 7]. Human renal biopsy data and an expanding number of animal studies

suggest that the expression of chemokine receptors on the surface of leukocytes infiltrating the glomerulus or tubulointerstitium are functional in the initiation and progression of renal disease [8–10]. The CC chemokine receptor CCR1 is expressed on circulating monocytes and lymphocytes, and has been consistently found to be up-regulated in a variety of progressive renal disease models [9, 10]. We have recently shown that blockade of CCR1 with the small-molecule antagonist BX471 (5-chloro-2-(2-[(2R)-4-(4-fluorobenzyl)-2-methylpiperazin-1-yl]-2-oxoethoxy phenyl) urea hydrogen chloride salt) reduced interstitial leukocyte infiltration and renal fibrosis in murine obstructive nephropathy [11]. An important role of CCR1-positive leukocytes in mediating tissue fibrosis has also been demonstrated in a pulmonary fibrosis model in mice [12]. In contrast, genetic targeting of CCR1 increased the severity of glomerulonephritis in knockout mice given nephrotoxic serum [13]. Thus, it is not known, whether CCR1 blockade is protective in the progression phase of chronic nephropathies (e.g., established glomerular disease with severe proteinuria and secondary interstitial damage). To investigate whether therapeutic CCR1 antagonism affects the course of glomerulosclerosis and interstitial fibrosis BX471 was studied in murine adriamycin nephropathy, a model of FSGS with persistent proteinuria and interstitial inflammation [14]. In this report, we demonstrate that CCR1 and its ligands CCL3 (MIP-1α) and CCL5 (RANTES) are up-regulated in adriamycin nephropathy. Blocking CCR1 with BX471 reduced interstitial inflammation and fibrosis, but did not alter glomerular pathology and proteinuria. Our results suggest that CCR1 blockade is an effective therapeutic approach to slow progression to ESRD in chronic proteinuric nephropathies, even if the primary glomerular pathology is not attenuated.

METHODS

Animals

Male inbred BALB/c mice weighing 22 to 25 g and aged 8 weeks were obtained from Charles River (Sulzfeld, Germany) and kept in macrolone type III cages under a 12-hour light and dark cycle. Water and standard chow (Sniff, Soest, Germany) were available ad libitum. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Experimental design

To induce progressive glomerulosclerosis and tubulointerstitial inflammation in mice we adapted a previously described protocol [14]. Mice received two intravenous tail vein injections of 13 mg/kg body weight adriamycin at day 0 and 14. This induced persistent proteinuria throughout the study period of 6 weeks, associated with chronic glomerular and tubulointerstitial lesions as seen in human FSGS. Initial dose-finding experiments revealed that, in our hands, a single dose of 11 mg/kg adriamycin induced transient proteinuria only, with a peak at day 14 and complete remission between 4 and 6 weeks. With this regimen histology at 6 weeks showed minimal glomerular changes and no interstitial pathology. A single dose of 13 mg/kg adriamycin resulted in a more severe, but still transient proteinuria, with inconsistent and highly variable histologic results at week 6. Injection of 15 mg/kg adriamycin was associated with high mortality.

Eight groups of mice were studied. In a first set of experiments mice were injected with a matched volume of isotonic saline (group 1) or with 13 mg/kg adriamycin (2 mg/mL doxorubicin hydrochloride) (Pharmacia & Upjohn, Erlangen, Germany) on day 0 and 14 (group 2). To test the effect of BX471 adriamycin-treated mice were additionally dosed subcutaneously with either 50 μL of vehicle solution (group 3) or 50 mg/kg BX471 in 50 μL vehicle solution (group 4) at 8-hour intervals. Two additional control groups received isotonic saline instead of adriamycin, and were treated subcutaneously with 50 μL of vehicle solution (group 5) or 50 mg/kg BX471 (group 6) at 8-hour intervals. Treatment with vehicle or BX471 was started at day 14, when proteinuria had developed in adriamycin-injected mice, and was given throughout the study until week 6. To assess glomerular and interstitial pathology at week 2, before BX471 treatment was started, additional saline (group 7) and adriamycin-injected mice (group 8) were sacrificed for renal histology and immunohistochemistry at week 2. In each experiment five to seven mice per group were examined. A detailed evaluation of the antagonistic properties, dosing and pharmacokinetics of BX471 in mice has been published [11]. The vehicle solution (40% cyclodextrin) (Sigma-Aldrich, Deisenhofen, Germany) and BX471 were prepared as previously described [11].

Renal functional parameters

Spot urine samples were obtained from each mouse at day 0 and at 2-week intervals thereafter until week 6. Urine albumin concentrations were determined by a double-sandwich enzyme-linked immunosorbent assay (ELISA) [15]. The 96-well plates were coated overnight with 5 μg/mL of an affinity-purified goat antimouse albumin monoclonal antibody (Bethyl Laboratories, Montgomery, TX, USA) diluted in 0.05 mol/L carbonate buffer, pH 9.5. Wells were blocked with phosphate-buffered saline (PBS) containing 0.05 mL/100 mL of Tween 20 and 0.5% bovine serum albumin (BSA) (both from Sigma-Aldrich). After washing three times with

PBS/Tween serial dilutions of samples or standard (10 to 1000 ng/mL of mouse serum albumin) (Sigma-Aldrich) in blocking solution were added for 2 hours at room temperature. Plates were washed three times, and wells were incubated for 2 hours at room temperature with horseradish peroxidase–labeled goat antimouse albumin antibody (Bethyl Laboratories) diluted in blocking solution. Bound secondary antibody was detected with o-phenylenediamine (Sigma-Aldrich) as the substrate. Absorbance was read at 490 nm with a microtiter plate reader. Urinary creatinine was quantitated spectrophotometrically with a commercially available kit (Sigma-Aldrich). Albumin excretion was expressed as microgramms of urinary albumin per milligram of urinary creatinine.

Blood from retro-orbital venous plexus sampling was collected from each animal at the end of the study period (week 6). Serum values for blood urea nitrogen (BUN), total protein, albumin, and cholesterol were obtained with a Hitachi autoanalyzer (Hitachi, Tokyo, Japan).

Renal morphology and immunohistochemistry

From each mouse cranial kidney halves were used for histologic assessment. Tissue was fixed in 10% neutral-buffered formalin, dehydrated in graded alcohols and embedded in paraffin. For routine histology and morphometric analysis 4 μm coronal sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS) reagent, and silver following the instructions of the supplier (Bio-Optica, Milano, Italy). For immunohistochemistry sections were deparaffinized, rehydrated, transferred into citrate buffer, and either autoclaved or microwave treated for antigen retrival. Sections were blocked with 3% H_2O_2, avidin, and biotin (Vector Blocking Kit) (Vector Laboratories, Burlingame, CA, USA) for 20 minutes each. After washing in PBS, slides were incubated with the primary antibody for 1 hour at room temperature. Monoclonal rat antibodies were used to stain for leukocytes (antimouse CD45, 1:200) (BD Pharmingen, San Diego, CA, USA), macrophages (ER-HR3 [16], 1:100) (BMA Biomedicals, Augst, Switzerland), and T lymphocytes (a cross-reacting antihuman CD3 antibody, 1:100) (Serotec, Oxford, UK). Activated fibroblasts were stained with a polyclonal antiserum (1:1000) against recombinant murine fibroblast-specific protein 1 (FSP1) generated in a New Zealand white rabbit as described elsewhere [17]. For mRANTES/CCL5 a polyclonal rabbit antimouse antibody (PeproTech, Rocky Hill, NY) (1:50) was used. To detect murine monocyte chemoattractant protein-1 (mMCP-1)/CCL2, a custom antipeptide antiserum against mMCP-1 was raised in rabbits as previously described [18]. Signals of all primary antibodies were detected with a commercial mouse link and label kit following the instructions of the supplier (BioGenex SuperSensitive) (BioGenex, San Ramon, CA, USA). 3-amino-9-ethylcarbazole (AEC) substrate was used for signal development, and sections were counterstained with hemalaun. As negative controls for immunohistochemistry primary antibodies were omitted or replaced with species-matched control antibodies with irrelevant specificity.

Histomorphometric analysis

The degree of glomerulosclerosis was assessed by computer-aided image analysis of PAS-stained kidney sections. Under a light microscope (Leitz DMR) (Leica Microsystems, Bensheim, Germany) at ×400 magnification, 20 randomly selected glomerular cross-sections per mouse were captured with a digital camera (Leica DC300) (Leica Microsystems) connected to the microscope. Quantitative analysis of the pictures was performed by using the Adope PhotoShop version 6.0 software, which allows counting of specifically stained pixels [19]. The outline of the glomerular tuft was traced, and the number of pixels within the outline was used as a measure of total gomerular area. The area covered by PAS-positive staining in the same glomerulus was determined by counting the pixels of PAS-specific red color. The degree of glomerulosclerosis for each glomerulus was calculated as percentage of specific colored pixels per total number of glomerular pixels. Interstitial volume expansion within interstitial lesions was quantitated as recently described [11]. Briefly, an interstitial volume index was determined by superposing a grid containing 100 (10 × 10) sampling points on pictures of 20 nonoverlapping fields (×200) of silver-stained tissue per animal. The number of points overlaying interstitial space were counted and expressed as percentage of all points. Accumulation of leukocytes and fibroblasts within interstitial lesions was analyzed by counting positive cells in 20 high power fields (×400) per mouse. Analyses were performed by operators unaware of the origin of each kidney section.

RNA preparation and renal chemokine expression

The lower half of each kidney was snap frozen in liquid nitrogen and stored at −80°C. Total RNA was prepared using the method of Chomczynski and Sacci [20]. Renal chemokine expression was analyzed using a ribonuclease protection assay (RPA). A RiboQuant multiprobe template set for murine chemokines (mCK5) was obtained from BD Pharmingen. Transcription of antisense riboprobes with α^{32}P-uridine triphosphate (UTP) (3000 Ci/mmol) (NEN, Cologne, Germany), hybridization and RNase treatment was performed according to the manufacturer's instructions. RNase protected hybridization products were separated on a 6% denaturating polyacrylamide gel. The intensity of the specific bands was quantitated using a Molecular Dynamics Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA,

USA) and a standard software program (ImageQuant) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression.

For in situ hybridization single-stranded ^{35}S-labeled riboprobes for mMCP-1/CCL2 and mRANTES/CCL5 were prepared by in vitro transcription of subcloned cDNA fragments, and hybridization was performed on 4 μm sections of paraffin-embedded renal tissue as previously described [18, 21].

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) for CCR1

CCR1 mRNA expression was determined in saline- or adriamycin-treated kidneys (week 6). In addition, CCR1 expression was analyzed in isolated leukocyte populations and cultured intrinsic renal cells. Splenic macrophages and T cells from BALB/c mice were obtained with an immunomagnetic cell seperation technique. Spleens were dissociated into single cell suspensions. Macrophages were labeled with a fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse F4/80 antibody (1:75) (Serotec) and isolated after incubation with paramagnetic anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) using separation columns (Miltenyi Biotec) according to the manufacturer's instructions. CD4+ T cells were seperated using anti-CD4 MicroBeads (Miltenyi Biotec). Cell purity after separation was verified to be >95%, and viability assessed by trypan blue exclusion was >90%. For isolation of primary renal fibroblasts small pieces of renal tissue from BALB/c mice were incubated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen) and 1% penicillin-streptomycin (DMEM-C10) for 21 days. Adherent cells were lifted with 1.5 mmol/L ethylenediaminetetraacetic acid (EDTA) and were depleted for leukocytes by immunomagnetic selection using FITC antimouse CD45 (BD Pharmingen) and anti-FITC MicroBeads (Miltenyi Biotec). A tubular epithelial cell line, mouse cortical tubular (MCT cells) [22] was maintained in DMEM-C10. Cell cultures were incubated for 12 hours without supplements before stimulation or standard medium for 24 hours. Tubular epithelial cells were stimulated with 0.1 μg/mL lipopolysaccharide (LPS) for 8 hours, renal fibroblasts with 1 ng/mL transforming growth factor-β (TGF-β) for 8 hours.

Total renal RNA or RNA isolated from cell lines were treated with DNase I (Qiagen, Hilden, Germany). Two micrograms of RNA underwent random primed reverse transcription for 1 hour at 42°C using a modified Moloney murine leukemia virus reverse transcriptase (Superscript) (Life Technologies, Karlsruhe, Germany). Negative control samples were prepared by performing the latter reaction in the absence of reverse transcriptase (RT-_control). Real-time RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) using a heat-activated TaqDNA polymerase (Amplitaq Gold) (PE Biosystems) as described [18]. Controls consisting of ddH_2O were negative for target or housekeeping genes. The following oligonucleotide primers (300 nmol/L) and probes (100 nmol/L) were used: 5′-TTAGCTTCCATGC CTGCCTTATA-3′ (sense), 5′-TCCACTGCTTCAGGC TCTTGT-3′ (antisense), 5′-ACTCACCGTACCTGTAG CCCTCATTTCCC-3′ [internal fluorescence-labeled probe (FAM)] for murine CCR1; and 5′-CATGGCCT TCCGTGTTCCTA-3′ (sense), 5′-ATGCCTGCTTCAC CACC-TTCT-3′ (antisense), 5′-CCCAATGTGTCCGT CGTGGATCTGA-3′ [internal fluorescence-labeled probe (VIC)] for murine GAPDH. In some experiments 18S RNA was chosen as the internal standard, with primers and probes purchased as predeveloped TaqMan assay reagents. All primers and probes were obtained from PE Biosystems. Specificity of the CCR1 primers and probe was tested on plasmids containing sequences of CCR1, CCR2, and CCR5.

Statistical analysis

Values are expressed as mean ± SEM or SD as indicated. Statistical analysis was performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Significance of differences was determined by the appropriate two-sided *t* test for single comparisons. Analysis of variance (ANOVA) with post hoc Bonferroni's correction was used for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

Proteinuria and renal damage in adriamycin-treated mice

All mice that received two doses of 13 mg/kg adriamycin developed severe albuminuria within 2 weeks after the first dose, with a further increase in urinary albumin excretion at week 4, two weeks after the second adriamycin injection, that persisted throughout the study period until week 6 (Fig. 1A). The severe proteinuria was accompanied by hypoproteinemia, hypoalbuminemia, and an increase of serum cholesterol levels (Fig. 1B), consistent with the development of a nephrotic syndrome. There was also a tendency toward elevated values of BUN (Fig. 1B) in adriamycin-treated mice compared to saline-treated controls, but this difference did not reach statistical significance ($P = 0.077$). Histology at 6 weeks revealed the typical pathologic abnormalities seen in FSGS. In most glomeruli there was a segmental to global accumulation of hyaline matrix, with collapse of the associated glomerular capillary loops (Fig. 2A to D). Quantitative evaluation of the glomerulosclerosis demonstrated

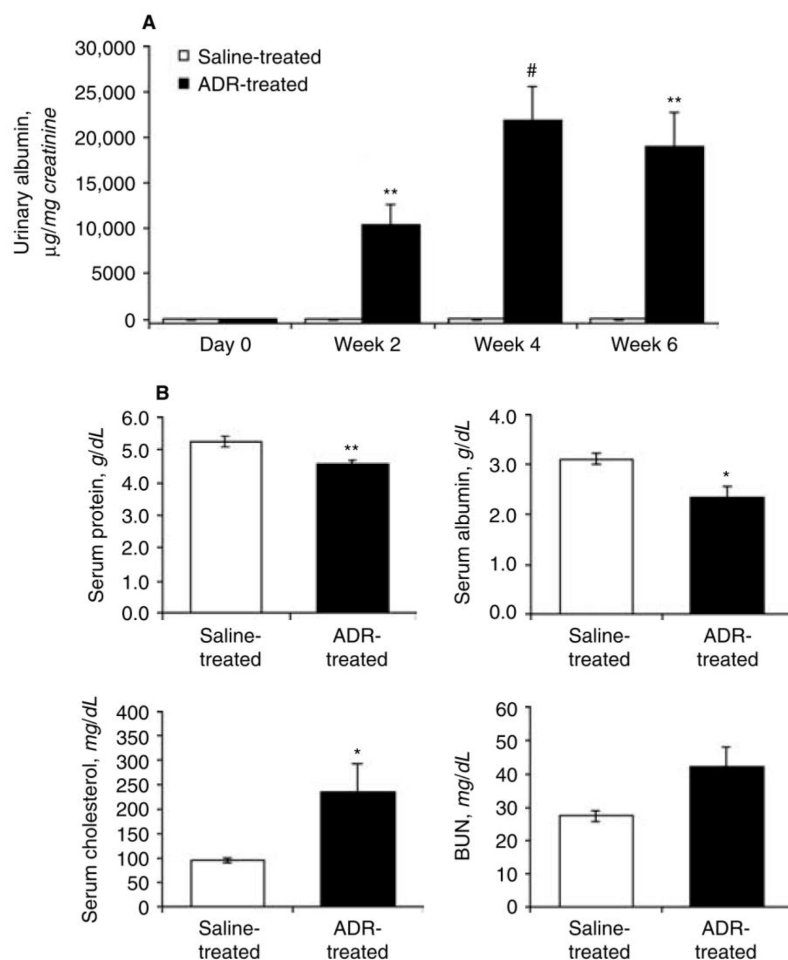

Fig. 1. Functional parameters in progressive adriamycin (ADR) nephropathy. Persistent, nephrotic-range proteinuria with decreased serum protein levels, hypalbuminemia and elevated serum cholesterol in adriamycin-treated mice. (*A*) Urinary albumin excretion was evaluated at day 0 and at week 2, 4, 6 after the first adriamycin injection in adriamycin-treated mice and saline-treated controls. (*B*) Levels of total serum protein, serum albumin, serum cholesterol, and blood urea nitrogen (BUN) in adriamycin-treated mice and saline-injected controls were determined at week 6. Values are expressed as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $\#P < 0.001$ compared to saline-treated.

a 2.7-fold increase of PAS-positive staining in glomeruli of adriamycin-treated mice compared to saline-injected controls, in which only glomerular basement membranes stained positive (44.0% ± 2.1% versus 16.3% ± 0.3% $P < 0.001$) (Fig. 3A). Glomerular pathology was accompanied by prominent tubulointerstitial changes, including tubular cell atrophy with brush border loss, tubular dilation, intraluminal cast formation, and expansion of the interstitial volume due to accumulation of mononuclear cells and deposition of extracellular matrix (Fig. 2B and D). Less frequently, glomeruli with minimal lesions embedded in normal tubules could be found adjacent to severely damaged areas, indicating the focal nature of the disease process.

Immunohistochemical staining detected a prominent accumulation of CD45+ leukocytes in interstitial lesions of adriamycin-treated mice at 6 weeks (7.5 ± 0.2 cells/high power field versus 0.3 ± 0.09 cells/high power field in saline-treated controls) ($P < 0.001$) (Figs. 2E and F and 3B). This interstitial infiltrate consisted of ER-HR3+ macrophages (5.9 ± 0.2 cells/high power field versus 0.2 ± 0.08 cells/high power field) ($P < 0.001$) and CD3+ T cells (3.0 ± 0.2 high power field versus 0.3 ± 0.03 cells/high power field) ($P < 0.001$) (Figs. 2G and H and 3C and D). In contrast, no glomerular leukocyte infiltration was detected at 6 weeks after induction of the disease. The accumulation of interstitial leukocytes was accompanied by fibrotic lesions in the cortex. There was a significant increase of interstitial FSP1+ fibroblasts (5.7 ± 0.3 cells/high power field versus 0.4 ± 0.07 cells/high power field) ($P < 0.001$) in cortical lesions of adriamycin-treated mice at 6 weeks (Figs. 2I and J and 3E). Quantitative analysis of silver-stained sections revealed an increase in the interstitial volume index by 3.1-fold in adriamycin-treated mice compared to saline-treated controls (19.2 ± 2.5 versus 6.1 ± 0.5) ($P < 0.001$) (Figs. 2K and L and 3F).

Expression of chemokines and the chemokine receptor CCR1 in adriamycin nephropathy

As locally expressed chemokines mediate renal leukocyte recruitment we investigated the expression

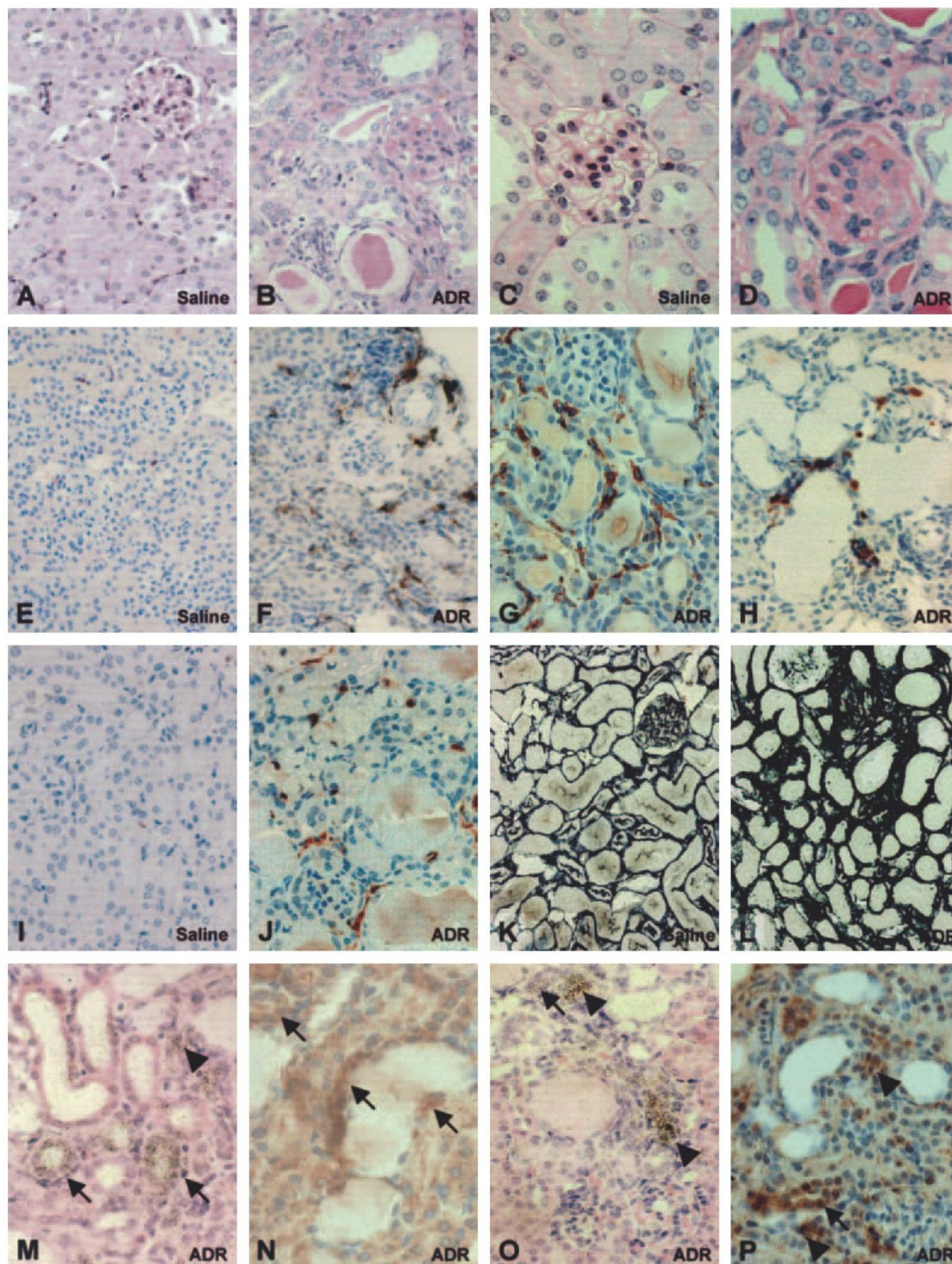

Fig. 2. Severe glomerulosclerosis, tubular injury, interstitial leukocyte accumulation, and interstitial fibrosis in kidneys with adriamycin (ADR) nephropathy at week 6. (*A* and *C*) Periodic acid-Schiff (PAS)-stained kidney sections of saline-treated contol mice showed normal renal morphology. (*B* and *D*) Adriamycin-injected mice developed severe glomerular lesions with segmental to global sclerosis, indicated by PAS-positive glomerular

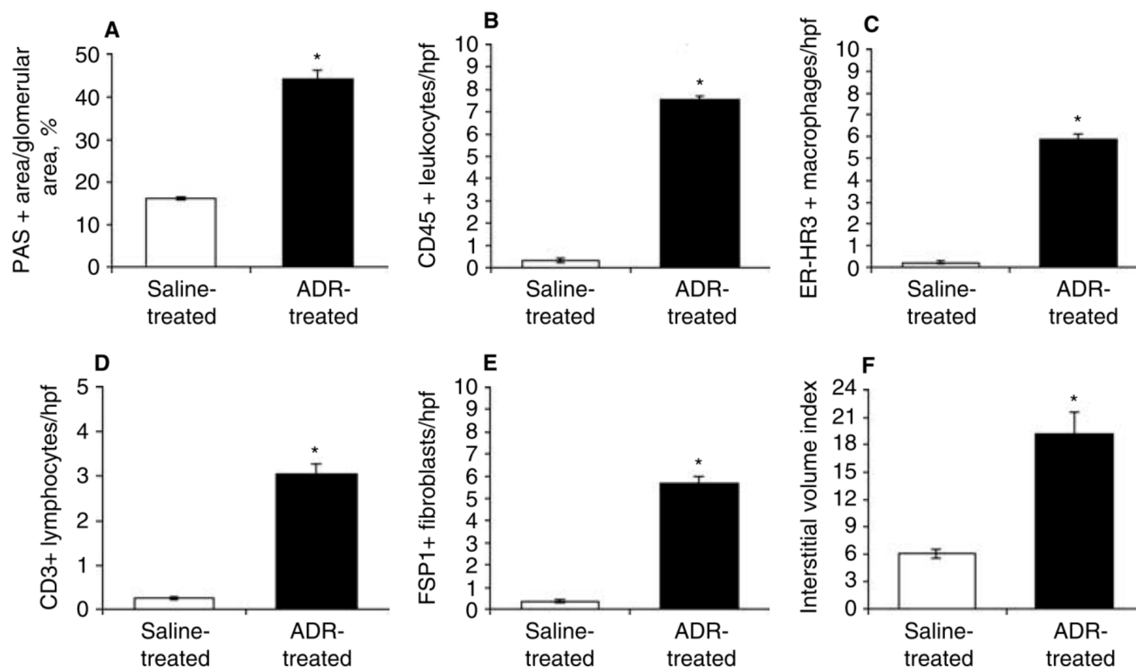

Fig. 3. Morphometric analysis of glomerulosclerosis, interstitial leukocyte accumulation, and interstitial fibrosis in kidneys with adriamycin (ADR) nephropathy compared to saline-treated controls at week 6. The degree of glomerulosclerosis (*A*), interstitial accumulation of CD45+ leukocytes (*B*), ER-HR3+ macrophages (*C*), CD3+ lymphocytes (*D*), fibroblast-specific protein 1 (FSP1)+ fibroblasts (*E*), and interstitial volume expansion (*F*) was analyzed as described in the **Methods** section. Values are expressed as means ± SD. $^*P < 0.001$ compared to saline-treated.

of chemokines in kidneys of mice with adriamycin nephropathy at 6 weeks. Whole-kidney mRNA samples were subjected to a ribonuclease protection assay containing probes for detection of the CC chemokines CCL1/TCA-3, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CCL11/eotaxin (Fig. 4A and B). All of these chemokines were up-regulated in kidneys of adriamycin-treated mice compared to saline-treated controls at week 6 (Fig. 4B). The mRNA expression of the CCR1 ligands CCL3/MIP-1α and CCL5/RANTES was increased by 11.5-fold and 3.5-fold, respectively. In addition, we detected increased mRNA levels of CCL2/MCP-1 (9.0-fold), CCL3/MIP-1β (10.8-fold), and CCL11/eotaxin (5.6-fold). There was also a small, but significant induction of CCL1/TCA-3 (1.3-fold) (Fig. 4B). To localize the chemokine mRNA expression within kidneys after adriamycin treatment we performed in situ hybridization studies with antisense probes for CCL2/MCP-1 and CCL5/RANTES on kidneys obtained from mice 6 weeks after the first adriamycin injection (Fig. 2M and O). CCL2 and CCL5 mRNA transcripts (black silver grains) both were abundantly detected in the cortical tubulointerstitium, but not in glomeruli of adriamycin-treated animals. CCL2 mRNA was prominently expressed in tubular epithelial cells (Fig. 2M, arrows), and, to a lesser extent, in interstitial cells. Tubular cells also expressed CCL5, but CCL5 mRNA was more abundantly detected in the interstitial infiltrate (Fig. 2O, arrowheads). Of note, the tubular expression of chemokine mRNA (and protein) localized

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deposits. Glomerular injury was accompanied by tubular damage with tubular dilation, intratubular cast formation, tubular cell atrophy, and necrosis. There was a marked expansion of the interstitium with infiltration of mononuclear cells and matrix deposition. (*E*) Immunohistochemical staining for CD45+ leukocytes in saline-treated control kidneys and (*F*) adriamycin-treated kidneys demonstrated a periglomerular and interstitial, but no glomerular accumulation of leukocytes after adriamycin injection. (*G*) The interstitial leukocyte infiltrate in the cortex of adriamycin-treated mice consisted of ER-HR3+ macrophages and (*H*) CD3+ lymphocytes. (*I*) Fibroblast-specific protein 1 (FSP1)+ cells were rarely detectable in control kidneys. (*J*) In contrast, FSP1+ cells as markers for active fibrosis accumulated in the interstitium of kidneys with adriamycin nephropathy. (*K*) Silver-stained cortical sections of control kidneys and (*L*) adriamycin-treated kidneys demonstrated interstitial volume expansion in adriamycin-injected mice. (*M*) (In situ hybridization and (*N*) immunohistochemistry for CCL2/monocyte chemoattractant protein 1 (MCP-1) localized most CCL2 expression to tubular epithelial cells (arrows), with some expression also present in the interstitial infiltrate (arrowhead). Note that CCL2 mRNA expression was clearly restricted to intact tubular cells, and did not occur in dilated tubules with flattened epithelium. (*O*) CCL5/RANTES mRNA and (*P*) protein expression localized more prominently to interstitial infiltrates (arrowheads). CCL5 protein was also found in intact, but not atrophic tubuli (arrows). CCL2 and CCL5 expression was absent in glomeruli. PAS stain (A to D); hematoxylin counterstain (E to J, N, and P); silver staining (K and L); hematoxylin-eosin counterstain (M and O) [original magnifications ×200 (A, B, and E to P); ×400 (C and D).

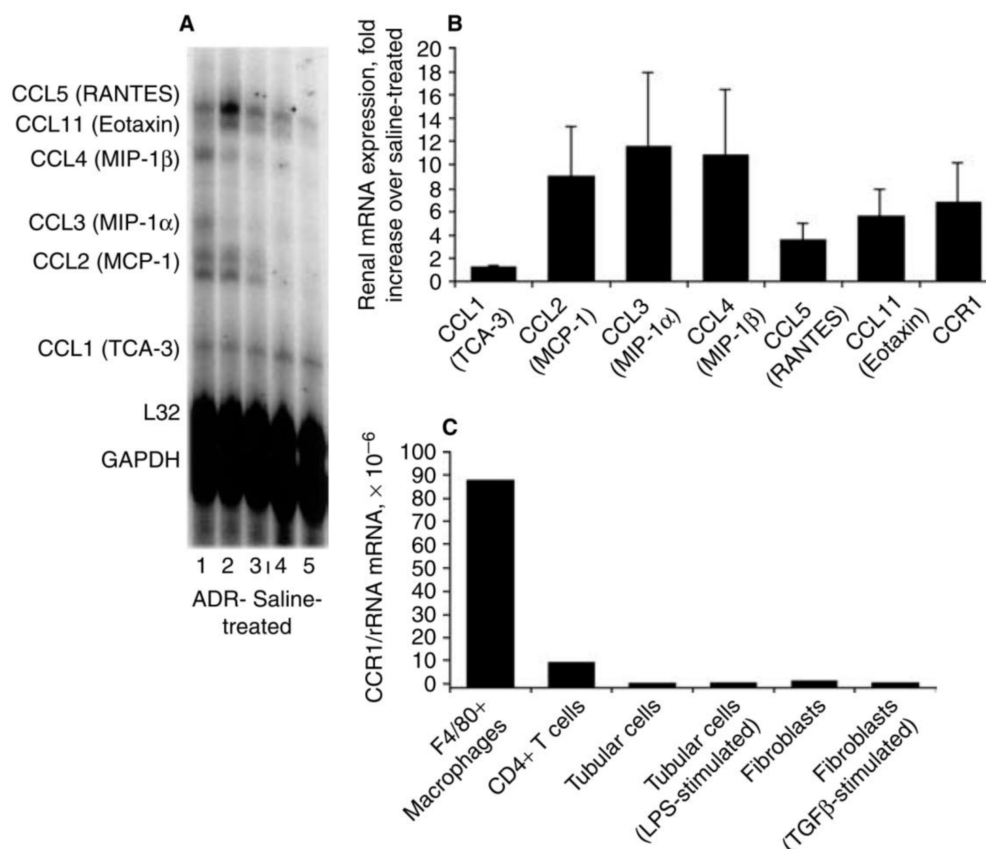

Fig. 4. Expression of proinflammatory chemokines and CCR1 in murine adriamycin (ADR) nephropathy at week 6. (*A*) mRNA expression of multiple CC chemokines was increased in kidneys of adriamycin-treated mice compared to saline-treated controls at week 6 as detected by RNase protection assay (RPA). A representative gel segment from three adriamycin-treated and two control kidneys is shown. (*B*) Chemokine mRNA was quantified by image densitometry after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. In addition, renal CCR1 expression was assessed by real-time reverse transcription-polymerase chain reaction (RT-PCR) and normalized to GAPDH expression as described in the **Methods** section. Renal expression of all CC chemokines tested, including the CCR1 ligands CCL3 (MIP-1α) and CCL5 (RANTES), was up-regulated in adriamycin-treated mice ($N = 5$) compared to saline-treated controls ($N = 3$). Similarly, expression of CCR1 was increased in adriamycin-treated kidneys ($N = 7$) compared to control kidneys ($N = 5$). Results are expressed relative to the levels in saline-injected controls and are given as mean ± SEM. $P < 0.05$ compared to saline treated controls. (*C*) To identify the potential cellular target population of the CCR1 antagonist BX471 expression of CCR1 was assessed by real-time RT-PCR in isolated leukocyte subsets and intrinsic renal cells. CCR1 mRNA was most abundantly detected in splenic macrophages isolated from BALB/c mice. Macrophages expressed tenfold more CCR1 than splenic CD4+ T cells. In contrast, CCR1 expression was not detectable in resting or lipopolysaccharide (LPS)-stimulated tubular epithelial cells. CCR1 expression was also absent in primary renal fibroblasts, either unstimulated or transforming growth factor-β (TGF-β) stimulated.

to intact tubular epithelial cells, but was not found in dilated tubules with flattened and atrophic epithelium (Fig. 2M to P). In kidneys from saline-treated control mice CCL2 and CCL5 transcripts were only occasionally found in few interstitial cells (data not shown). No signals were detected with the use of CCL2 or CCL5 sense sequence templates as a negative control. Immunostaining for CCL2 (Fig. 2N) and CCL5 (Fig. 2P) localized the proteins in a similar distribution as the respective mRNA transcripts. Particularly, no significant glomerular staining for the two chemokines could be detected in adriamycin-treated mice.

Because we intended to study effects of the CCR1 antagonist BX471 in adriamycin nephropathy we first determined the expression of CCR1 in this model. Using real-time RT-PCR a 6.7-fold induction of CCR1 mRNA expression could be detected in kidneys with adriamycin nephropathy (Fig. 4B). Unfortunately, due to the lack of appropriate antibodies and probes for in situ hybridization cell-specific localization of renal CCR1 expression by immunostaining or in situ hybridization is not feasible at the moment. We therefore performed studies with leukocyte subsets, a murine tubular epithelial cell line, and primary renal fibroblasts to identify cell types which express CCR1, and therefore would be the potential target for renal BX471 action in the kidney. Both macrophages and CD4+ T cells isolated from spleens of BALB/c mice expressed CCR1 mRNA. Macrophages expressed CCR1

at a 10-fold higher level than CD4+ T cells (Fig. 4C). No CCR1 mRNA could be detected in renal tubular epithelial cells, with or without LPS stimulation (Fig. 4C). Similarly, CCR1 mRNA expression was not detectable in unstimulated or TGF-β–stimulated renal fibroblasts (Fig. 4C). These results suggest that infiltrating mononuclear cells, in particular of the monocyte/macrophage lineage, are the major source of renal CCR1 expression in adriamycin nephropathy. In contrast, activated renal tubular cells and interstitial fibroblasts do not express CCR1 and therefore do not contribute to the renal CCR1 expression.

CCR1 blockade does not affect glomerulosclerosis and proteinuria in adriamycin nephropathy

In order to study effects of the CCR1 blockade on the progression of chronic FSGS rather than on its initiation, treatment with the CCR1 antagonist BX471 was started at the time of the second adriamycin injection at week 2, when gross albuminuria was already present (Fig. 5A). At this time point adriamycin-injected mice had already developed substantial glomerular pathology, as indicated by deposition of PAS-positive material in the glomeruli. The glomerular area with PAS-positive staining was 34.5% ± 3.5% in adriamycin-injected mice at week 2, compared to 16.3% ± 1.7% in saline-injected controls ($P < 0.001$) (Fig. 7A). Mice with established adriamycin nephropathy received either vehicle solution or BX471 from week 2 to 6. In vehicle-treated mice there was a further increase in the urinary albumin excretion from week 2 to 4, following the second adriamycin injection, and this degree of albuminuria persisted until the end of the study period at week 6 (Fig. 5A). Mice that received BX471 instead of the vehicle showed a similar response in the albuminuria, without significant differences throughout the experimental period (Fig. 5A). Consistent with the same degree of urinary albumin loss there were no significant differences in the serum values for total protein, albumin, and cholesterol between vehicle-treated and BX471-treated mice with adriamycin nephropathy at 6 weeks. Serum protein and albumin levels decreased, and cholesterol levels increased to a similar extent in both groups compared to mice without adriamycin treatment, which received either vehicle or BX471 from week 2 to 6 (Fig. 5B). The latter two groups of mice served as controls and demonstrated that neither treatment with the vehicle solution nor BX471 allone (without injection of adriamycin) resulted in albuminuria (Fig. 5A) or altered serum values (Fig. 5B). BUN levels were not significantly different between all four groups of mice.

A similar degree of albuminuria in vehicle- and BX471-treated mice suggested that treatment with the CCR1 antagonist did not alter the extent of glomerular damage induced by the adriamycin injections. In both groups of mice the extent of glomerular damage further increased from week 2 to 6 (Figs. 6A and B and 7A). Consistent with the functional data the extent of glomerulosclerosis was similar in vehicle-treated and BX471-treated mice on histologic evaluation of PAS-stained sections (Fig. 6A and B). The glomerular area with PAS-positive staining was 43.3% ± 1.8% and 44.3% ± 2.9% in vehicle-treated and BX471-treated mice with adriamycin nephropathy, respectively, without significant differences between the two groups (Fig. 7A). In contrast, glomerular PAS staining was significantly lower in mice which received either vehicle or BX471, but no adriamycin, and it was in a similar range as in the saline-treated control mice at week 2 (Fig. 7A). Glomerular leukocyte infiltration was not seen in any of the six groups of mice. In summary, these data indicate that BX471, given from week 2 to 6, did not affect the glomerular injury and the developing nephrotic syndrome in adriamycin nephropathy.

CCR1 blockade reduces interstitial leukocyte recruitment in adriamycin nephropathy

Adriamycin nephropathy is characterized by progressive interstitial lesions consisting of immune cell infiltrates, fibroblast accumulation, and matrix deposition. As reported by Wang et al [14] in their original characterization of the murine adriamycin nephropathy model interstitial accumulation of macrophages appears to be most prominent in early phases of the disease, with a dramatic decrease in later stages. In contrast, a gradual increase in the number of interstitial T lymphocyte infiltrates was noted until week 6 [14]. As CCR1 is involved in immune cell migration, we compared renal interstitial leukocyte recruitment between BX471- and vehicle-treated mice with adriamycin nephropathy. At 6 weeks, BX471 treatment reduced the amount of CD45+ leukocytes in interstitial lesions by 37% compared to vehicle-treated mice with adriamycin nephropathy (4.6 ± 0.4 cell/high power field versus 7.3 ± 0.2 cells/high power field) ($P < 0.001$) (Fig. 7B). Consistent with the previous report [14] we found the most prominent macrophage infiltration in adriamycin-injected mice at week 2 (8.9 ± 1.9 ER-HR3+ cells/high power field), with a subsequent decrease in vehicle-treated control mice until week 6 (5.6 ± 0.2 cells/high power field) ($P < 0.05$) (Fig. 7C). Importantly, in this later phase BX471 treatment from week 2 to 6 significantly reduced the tubulointerstitial infiltration of macrophages by 51% compared to vehicle-treated mice (2.7 ± 0.2 cells/high power field versus 5.6 ± 0.2 cells/high power field) ($P < 0.001$) (Figs. 6C and E and 7C). At week 2 significant numbers of interstitial CD3+ lymphocytes were present in adriamycin-injected mice (1.1 ± 0.2 cells/high power field), and these increased further in vehicle-treated mice until week 6 (3.2 ± 0.6 cells/high power field) ($P < 0.05$) (Fig. 7D). BX471 treatment reduced this gradual accumulation of interstitial CD3+ lymphocytes by 22% (2.5 ± 0.1 cells/high power field

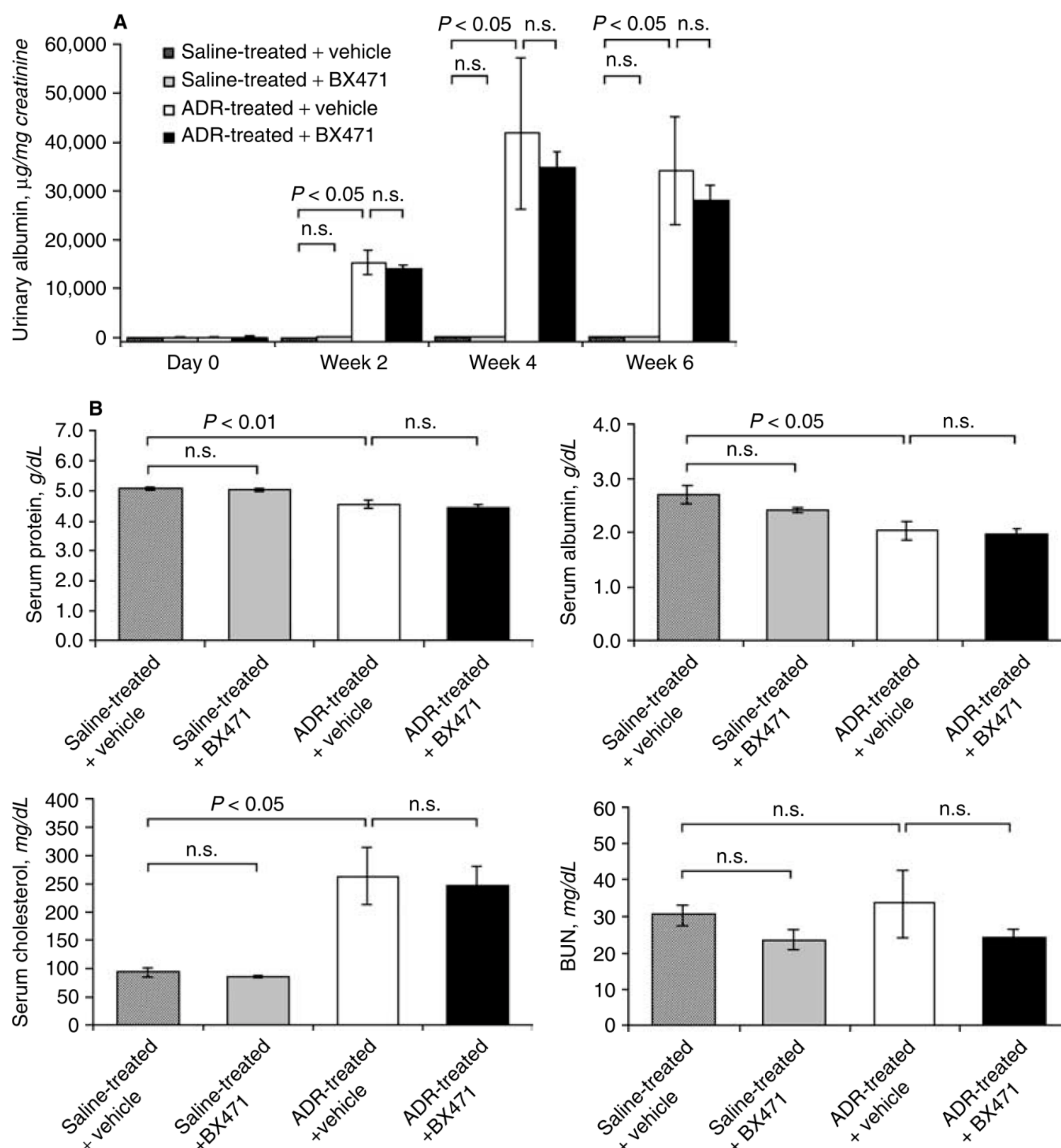

Fig. 5. BX471 treatment of mice with established adriamycin (ADR) nephropathy did not affect proteinuria and serum parameters. (*A*) Urinary albumin excretion was evaluated at day 0 and at week 2, 4, and 6 in vehicle- or BX471-treated mice without or with induced adriamycin nephropathy. (*B*) Levels of total serum protein, serum albumin, serum cholesterol, and blood urea nitrogen (BUN) in BX471- or vehicle-treated mice with or without adriamycin nephropathy were determined at week 6. Values are expressed as means ± SEM. NS is not significant.

versus 3.2 ± 0.6 cells/high power field in BX471-treated and vehicle-treated mice at 6 weeks, respectively) ($P < 0.05$), (Figs. 6E and F and 7D). Control mice, which did not receive adriamycin, but were treated with vehicle or BX471 alone, did not show leukocytic cell infiltrates in the interstitial compartment (Fig. 7B to D). Taken together, these data demonstrate that CCR1 blockade with BX471 reduced interstitial recruitment of macrophages and lymphocytes in adriamycin nephropathy.

CCR1 blockade reduces interstitial fibrosis in adriamycin nephropathy

As a cellular marker of fibrosis the amount of FSP1+ fibroblasts was assessed by immunohistochemistry in

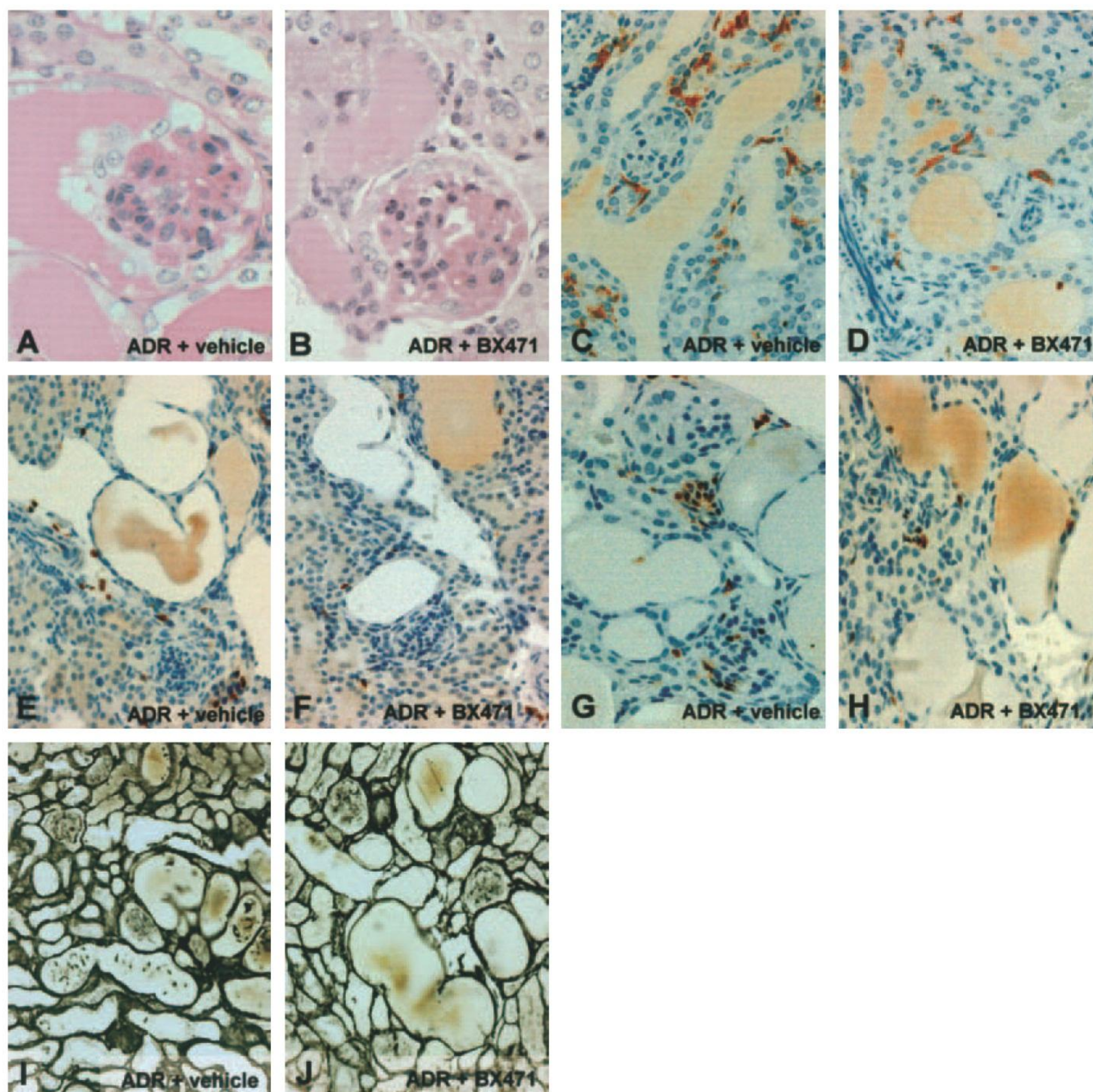

Fig. 6. Glomerulosclerosis, interstitial leukocyte accumulation, and interstitial fibrosis in vehicle- or BX471-treated mice with adriamycin (ADR) nephropathy at week 6. Periodic acid-Schiff (PAS)-stained kidney sections from vehicle-treated mice (*A*) and BX471-treated mice (*B*) revealed a similar degree of glomerulosclerosis after adriamycin injections. Interstitial infiltration of ER-HR3+ macrophages in vehicle-treated mice with adriamycin nephropathy (*C*) was reduced in BX471-treated mice (*D*). Interstitial CD3+ lymphocytes were more abundant in vehicle-treated ADR mice (*E*) than in BX471-treated mice (*F*). The accumulation of fibroblast-specific protein 1 (FSP1)+ fibroblasts in interstitial lesions of vehicle-treated adriamycin mice (*G*) was more prominent than in BX471-treated mice (*H*). Silver staining revealed a more severe expansion of the interstitial volume in vehicle-treated mice with adriamycin nephropathy (*I*) compared to the BX471-treated group (H) [original magnifications ×400 (A through J)].

interstitial lesions of vehicle- and BX471-treated mice with adriamycin nephropathy. The progressive accumulation of fibroblasts from week 2 (2.9 ± 0.8 cells/high power field) to week 6 (5.3 ± 0.2 cells/high power field in vehicle-treated mice) ($P < 0.05$) was completely blocked in BX471-treated mice (2.8 ± 0.1 cells/high power field) ($P < 0.001$ compared to vehicle-treated controls), resulting in a 48% reduction of fibroblast accumulation in BX471-treated mice at week 6 (Figs. 6G and H and 7E). Furthermore, BX471 treatment reduced the

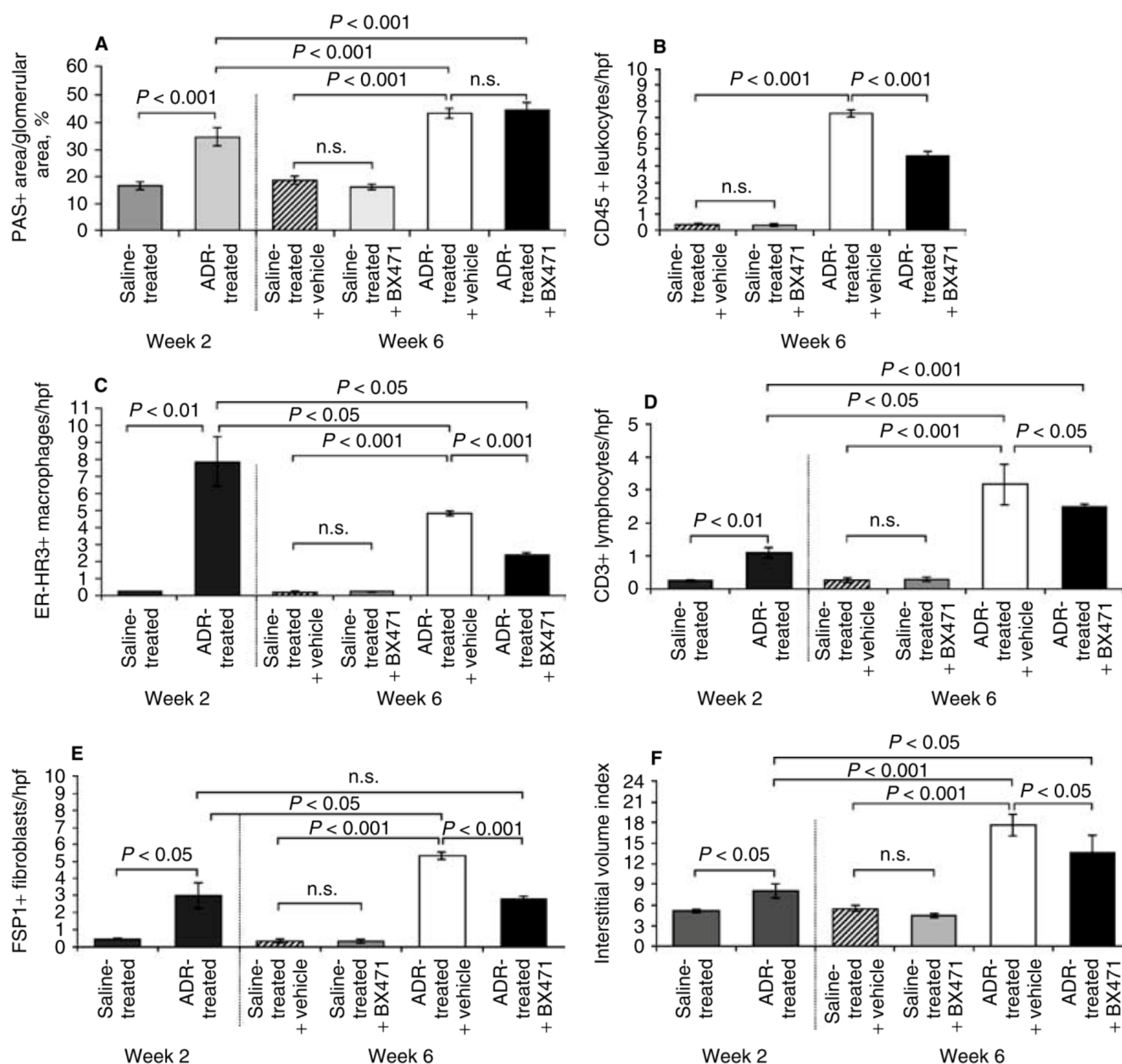

Fig. 7. Morphometric analysis of glomerulosclerosis, interstitial leukocyte accumulation, and interstitial fibrosis in vehicle- or BX471-treated mice with adriamycin (ADR) nephropathy at week 6. The degree of glomerulosclerosis (*A*), interstitial accumulation of CD45+ leukocytes (*B*), ER-HR3+ macrophages (*C*), CD3+ lymphocytes (*D*), fibroblast-specific protein (FSP1)+ fibroblasts (*E*), and interstitial volume expansion (*F*) was analyzed in saline- or adriamycin-treated mice at week 2 (when BX471 treatment was started) and in vehicle- or BX471-treated mice with adriamycin nephropathy at week 6. As control groups, vehicle- or BX471-treated mice without adriamycin injections were also evaluated. Values are expressed as means ± SD. NS is not significant.

progressive interstitial volume expansion from week 2 (interstitial volume index 8.0 ± 1.0) to week 6 (17.6 ± 1.6 in vehicle-treated controls) ($P < 0.001$) by 23% (13.6 ± 2.7 in BX471-treated mice) ($P < 0.05$ compared to vehicle-treated controls) (Figs. 6I and J and 7F). No changes in interstitial cell counts or morphometric parameters were observed in vehicle- or BX471-injected control mice without adriamycin treatment (Fig. 7B to F). Thus, BX471 treatment significantly reduced not only the interstitial leukocyte influx, but also the extent of interstitial fibrosis in mice with adriamycin nephropathy.

DISCUSSION

In the kidney, the extent of interstitial leukocyte infiltration and tubulointerstitial fibrosis are strong prognostic factors for the progression to ESRD, independent of the primary glomerular or interstitial lesion [1, 5]. We have previously shown that interstitial expression of the proinflammatory chemokine receptors CCR1, CCR2, and CCR5 on leukocyte infiltrates and their respective chemokine ligands is induced in various models of progressive renal injury, including obstructive nephropathy after unilateral ureteral ligation and immune complex

nephritis in MRL lpr/lpr mice [21, 23]. Therefore, interfering with renal leukocyte recruitment (e.g., by blocking CCR1) might reduce the inflammatory response in the kidney and prevent the progression of renal fibrosis [24]. We used adriamycin-induced nephropathy in mice as a model of FSGS to examine whether BX471, a small molecule CCR1 antagonist, may reduce renal leukocyte recruitment and subsequent interstitial fibrosis, a hypothesis supported by our results. These findings are of particular relevance to the potential therapeutic role of CCR1 antagonism in progressive human nephropathies, as the adriamycin-induced glomerulosclerosis and subsequent interstitial inflammation and fibrosis resembles the course of glomerular and interstitial changes in human chronic proteinuric renal disease [14].

CCR1 blockade with BX471 has been shown to inhibit leukocyte adhesion and transmigration through endothelium in a dose-dependent manner under conditions of shear flow in vitro [25, 26]. In the obstructive nephropathy model we have recently shown that CCR1 blockade with BX471 reduced the amount of leukocyte infiltration into the renal interstitium [11]. It might appear that the report of worsening of glomerulonephritis in CCR1-deficient mice with nephrotoxic serum nephritis would be in contrast to these findings [13]. However, in these mice an enhanced Th1 immune response was noted, so that CCR1 deficiency might result in an overall change in the immune response toward the nephrotxic serum, altering the initiation of the disease [13]. Here, we used adriamycin nephropathy as a model of progressive renal disease induced by a nonimmune-mediated glomerular injury. We found CCR1 to be prominently expressed in kidneys during adriamycin nephropathy, and renal expression of multiple proinflammatory chemokines was up-regulated, including the CCR1 ligands CCL3/MIP-1α and CCL5/RANTES. Our results clearly demonstrate that CCR1 blockade with BX471 in the advanced stage of this model reduced renal interstitial leukocyte recruitment and progression of renal fibrosis. These observations are consistent with the beneficial effects of CCR1 blockade on the extent of leukocyte infiltration and disease outcome in other models, including obstructive nephropathy [11], renal and heart transplantation [27, 28], pulmonary fibrosis [12], and experimental encephalomyelitis [25, 29]. Together these data argue for an important role of CCR1-mediated leukocyte recruitment into the tubulointerstitium in vivo. Surprisingly CCR1 blockade had no measurable effect on glomerular pathology and proteinuria, indicating different mechanisms for leukocyte infiltration of the glomerulus and tubulointerstitium.

BX471 treatment most effectively reduced the accumulation of interstitial macrophages, being consistent with the known expression of CCR1 on blood monocytes and tissue macrophages. CCR1 and responsiveness to its ligand CCL3/MIP-1α is up-regulated on human monocytes upon transition to the macrophage phenotype [30], and CCR1 mediates human monocyte adhesion and transendothelial migration under flow [26]. Furuichi et al [8] analyzed CCR1 expression in glomerular and interstitial lesions of human nephropathies by immunohistochemistry. These investigators noted that most CCR1-positive cells in the interstitium were macrophages, and that no CCR1 expression was detectable in intrinsic renal cells. Moreover, the number of CCR1-positive cells in the interstitium correlated with the intensity of interstitial fibrosis [8]. We found an abundant expression of CCR1 on splenic macrophages isolated from BALB/c mice, but not on tubular epithelial cells or primary renal fibroblasts. Taken together, these data suggest that circulating monocytes and/or infiltrating renal macrophages are a major cellular target of the CCR1 blockade by BX471. There was also a decreased accumulation of CD3+ lymphocytes in interstitial lesions of adriamycin mice, albeit less prominent than the reduction in macrophages. This may relate to the lesser expression of CCR1 mRNA demonstrated in splenic CD4+ T cells. Therefore, the decreased interstitial T cell accumulation in BX471-treated mice might be either the consequence of a direct antagonism of CCR1 expressed on infiltrating T cells, or an effect secondary to the decreased interstitial macrophage infiltration with subsequent down-regulation of the inflammatory response in the interstitium.

The reduced interstitial leukocyte infiltration was associated with a reduction of fibrosis in interstitial lesions of BX471-treated mice with adriamycin nephropathy. BX471 treatment from week 2 to 6 completely prevented any further accumulation of interstitial fibroblasts, without reversal of the already increased numbers present at week 2. The progressive expansion of the interstitial volume, which could already be noted at week 2 and increased until week 6, was significantly diminished in BX471-treated mice, but could not be completely prevented. We did not find any CCR1 mRNA expression in tubular epithelial cells or fibroblasts. This argues against a direct BX471 effect on CCR1 signaling in epithelial-mesenchymal transformation or fibroblast activation. It is most likely that the reduction of infiltrating mononuclear cells in response to BX471 treatment caused less fibroblast proliferation and activation. Leukocytes, and particularly macrophages, secrete profibrotic cytokines such as TGF-β, platelet-derived growth factor, basic fibroblast growth factor, connective tissue growth factor, and platelet activating factor, which mediate epithelial-mesenchymal transformation, fibroblast proliferation, and matrix synthesis [4, 31]. This interpretation is consistent with our previous findings in the obstructive nephropathy model demonstrating a similar reduction of mononuclear cell infiltrates and subsequent

interstitial fibrosis by BX471 [11]. Similar effects of CCR1 blockade were also reported in bleomycin-induced pulmonary fibrosis with reduced mononuclear cell infiltrates and collagen deposition in the lung, and an improvement of survival [12]. The beneficial effects of BX471 in the interstitium of adriamycin nephropathy are particularly interesting in view of the severe proteinuria, which was not affected by BX471 treatment. The persistent proteinuria should provide a sustained stimulus for tubular cell activation, which may result in secretion of chemokines and profibrotic cytokines [32]. However, our data show that persistent proteinuria per se was insufficient to fully promote interstitial fibrosis when the interstitial infiltration of leukocytes was reduced in BX471-treated mice.

BX471 treatment had no effects on the degree of glomerular damage induced by adriamycin, as functional and histologic parameters were not different in vehicle- or BX471 treated mice with adriamycin nephropathy. A direct cytotoxic effect of adriamycin on podocytes is thought to induce the initial glomerular damage. The progressive glomerulosclerosis is unlikely to be caused by a toxic effect of chronic accumulation of adriamycin because adriamycin is rapidly cleared from blood and kidney [33, 34]. The progressive glomerular sclerosis may relate to podocyte damage resulting in a self-perpetuating process. These and any potential immunologic mechanisms leading to the progressive glomerular injury after adriamycin are apparently independent of CCR1, as its blockade had no beneficial effects on the glomerular lesions and proteinuria. Consistently with this view we could not detect any significant glomerular infiltration of leukocytes at week 2 and 6. In addition, we could not detect glomerular expression of the chemokines CCL2/MCP-1 or CCL5/RANTES in adriamycin mice at 6 weeks, which is in marked contrast to prominent expression of these chemokines in the tubulointerstitial compartment. Taken together, these data indicate that the beneficial effects of BX471 are restricted to sites of recruitment of CCR1-positive leukocytes. Furthermore, they provide evidence for different mechanisms of glomerular and tubulointerstitial disease processes and inflammatory leukocyte influx in these compartments.

CONCLUSION

Blockade of CCR1 with BX471 substantially reduced interstitial leukocyte accumulation and subsequent renal fibrosis in a murine model of FSGS with progressive interstitial injury. This occurred in spite of persistent proteinuria and glomerular damage. These findings suggest a role for CCR1 in leukocyte recruitment to the interstitium and a role of these CCR1-positive leukocytes in the progressive renal interstitial fibrosis. CCR1 blockade may therefore represent a promising therapeutic strategy also in chronic proteinuric nephropathies that progress to ESRD.

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3.4 Late-onset endothelin-A receptor blockade reduces podocyte injury in homozygous Ren-2 rats despite severe hypertension

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Endothelin and Renal Injury

Late-Onset Endothelin-A Receptor Blockade Reduces Podocyte Injury in Homozygous Ren-2 Rats Despite Severe Hypertension

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Abstract—We have recently found in male homozygous hypertensive Ren-2 transgenic rats (TGRs) fed a high-salt diet that early onset selective endothelin (ET) A (ET_A) or nonselective ET_A/ET B (ET_B) receptor blockade improved survival rate and reduced proteinuria, glomerulosclerosis, and cardiac hypertrophy, whereas selective ET_A receptor blockade also significantly attenuated the rise in blood pressure. Because antihypertensive therapy in general is known to be more efficient when started at early age, our study was performed to determine whether onset of ET receptor blockade at a later age in animals with established hypertension will have similar protective effects as does early-onset therapy. Male homozygous TGRs and age-matched normotensive Hannover Sprague–Dawley rats were fed a high-salt diet between days 51 and 90 of age. TGRs received vehicle (untreated), the selective ET_A receptor blocker atrasentan (ABT-627), or the nonselective ET_A/ET_B receptor blocker bosentan. Survival rates in untreated and bosentan-treated TGRs were 50% and 64%, respectively, whereas with atrasentan, survival rate of TGR was 96%, thus, similar to 93% in Hannover Sprague–Dawley rats. From day 60 on, systolic blood pressure in atrasentan-treated TGRs was transiently lower ($P<0.05$) than in untreated or bosentan-treated TGRs. Glomerular podocyte injury was substantially reduced with atrasentan treatment independent of severe hypertension and strongly correlated with survival ($P<0.001$). Our data indicate that in homozygous TGR ET receptors play an important role also in established hypertension. Selective ET_A receptor blockade not only reduces podocyte injury and end-organ damage but also improves growth and survival independently of hypertension. **(*Hypertension*. 2006;48:965-971.)**

Key Words: endothelin-1 ■ ET_A and ET_B receptors ■ bosentan ■ atrasentan (ABT-627), homozygous transgenic Ren-2 rats ■ hypertension ■ end-organ damage ■ survival rate

The hypertensive rat strain transgenic for the mouse Ren-2 renin gene ([TGR] strain name TGR[mRen2]27)[1] is a valuable monogenetic model of renin-dependent and thus angiotensin II (Ang II)–dependent hypertension, which exhibits typical signs of fulminant hypertension, that is, reduced glomerular filtration rate and proteinuria associated with glomerulosclerosis.[2,3] Moreover, it carries a salt-sensitive component.[4]

Endothelin (ET)-1 is known to be one of the most powerful vasoconstrictors[5,6] and also a mitogen in vivo and in vitro.[7] The beneficial effects of ET receptor blockers in modulating target organ damage are attributed to their antiproliferative actions.[8] Numerous studies have shown that the ET system plays an important role in the pathogenesis of high blood pressure (BP) in salt-sensitive models of hypertension and in associated end-organ damage.[9] For the detrimental effects of ET-1 in the development of hypertension, activation of ETA (ET_A) receptors may be responsible, whereas the role of ET B (ET_B) receptors may be the mediation of peripheral vasorelaxation and the renal tubular natriuresis. Nonselective blockade, therefore, inhibits not only the deleterious effects of ET-1 mediated by ET_A receptors but also concomitantly blocks its antihypertensive effects mediated by ET_B receptors. However, because at present only conflicting data regarding selective ET_A and nonselective $ET_{A/B}$ receptor blockade are available, the relative beneficial effects of selective versus nonselective ET receptor blockade remain to be elucidated.

Several lines of evidence indicate that Ang II stimulates the release of ET-1, and it is known that the ET system plays an important role in the pathogenesis of hypertension and accompanying end-organ damage in salt-dependent and in Ang II–dependent models of hypertension induced by exogenous administration of Ang II.[10]

Dietary sodium plays an important role in the pathogenesis of hypertension not only in humans[11] but also in salt-sensitive

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models of hypertension.[12] Thus, on one hand, the increased sodium intake exerts detrimental cardiac effects and leads to dysfunction of vascular endothelium, which, through the release of ET-1, contributes to vascular changes found in salt-sensitive hypertension.[13] On the other hand, selective ET_A receptor blockade lowers BP predominantly in salt-dependent models of hypertension. Moreover, a growing body of evidence shows that on high salt intake there is a substantial impact of ET_B receptors in promoting higher sodium excretion from the body.[14]

Proteinuria may be caused by defects of podocytes that maintain intact filtration barrier and control glomerular basement membrane (GBM) turnover under normal conditions,[15] of the endothelial cells, and of the GBM itself. Podocytes are reported to be injured in many types of proteinuric renal diseases, including nephrotic syndrome, diabetic nephropathy, and lupus nephritis.[16,17] Only a few studies explored the involvement of podocyte damage in experimental hypertensive glomerulopathy.[18]

In our previous study, we have shown that early treatment both with bosentan, a nonselective ET_A/ET_B receptor antagonist, and with atrasentan, a selective ET_A receptor blocker, improved survival rate and ameliorated end-organ damage in homozygous male TGRs fed a high-salt diet (HS), but only atrasentan attenuated the rise in BP.[19] It is well known that young animals are more susceptible to various hypertensive stimuli than adult animals[20]; therefore, interventions made in early life are usually more effective.[21]

Thus, in the present study, our objective was, first, to evaluate whether selective ET_A or nonselective $ET_{A/B}$ receptor blockade in homozygous TGRs on a high-salt regimen will have similar protective effects on survival, end-organ damage, and BP when started in adult rats with established hypertension as when applied at an early stage before BP had risen and, second, whether selective ET_A receptor blockade is superior to nonselective ET_A/ET_B receptor blockade under these experimental conditions.

Methods

The protocols in the present study are in adherence to the Guide for the Care and Use of Laboratory Animals and were approved by Czech Animal Care and Use Committee (protocols 79/2001 and 923/2003).

Animals

Homozygous male TGRs and their normotensive Hannover Sprague–Dawley control rats (HanSD) were housed at 25°C under a 12 hour light/dark cycle and had free access to chow and water. All of the animals used in this study were bred at the Center for Experimental Medicine of the Institute for Clinical and Experimental Medicine from stock animals supplied from the Max Delbrück Center for Molecular Medicine (Berlin, Germany).

Experimental Design

At the age of 51 days, HanSD rats and 3 groups of TGRs were placed on a HS diet (2% NaCl). At the same time, 1 group of TGRs received no drug (untreated TGRs), the 2 other groups of TGRs received treatment with either the nonselective ET_A/ET_B receptor blocker bosentan (Actelion) or the selective ET_A receptor blocker atrasentan (ABT-627; Abbott). Bosentan was mixed into the diet in an amount depending on the actual food intake to achieve a final consumption of 100 mg kg^{-1} day^{-1}.[9] This dose was validated previously in our laboratory to effectively block ET receptors.[22] Atrasentan was added to the drinking fluid; the dose was adjusted weekly to provide a dose of 5 mg kg^{-1} day^{-1},[23] which is accepted and confirmed by various groups of investigators to effectively block ET_A receptors.[24,25] The following experimental groups were investigated: (1) male HanSD rats+HS (n=14); (2) male TGR+HS, untreated (n=18); (3) male TGR+HS+bosentan (n=14); and (4) male TGR+HS+atrasentan (ABT-627; n=24).

Determination of BP, Protein Excretion, and Tissue Weight

From the age of 32 days on, rats were weighed and systolic BP (SBP) was measured once weekly by the tail-cuff method validated previously in our laboratory.[26] At the age of 50 and 80 days, animals were individually housed in metabolic cages, and measurements of fluid consumption and urine excretion, as well as protein excretion, were monitored over 24 hours. Urinary protein determination was performed using the biuret method (Lachema).

On termination of the experiment (day 90), animals were weighed and anesthetized with thiopental sodium (50 mg kg^{-1}), and mean arterial pressure (MAP) was monitored directly in the carotid artery using the data acquisition system PowerLab (ADInstruments). Kidneys and hearts were rapidly removed and weighed. Ratios of kidney weight (KW)/body weight (BW) and heart weight (HW)/BW were used as indices of organ hypertrophy.

Determination of Tissue ET-1 Concentrations

After dissection on ice-cold plates, tissues from the left cardiac ventricle and right kidney cortex were immediately frozen in liquid nitrogen for ET-1 determination by ELISA (Amersham).

Histological Examination

The left kidney was quickly removed, fixed in 4% buffered formaldehyde, dehydrated, and embedded. Paraffin sections were stained with hematoxylin/eosin and periodic acid-Schiff reaction and examined using a Nicon Eclipse E 600 light microscope. Slides were evaluated in a blind fashion. As described previously,[27] 100 glomeruli per section were selected randomly, and the degree of glomerular damage was evaluated using a semiquantitative scoring method (grade 0: normal glomeruli; grade 1: sclerotic area ≤25% of total glomerular area or distinct adhesion present between capillary tuft and Bowman's capsule; grade 2: sclerotic area between 25% and 50%; grade 3: sclerotic area 50% to 75%; and grade 4: sclerotic area 75% to 100% of total glomerular area). The glomerulosclerosis index was calculated using the following formula: glomerulosclerosis index= $(1\times n_1)+(2\times n_2)+(3\times n_3)+(4\times n_4)/n_0+n_1+n_2+n_3+n_4$, where n_x is the number of glomeruli in each grade of glomerulosclerosis.

Electron Microscopy and Measurement of GBM Thickness

Small portions of the renal cortex of formalin-fixed and paraffin-embedded kidneys were selectively removed and used for electron microscopic examinations. Two animals from each group were examined. Samples were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined using a Philips EM 286 /Morgagni transmission electron microscope (FEI Company). Evaluations were performed by 2 independent observers in a blinded fashion. Thickness of the GBM was measured in 5 glomeruli per rat. The average of 50 measurements per glomerulus was taken. The perpendicular distance from the endothelial cell boundary to the epithelial cell boundary of the peripheral basement membrane was measured. Areas of wrinkled GBM and bevelled sections were excluded. The GBM measurements were taken using Analysis version 3.2 (Build 765 GmbH).

Statistical Analysis

Statistical analysis of data were performed using Graph-Pad Prism software (Graph Pad Software). Group comparisons were determined by 2-way ANOVA. Statistical comparisons of the results obtained for HWs and KWs and for ET-1 concentrations were made by 1-way ANOVA. The relationship between podocyte damage and

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survival rate was evaluated using least-squares linear regression analysis. Unless otherwise stated, values are expressed as mean±SEM, and "n" represents the number of animals. A P value <0.05 was considered significant.

Results

Systolic and Mean Arterial BP

In HanSD rats, SBP remained within the normotensive range throughout the whole experimental period (Figure 1A). In the 3 groups of TGRs, SBP rose gradually after weaning until the age of 46 days. Thus, just before changing to a high salt intake on day 51, SBP was 198.1±6.1 mm Hg in subsequently untreated rats, 201.0±5.4 mm Hg in rats subsequently treated with bosentan, and 192.0±3.8 mm Hg in rats subsequently treated with atrasentan. At the age of 60 days, SBP was 198.6±5.5 and 209.2±5.6 mm Hg in untreated and bosentan-treated TGRs, respectively, whereas treatment with atrasentan resulted in a significantly lower SBP (173.9±4.3 mm Hg; $P<0.05$) but did not reach SBP of HanSD rats (151.3±5.5 mm Hg). At day 81 of age, SBP was 151.3±5.2 mm Hg in HanSD rats and 210.8±3.4, 210.7±8.5, and 200.0±5.2 mm Hg in the surviving untreated, bosentan-treated, and atrasentan-treated TGRs, respectively.

At the end of the experiment on day 90, MAP in the surviving rats was not different among HanSD, bosentan-treated, and atrasentan-treated TGRs (153.6±6.5, 159.3±10.5, and 165.3±5.2 mm Hg, respectively). In untreated TGRs, MAP of 200.7±18.9 mm Hg was higher but not significantly different from the other groups of TGRs.

Survival Rate

Survival rate in HanSD rats was 93%, and in atrasentan-treated TGRs it was 96%, being significantly different from untreated and bosentan-treated TGRs ($P<0.05$; Figure 1B), which started to die progressively from days 60 and 53 of age on, respectively. Survival rates were 50% and 64%, respectively, on termination of the experiment.

Protein Excretion

There were no significant differences in protein excretion between the TGR groups at the age of 50 days before treatment (Figure 2A). At the age of 80 days, untreated TGRs had significantly greater proteinuria than HanSD rats (32.95±2.0 versus 13.75±2.22 mg of protein per 24 hours; $P<0.05$; Figure 2B). Both bosentan and atrasentan decreased protein excretion to

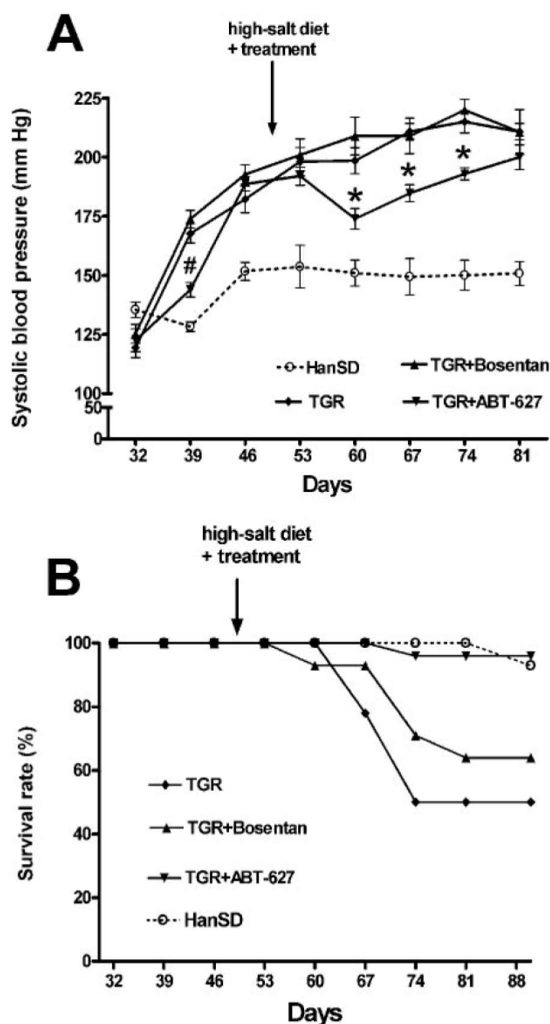

Figure 1. SBP (A) and survival rates (B) during the course of the experiment in homozygous male Ren-2 TGRs (+/+) on high salt intake (HS). #$P<0.01$ vs unmarked values, $P<0.05$; *$P<0.05$ atrasentan (ABT-627) vs untreated and bosentan-treated TGRs.

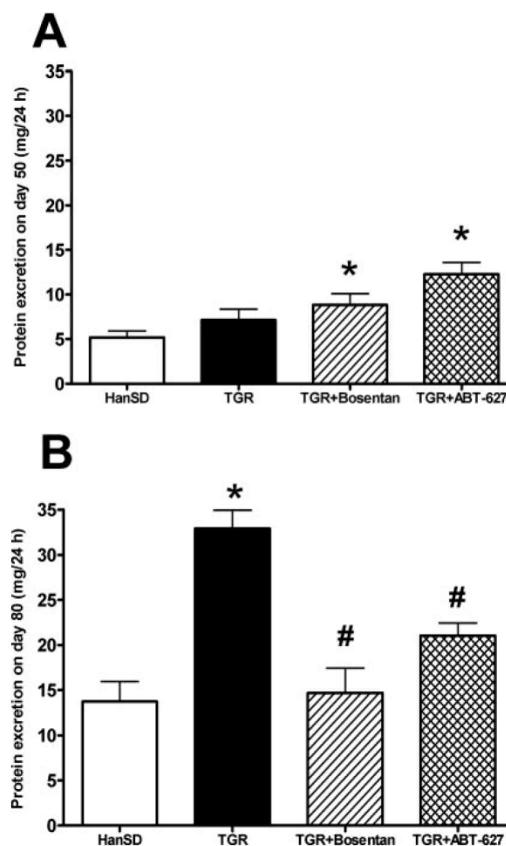

Figure 2. Protein excretion at the age of 50 days before treatment (A) and at the age of 80 days (B) in homozygous male Ren-2 TGRs (+/+) on high salt intake (HS) during treatment. *$P<0.05$ vs normotensive HanSD (−/−); #$P<0.05$ vs untreated TGRs.

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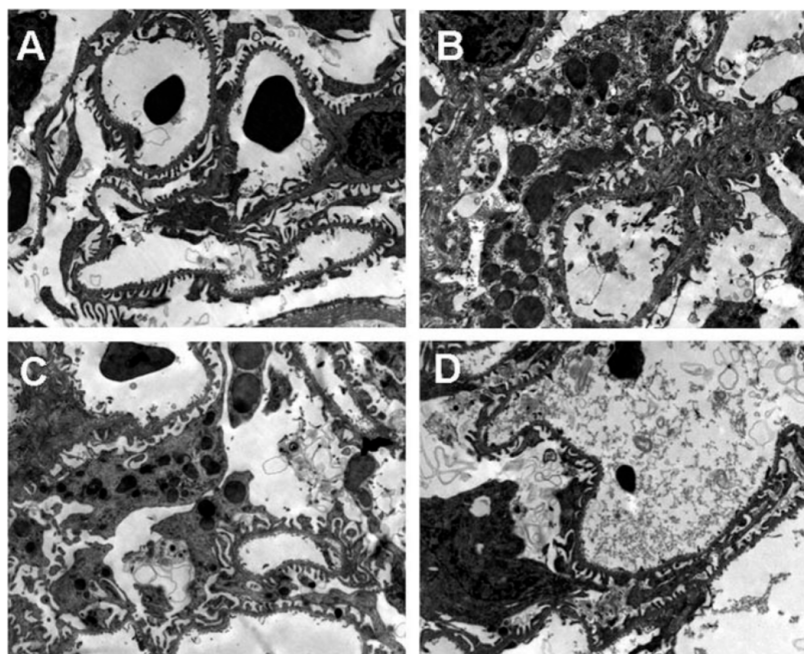

Figure 3. Representative transmission electron micrographs of podocytes and GBMs in HanSD rats (A) and untreated (B), bosentan-treated (C), and atrasentan (ABT-627)-treated (D) TGRs (×4400 magnification).

that found in control HanSD rats (14.70±2.75 and 21.06±1.38 mg of protein per 24 hours, respectively; *P* value not significant).

BW

During the study, HanSD rats weighed statistically more than each group of TGRs. Among the TGR groups, BWs of untreated TGRs were the lowest with reduced weight gain starting on day 39 of age. By day 90 of age, BW of HanSD rats (354.3±8.2 g) was significantly ($P<0.01$) higher than that of untreated and bosentan-treated TGRs (227.6±18.1 and 240.0±10.1 g, respectively). In atrasentan-treated TGRs, it was higher than in the other groups of TGRs (310.7±5.7 g; $P<0.01$) but did not reach that of HanSD rats.

HWs and KWs

In untreated TGRs, the ratio HW/BW was 4.15±0.08 as compared with 3.13±0.09 in HanSD rats ($P<0.01$), but its increase was significantly attenuated by bosentan or atrasentan treatment (3.67±0.04 and 3.61±0.06, respectively; $P<0.01$). Similarly, the ratio of left ventricular weight to BW was 3.08±0.09 in untreated TGRs versus 2.21±0.08 in HanSD rats, and its rise was significantly attenuated by bosentan or atrasentan treatment (2.65±0.04 and 2.58±0.06, respectively; $P<0.01$). There was no difference in the ratios of KW/BW among all of the TGR groups of rats at the end of the experiment (4.74±0.18, 4.62±0.22, and 4.11±0.15 in untreated, bosentan-treated, and atrasentan-treated TGRs, respectively).

Glomerulosclerosis Index

At the end of the experiment, we did not find statistically significant differences in the glomerulosclerosis indices between HanSD rats (0.149±0.016) and the surviving untreated (0.152±0.024), bosentan-treated (0.140±0.018), and atrasentan-treated TGRs (0.160±0.022). Only mild changes of renal parenchyma and mild hyalinosis of afferent arterioles were observed in all 4 groups of rats. Glomerular involvement ranged from slight basement membrane wrinkling to mild focal mesangial expansion. Total glomerular collapse was present only sporadically.

Ultrastructural Examination and GBM Measurement

The only major morphological differences between the groups were seen in the thickness of GBM and in podocyte alterations. As compared with HanSD rats (Figure 3A), TGRs fed a HS diet showed the most striking glomerular changes on the ultrastructural level; that is, irregular thickening, partial disintegration, and focal wrinkling of the GBM were found. The foot processes of podocytes seemed wider than normal and revealed patchy fusion. Sporadically, podocytes contained electron dense granules and lipid droplets in their basal parts (Figure 3B). TGRs treated with bosentan showed only moderate podocyte abnormalities; fusion of foot processes was nearly absent. Foot processes seemed to be thinner and longer. The appearance of lipid droplets and vacuoles within the podocyte cytoplasm was not prevented by bosentan (Figure 3C). Podocytes of atrasentan-treated animals showed a normal structure and an orderly arrangement (Figure 3D) and resembled those of control HanSD rats. A strong correlation ($P<0.001$) between podocyte injury (x=GBM width) and survival rate (y) in rats from all 4 groups has been found ($y=168-0.69\ x$; $r^2=0.7295$; $P<0.0001$).

The width of the GBM on electron microscopic sections in untreated (157.9±2.0 nm) and bosentan-treated (148.6±2.0 nm) TGRs was significantly greater than in HanSD rats (111.0±0.9 nm). We did not find significant differences between atrasentan-treated TGRs (115.2±1.1 nm) and HanSD rats.

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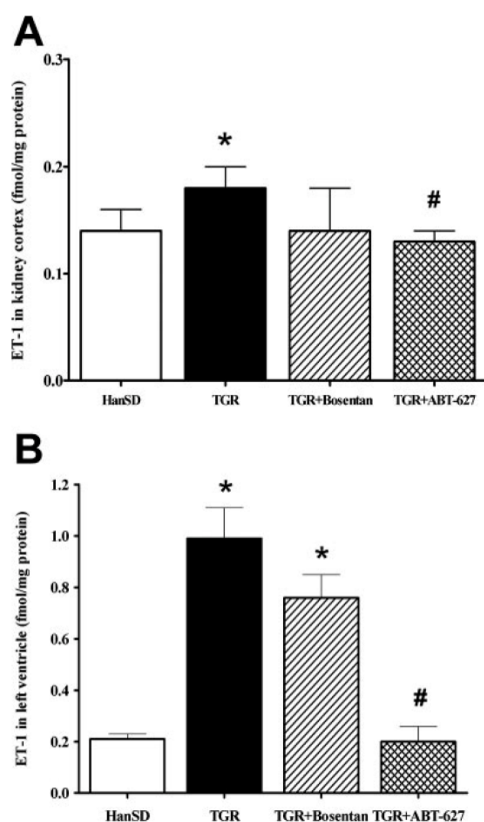

Figure 4. ET-1 concentrations in kidney cortex (A) and in left heart ventricle (B) in HanSD rats (−/−) and in homozygous male Ren-2 TGRs (+/+) on high salt intake (HS) without and with treatment with bosentan or atrasentan (ABT-627). *$P<0.05$ vs normotensive HanSD (−/−); #$P<0.05$ vs untreated TGRs (+/+).

ET-1 Tissue Concentration

Kidney cortex ET-1 content was increased in surviving untreated TGRs when compared with HanSD rats (0.18±0.02 versus 0.14±0.02 fmol/mg protein; $P<0.05$). In bosentan- and atrasentan-treated TGRs, cortical ET-1 concentrations were similar to those in HanSD rats (0.14±0.04 and 0.13±0.01 fmol/mg protein; respectively; Figure 4A).

Left heart ventricular ET-1 content in untreated TGRs exceeded that of HanSD rats >4-fold (0.99±0.12 versus 0.21±0.02 fmol/mg protein; $P<0.05$). In bosentan-treated TGRs, left ventricular ET-1 concentration was moderately but significantly reduced to 0.76±0.09 fmol/mg protein ($P<0.05$) when compared with untreated TGRs, whereas in atrasentan-treated TGRs, it was 0.20±0.06 fmol/mg protein and, thus, similar to that in HanSD rats (Figure 4B).

Discussion

The effects of pharmacological interruption of the ET system have not been studied in homozygous Ren-2 transgenic rats at a time when arterial hypertension is firmly established. In the present study we, therefore, addressed 2 questions: (1) whether ET receptor blockade has a similar protective effect on hypertension, end-organ damage, and survival in homozygous TGRs on a high salt intake when started at a later stage in life as when started immediately after weaning; and (2) whether nonselective ET_A/ET_B or selective ET_A receptor blockade was more effective.

It is generally accepted that increased dietary sodium intake causes hypertension in salt-sensitive humans and animals and induces endothelial dysfunction through enhanced ET-1 production, which may be stimulated by Ang II. Although increased sodium intake should decrease Ang II production, our previous experiments have shown that it does not decrease plasma or kidney Ang II levels in conscious male TGRs between 32 and 90 days of age.[19] Thus, we conclude that the activated circulating and tissue renin–angiotensin system in conscious TGRs is not modulated by or is relatively unresponsive to changes in sodium balance. We also suggest that in TGRs, the enhanced plasma and kidney Ang II would increase ET-1 production, which could be one of the potential mechanisms contributing to the development of hypertension and/or organ damage in TGRs.

We have demonstrated recently[19] that treatment both with the unselective ET_A/ET_B receptor blocker bosentan or with the selective ET_A receptor blocker atrasentan, when started immediately after weaning, markedly improved survival rates of homozygous TGRs and that atrasentan but not bosentan, in addition, induced a significant decrease in BP. Also, selective ET_A blockade had substantial nephroprotective and cardioprotective effects that were superior to unselective receptor blockade. Our present findings are partly compatible with the results of our previous study.[19] ET_A receptor blockade with atrasentan substantially prolonged survival, improved BW, and, in contrast to nonselective blockade with bosentan, also transiently decreased SBP. This only temporary decrease of BP is surprising, because the dose used is generally accepted as sufficient to block ET_A receptors.[24,25] Both atrasentan and bosentan partially reduced cardiac hypertrophy and normalized protein excretion. The most important difference from our previous findings[19] is that, in the present study, bosentan was much less effective in reducing the mortality rate than atrasentan. However, this finding is in accordance with previous results of Rothermund et al[3] who found no improvement of survival in heterozygous Ren-2 rats receiving a nonselective ET_A/ET_B receptor blocker. This lack of effect may be related either to the later onset of drug treatment and/or, more likely, the later introduction of the high-salt regimen, which resulted in a lesser rise in tissue ET-1 concentration. The greater efficacy of selective ET_A receptor than nonselective ET_A/ET_B receptor blockade in improving survival rate may have resulted rather from its antiproliferative than from its antivasoconstrictor actions.[8] There exist 2 types of ET_B receptors, namely, ET_{B1} and ET_{B2} receptors, mediating either vasodilation or vasoconstriction, respectively.[28] Our results favor the possibility that vasoconstrictory ET_{B2} receptors may play a less important role in BP regulation, because concomitant blockade of ET_A and ET_B receptors did not lower BP in contrast to the effect of the ET_A antagonist, which suggests a substantial effect of vasodilatory ET_{B1} receptors. However, conflicting results concerning this issue have been obtained. Although Matsumura et al[29] found in deoxycorticosterone acetate salt rats that selective ET_B blockade has no vasomotor effect, referring to the negligible impact of both vasodilatory ET_B receptors on endothelium

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and vasoconstrictory ET_B receptors on vascular smooth muscle cells, a major role for vasodilatory ET_B receptors was found in hamsters[30] rat[31] and humans,[32] where their blockade resulted in hypertension.

Although our TGR developed strong proteinuria, on light microscopy only moderate alterations in the renal parenchyma were observed in TGRs. By electron microscopy, however, we found significant morphological differences between the experimental groups. They included thickening of the GBM and degenerative changes of podocytes. We found that treatment with atrasentan normalized the thickness of the GBM and reduced the extent of podocyte alterations. Moreover, there was a strong correlation between podocyte injury and survival, showing a great dependency of proper podocyte function on further survival of the animals. Our results are in agreement with studies of Barton et al,[33] which have shown positive effects of selective ET_A blockade on vascular structure. Similar results were shown by Ortmann et al[34] in aged Wistar rats in vivo. Our findings are also compatible with those of Matsumura et al,[29] who found with ET_A receptor blockade a reduction of the histopathologic changes induced by the deoxycorticosterone acetate salt regimen and support their suggestion that ET_A receptor blockade has beneficial structural effects on GBM thickness and the extent of glomerulosclerosis and proteinuria. Podocytes seem, therefore, to be target cells for ET-1 as shown previously by Rebibou et al.[35] However, the exact mechanism(s) leading to the reduction of proteinuria by ET_A receptor blockade are not yet known. On one hand, a plausible explanation for the discrepancy between undetectable glomerulosclerotic changes despite increased proteinuria may be that protein excretion rather correlates with the ultrastructural changes that precede the light microscopic changes. This is supported by the findings of Boffa et al,[36] who found glomerulosclerotic changes not before 4 weeks' duration of hypertension induced by NO deficiency. Moreover, one cannot exclude the possibility that markedly affected animals died before the end of the experiment, and only slightly affected animals survived. Although we did not find a significant proteinuria at the age of 50 days, Springate et al[37] found increased albuminuria already at the age of 2 months in heterozygous TGRs; unfortunately, they did not evaluate the presence of glomerulosclerosis at this time point. On the other hand, the effects of angiotensin-converting enzyme inhibitors cannot be attributed to measurable changes in ultrastructural components of the capillary wall but may rather be related to changes in intrinsic functional properties as shown by Macconi et al.[38] We suppose that pathologic changes of podocytes and of the GBM occur early in the natural course not only of diabetic nephropathy[17] but also of hypertension and may play an important role in microalbuminuria and macroalbuminuria. One mutual trigger mechanism for these events seems to be redox stress.[17] Our findings are in agreement with Nagase et al,[18] who reported immunohistochemically first podocyte impairment at 2 weeks during salt loading in Dahl-sensitive rats when proteinuria was only moderately increased. Ultrastructural changes appeared 3 weeks later. These early ultrastructural changes may bear an important role in the long-term remodeling process of glomerulosclerosis. The close association between podocyte injury and proteinuria suggests that podocyte impairment underlies proteinuria and glomerulopathy in hypertensive homozygous Ren-2 rats. It is postulated that mechanical and oxidative stress are key mediators of podocyte damage.[15]

It is of further interest that TGRs in the present study revealed lower concentrations of ET-1 both in the renal cortex and in the left ventricle than in our previous study.[24] The most plausible explanation for this phenomenon may be that in the present study, TGRs developed a milder form of hypertension and hypertension-related damage because of later onset of high salt intake. This is in line with the results of Whitworth et al,[39] who found significantly increased prepro–ET-1 mRNA expression only in animals with severe hypertension. Interestingly, ET_A blockade strongly lowered ventricular tissue ET-1 content probably because of displacement of ET-1 from the predominating ET_A receptors in this tissue in contrast to the renal tissue with predominant ET_B receptors. Also, in another hypertensive rat model, namely, in the Dahl salt-sensitive rat, Barton et al[33] demonstrated a decrease of ET-1 protein after treatment with the ET_A blocker. Renal ET-1 concentration was not affected by selective ET_A or nonselective ET receptor blockade probably because, in this organ, ET_B receptors dominate, and decreased clearance of ET-1 through ET_B receptors may compensate for the displacement of ET-1 from ET_A (and ET_B) receptors.

Collectively, in the present study BP, protein excretion, glomerulosclerosis index, HW, and tissue ET-1 content in adult TGRs were all lower than in the young TGRs of our preceding study.[19] These findings are compatible with the idea that young animals are more susceptible to hypertensive stimuli[20] (eg, HS intake) and may develop more severe hypertension and hypertension-related end-organ damage and confirm previous suggestions that the ET system is involved in hypertension and hypertension-related organ damage in TGRs on a high salt intake. Most importantly, our results also show that ultrastructural changes of podocytes precede light microscopic histological disturbances and closely correlate with survival rate. They can, therefore, serve as marker of future injury long before manifestation of proteinuria. Chronic selective ET_A blockade starting at the time of established hypertension in homozygous TGRs on a high salt diet proved to have substantial protective effects on survival rate and growth with a transient attenuation of the rise in BP.

Perspectives

This study provides further evidence that selective ET_A receptor blockade is preferential to nonselective ET receptor blockade and implies the possibility of using ultrastructural changes of podocytes as a possible prognostic marker of renal injury.

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Disclosures

None.

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3.5 Early-onset endothelin receptor blockade in hypertensive heterozygous Ren-2 rats.

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Early-onset endothelin receptor blockade in hypertensive heterozygous Ren-2 rats

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Abstract

Male heterozygous Ren-2 transgenic rats and Hannover Sprague–Dawley rats fed a normal or high-salt diet were either untreated or treated with the nonselective receptor ET_A/ET_B receptor blocker bosentan or the selective ET_A receptor blocker, ABT-627, known as atrasentan. Survival rate was partly increased by bosentan and fully normalized by atrasentan. Bosentan did not significantly influence the course of hypertension in TGR, whereas atrasentan significantly decreased BP on both diets. Atrasentan substantially reduced proteinuria, cardiac hypertrophy, glomerulosclerosis and left ventricular ET-1 tissue concentration on both diets. Our data indicate that ET_A receptor blockade is superior to nonselective blockade in attenuating hypertension, end-organ damage and improving survival rate.
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Keywords: Endothelin receptors; Bosentan; Atrasentan; Ren-2 rats; End-organ damage

1. Introduction

Almost two decades ago, the potent vasoconstrictor peptide endothelin-1 (ET-1) was discovered (Yanagisawa et al., 1988). Its action is mediated by two types of receptors, namely ET_A and ET_B receptors. While ET_A receptors in the vascular system mediate vasoconstriction and are localized on vascular smooth muscle cells, the major function of ET_B receptors, localized mainly on endothelial cells, seems to be vasodilation in addition to its clearance function. Moreover, two distinctive ET_B receptors–ET_{B1} and ET_{B2}–with quite opposing function were identified in the rat (Gellai et al., 1996).

Since its discovery, a growing body of evidence has been accumulated showing that ET-1 plays a pivotal role in several cardiovascular diseases, including chronic heart failure, ischemic heart disease, hypertension, atherosclerosis, pulmonary hypertension and chronic heart failure. In these disease states, the levels of circulating ET-1 are increased, and treatment with ET inhibitors proved to be advantageous (Masaki, 2004). Thus, e.g. the nonspecific ET receptor blocker bosentan has been approved as a therapeutic agent to treat pulmonary hypertension (Kenyon and Nappi, 2003). However, despite the increasing evidence that the ET system plays an important role in the pathogenesis of systemic arterial hypertension, its mechanism of action is still not well understood.

Our present experiments were therefore performed, first, to evaluate the role of ET-1 in the onset and maintenance of hypertension in heterozygous Ren-2 transgenic rats. Heterozygous rats transgenic for the mouse renin gene (TGR) (strain name TGR(mRen2)27), a model of monogenetically defined hypertension (Mullins et al., 1990), exhibit a salt-sensitive component (Callahan et al., 1996). Special emphasis was given to the difference in action between the nonselective ET_A/ET_B and the specific ET_A receptor blockade. The benefit of specific

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ET_A receptor blockade may relate to the fact, that, contrary to the nonspecific receptor blockade, it does not inhibit the vasodilatory (de Nucci et al., 1988) and natriuretic (Konishi et al., 2002) response to stimulation of ET_B receptors. Besides their vasoconstrictory function, ET_A receptors also mediate cell proliferation of various cell types, especially of vascular smooth muscle cells (Hirata et al., 1989). Therefore, the blockade of ET_A receptors may be beneficial in attenuating vascular alterations leading to end-organ damage. This antiproliferative action of ET_A receptor blockers has even attracted the attention of researchers in the field of cancer research (Salani et al., 2002; Nelson, 2003).

Since dietary sodium plays an important role in the pathogenesis of hypertension not only in humans (Weinberger, 1996) but also in salt-sensitive models of hypertension (Dahl et al., 1968), our second aim was to evaluate the influence of high-salt intake on the course of hypertension, end-organ damage and survival, as well as the potential role of ET-1 in TGR under these conditions. It is generally accepted that young animals are more susceptible to various hypertensinogenic stimuli (Zicha and Kunes, 1999) and also that therapeutic interventions made in these early periods of life are more effective. However, discrepant results were reported in heterozygous TGR, i.e. either no effect (Rothermund et al., 2003a) or hypotensive (Gardiner et al., 2000) effects of nonselective or selective ET receptor blockade were found in adult animals, whereas no effect was found in young animals (Whitworth et al., 1995; Rossi et al., 2000). Therefore, in the present study we evaluated the effects of nonselective as compared to selective ET receptor blockade in TGR on different sodium diets when treatment with receptor blockers was started early in their life.

2. Materials and methods

The protocols in the present study were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" and were approved by Czech Animal Care and Use Committee (Protocols 79/2001 and 923/2003).

2.1. Animals

We used male heterozygous rats transgenic for the mouse renin gene [TGR; strain name TGR(mRen2)27] and male Hannover Sprague–Dawley rats (HanSD) as normotensive controls. Animals were housed under standard conditions and had free access to chow and water. All animals used in this study were bred at the Center for Experimental Medicine of the Institute for Clinical and Experimental Medicine from stock animals supplied from Max Delbrück Center for Molecular Medicine in Berlin, Germany.

2.2. Experimental protocol

Animals were fed either a normal salt (NS, 0.45% NaCl) or high-salt diet (HS, 2% NaCl) starting on day 29 of age. At this time point, either nonselective ET_A/ET_B receptor blockade by bosentan, or selective ET_A receptor blockade by atrasentan was initiated. Bosentan (Actelion, Alschwil, Switzerland) was added to the diet. The concentration in the food was calculated to deliver a dose of 100 mg kg^{-1} day^{-1} (Roux et al., 1999). This dose was previously validated in our laboratory to effectively block ET receptors (Dvorak et al., 2004). The selective ET_A receptor blocker atrasentan (Abbott, Chicago, USA) was added to the drinking fluid; the dose was adjusted weekly to provide a concentration of 5 mg kg^{-1} day^{-1} (Mulder et al., 2000), which is generally accepted to effectively block ET_A receptors (Opgenorth et al., 1996; D'Angelo et al., 2005). At the start of the experiments, the animals were allotted to eight groups receiving either normal salt (NS) or high-salt diet (HS). As controls age-matched HanSD rats on the same regimens were investigated.

The following experimental groups were studied:

HanSD + NS (n = 24)
TGR + NS (n = 18)
TGR + NS + bosentan (n = 18)
TGR + NS + atrasentan (n = 18)
HanSD + HS (n = 24)
TGR + HS (n = 24)
TGR + HS + bosentan (n = 18)
TGR + HS + atrasentan (n = 23).

2.3. Blood pressure, proteinuria and tissue weight

From day 29 onwards, regular measurements of body weight and systolic BP (SBP) were made at weekly intervals using the tail plethysmography method (Hatteras Instruments, Cary, North Carolina, USA). At the age of 50 and 80 days, animals were housed in metabolic cages so that fluid intake could be monitored and urine collected. Urinary protein concentration in 24 h urine was measured by a biuret method (Lachema, Czech Republic).

By day 90, animals were weighed, anesthetized with thiopental sodium (50 mg kg^{-1}) and mean arterial pressure (MAP) was monitored directly in the carotid artery using the data acquisition system PowerLab (ADInstruments, Mountain View, California, USA). Kidneys and hearts were weighed. Ratios of kidney weight/body weight (KW/BW) and heart weight/body weight (HW/BW) were used as indices of organ hypertrophy.

2.4. Tissue ET-1 concentrations

Left heart ventricles were rapidly removed and cortex from the right kidney was quickly dissected. Both tissues were immediately frozen in liquid nitrogen for ET-1 determination using an enzyme-linked immunosorbent assay test (ELISA) (Amersham, Braunschweig, Germany).

2.5. Histological examination

The left kidney was quickly removed, fixed in 4% buffered formaldehyde, dehydrated and embedded. Paraffin sections

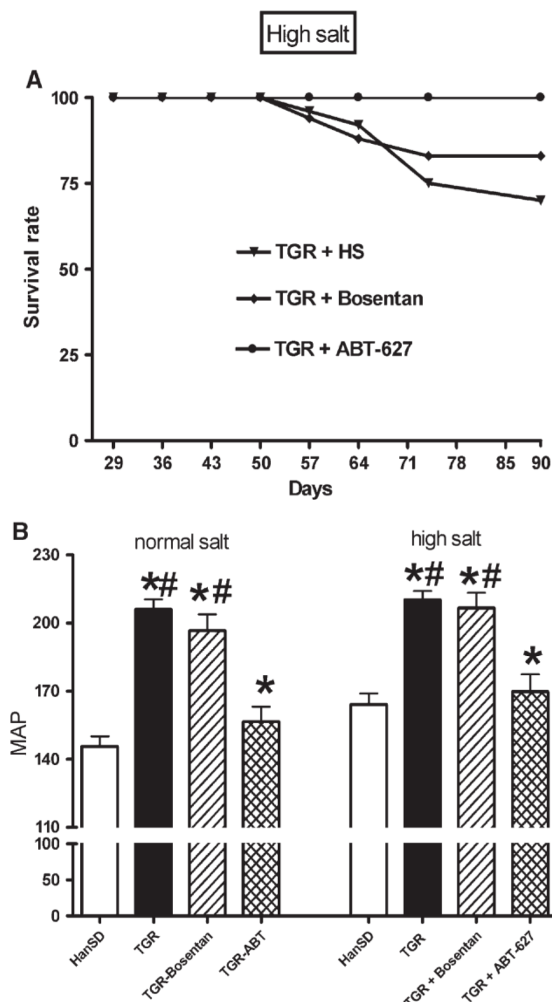

Fig. 1. Survival rate during the course of the experiment on high-salt diet (A) and mean arterial pressure on termination of the experiment (day 90) (B) in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal or high-salt intake. $*p<0.05$ versus unmarked values, $^{\#}p<0.05$ versus all other values.

were stained with hematoxylin eosin and periodic acid-Schiff reaction and examined using a Nicon Eclipse E 600 light microscope. Slides were evaluated in a blind fashion. As described previously (Yagil et al., 2002), fifty glomeruli were examined on a semi-quantitative scale: grade 0 = all glomeruli normal; grade 1 = 1–2 glomeruli affected; grade 2 = more than 2 but less than 17 glomeruli affected; grade 3 = 17 or more glomeruli affected.

2.6. Statistical analysis

Statistical analysis of data was performed using Graph-Pad Prism software (Graph Pad Software, San Diego, California, USA). The data were evaluated by two-way ANOVA with repeated measures. We have two grouping factors (diet and therapy) and one within factor (measurement). Newman–Keuls post hoc test was used for multiple comparison. Statistical comparisons of the results obtained for heart and kidney weights and for ET-1 concentrations were made by one-way ANOVA. Unless noted, values are expressed as mean ± S.E.M. and n represents the number of animals. A p-value less than 0.05 was considered significant.

3. Results

3.1. Survival rate

All TGR groups on NS diet survived to the end of the experiment. Untreated and bosentan-treated TGR on the HS diet started to die on day 50 (Fig. 1A) and their survival rates on termination of the experiment were 70% and 83%, respectively. In contrast, the administration of atrasentan markedly improved survival to 100% at the end of the experiment. Zero mortality was found in HanSD rats on both diets.

3.2. Systolic and mean arterial blood pressure

SBP of untreated and bosentan-treated TGR rose constantly throughout the course of the experiment on both diets, reaching on day 78 on the NS diet 234.6 ± 3.6 and 214.2 ± 4.1 mm Hg,

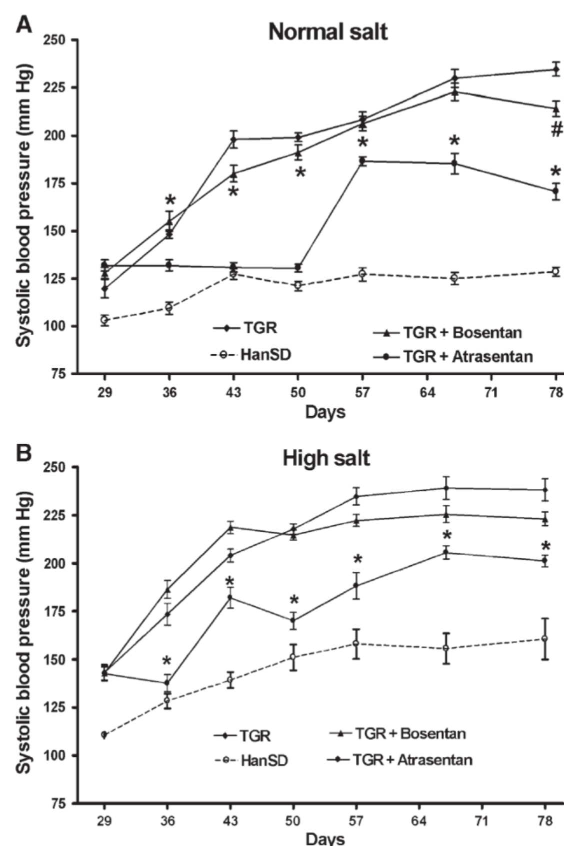

Fig. 2. Systolic blood pressure during the course of the experiment in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal (A) or high-salt (B) intake. $*p<0.05$ unmarked values, $^{\#}p<0.05$ bosentan-treated versus

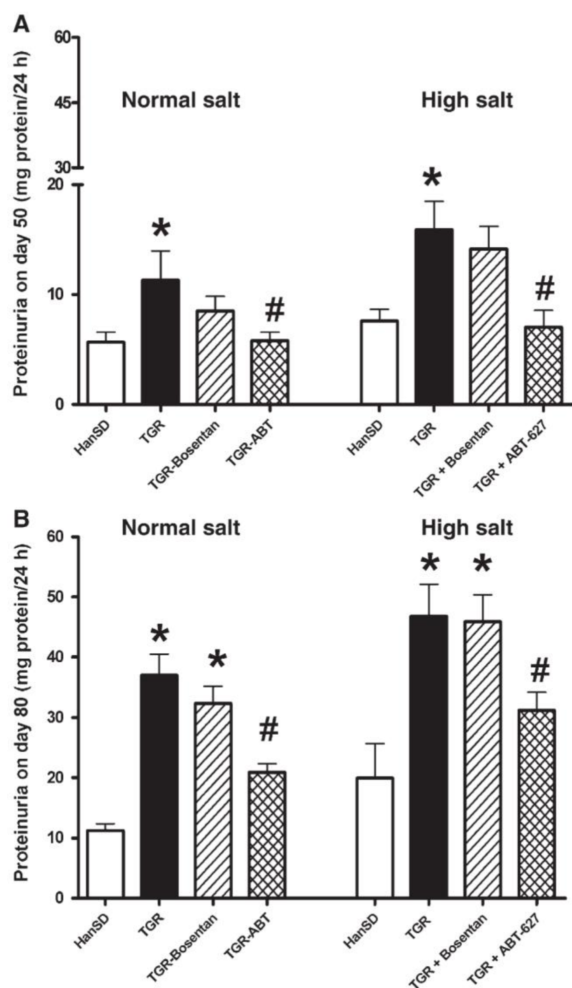

Fig. 3. Proteinuria at the age of 50 days (A) and 80 days (B) in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal or high-salt intake. *$p<0.01$ versus control HanSD, #$p<0.01$ versus untreated and/or bosentan-treated TGR.

respectively ($p<0.05$) (Fig. 2A) and on HS diet 238.2±5.8 and 223.1±3.5 mm Hg, respectively (n.s.) (Fig. 2B). From day 36 onwards (i.e. within 1 week of commencement of atrasentan treatment), SBP in atrasentan treated TGR on both diets was significantly lower than in untreated and bosentan-treated TGR, reaching on day 78 on the NS diet 170.7±4.3 mm Hg and on the HS diet 201.2±3.0 mm Hg ($p<0.01$). A highly significant increase in BP during the whole experiment induced by HS diet was found in all examined groups. SBP in control HanSD was significantly lower than in corresponding TGR groups on both diets and remained in the normotensive range during the whole experimental period, with 128.6±2.4 on the NS diet and 140.6±10.7 mm Hg on the HS diet. At the end of the experiment, MAP was significantly higher in untreated and bosentan-treated TGR than in their normotensive controls either on the NS (206.0±4.4 and 196.6±7.2 mm Hg, respectively; n.s.) or on the HS diet (210.2±3.9 and 206.6 ±6.7; n.s.) (Fig. 1B). On the contrary, atrasentan treatment resulted in a substantially lower MAP (156.6±6.5 on the NS and 169.9±7.4 mm Hg on the HS, $p<0.01$) when compared with untreated and bosentan-treated animals.

3.3. Proteinuria

HanSD on the NS diet exhibited low proteinuria on day 50 (5.6±0.92 mg protein/day) (Fig. 3A), which was only moderately increased by the HS diet (7.59±1.05 mg protein/day), and, as expected, both values had further increased on day 80 (11.56±1.15 on NS and 19.96± 5.78 mg protein/day on HS, respectively) (Fig. 3B). In contrast, protein excretion of untreated TGR on NS and HS diets was significantly increased on days 50 and 80, being more prominent on day 80. With bosentan treatment, there was a tendency for a partial reduction of proteinuria, while atrasentan treatment normalized protein excretion almost to the levels observed in HanSD animals.

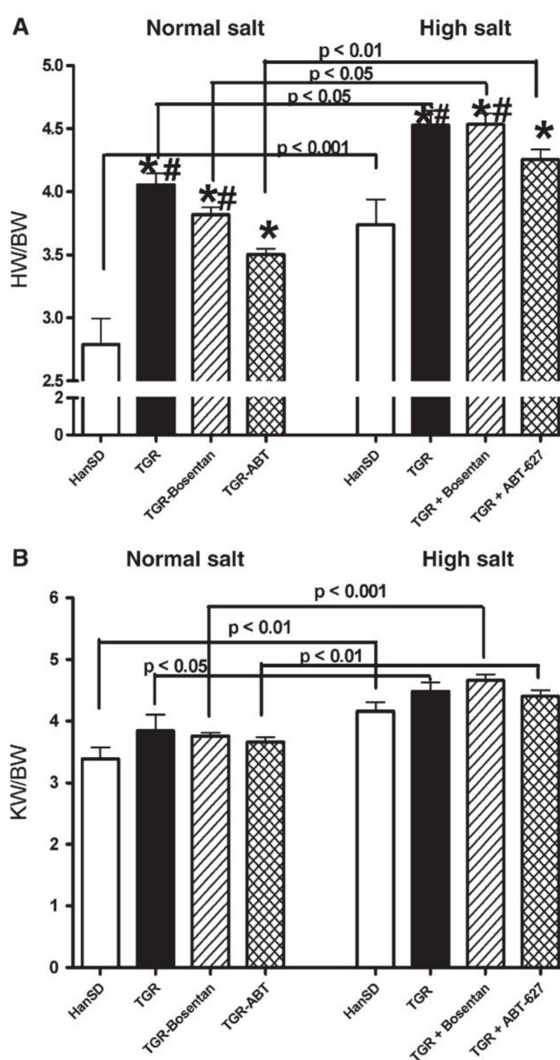

Fig. 4. Indices of HW/BW (A) and KW/BW (B) in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal or high-salt intake. Indices given in arbitrary units. *$p<0.05$ versus unmarked values, #$p<0.05$ versus all other values.

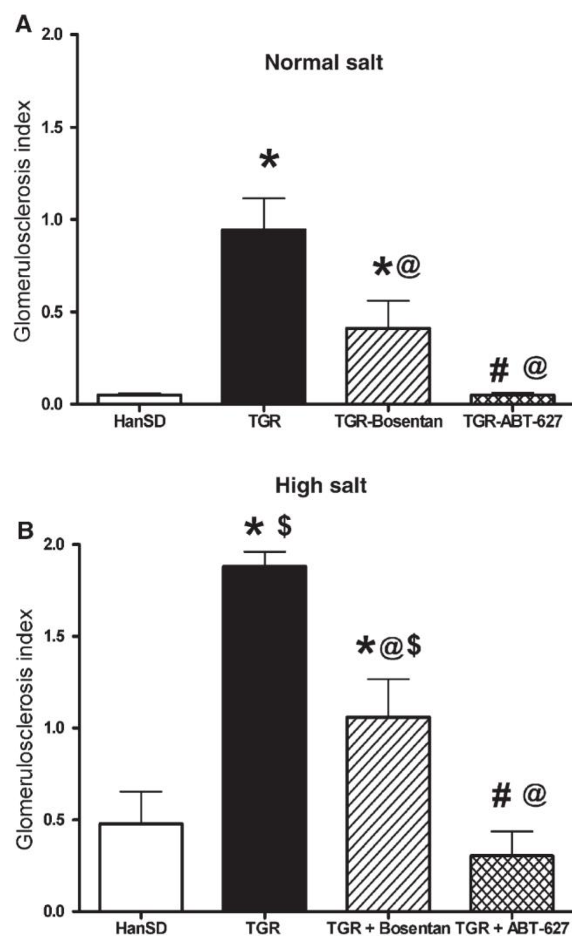

Fig. 5. Glomerulosclerosis indices in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal (A) or high-salt (B) intake. $*p<0.05$ versus control HanSD, $^{\#}p<0.05$ versus bosentan-treated TGR, $^{@}p<0.05$ versus untreated TGR, \$ versus corresponding group on NS diet.

3.4. Body and organ weights

No significant differences in body weight gains were observed between TGR groups throughout the course of the experiment; moreover, the dietary regimens had no influence on body weight gain (data not shown). Indices of HW/BW and KW/BW are depicted in Fig. 4. As expected, untreated TGR showed a significantly higher index of HW/BW than HanSD (4.06±0.09 versus 2.79±0.21, respectively, on NS and 4.53±0.12 versus 3.74±0.20, respectively, on HS diet) (Fig. 4A). Treatment with bosentan had no effect on HW/BW ratio (3.82±0.06 on NS and 4.53±0.09 on HS). On the contrary, administration of atrasentan caused a substantial decrease of HW/BW ratio both on NS (3.54±0.05) and on HS diet (4.26±0.08). HS diet induced greater cardiac hypertrophy than NS diet in all groups of rats.

No significant differences in the KW/BW indices among all examined groups were observed either on NS or HS diets; however, indices of KW/BW were significantly increased in rats on the HS as compared to rats on the NS diet (Fig. 4B).

3.5. Glomerulosclerosis index

There were almost no signs of glomerular injury in control HanSD rats on both diets (Fig. 5A and B). However, a substantial increase in glomerulosclerosis was found in untreated TGR on the NS diet and that increase was further intensified by HS intake. Bosentan treatment partly decreased, whereas atrasentan fully attenuated these organ changes.

3.6. ET-1 tissue concentration

As shown in Fig. 6A, HS diet significantly increased left ventricular ET-1 content in all groups of animals. Left ventricle ET-1 content of untreated TGR substantially exceeded that of HanSD. Both, bosentan and especially atrasentan, significantly reduced ET-1 concentrations in heart ventricles even below HanSD. Similarly, ET-1 in the kidney cortex (Fig. 6B) was

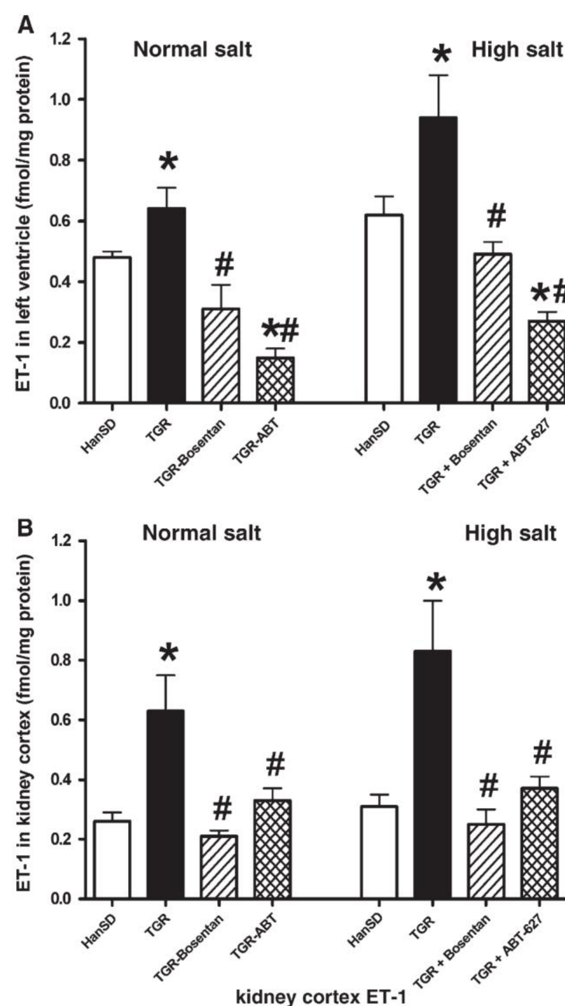

Fig. 6. Endothelin-1 concentrations in the left ventricle (A) and in the kidney cortex (B) in HanSD rats (−/−) and in heterozygous male Ren-2 transgenic rats (TGR; +/−) on normal or high-salt intake without and with bosentan or atrasentan treatment. $*p<0.05$ versus control HanSD, $^{\#}p<0.05$ versus untreated TGR.

significantly higher in untreated TGR than in HanSD. Interestingly, a more pronounced decrease in kidney ET-1 concentration was found in bosentan-treated than in atrasentan-treated animals. No significant difference was observed between rats on the HS and rats on the NS intake.

4. Discussion

Heterozygous TGR provide a suitable model of hypertension, since–in contrast to homozygous TGR who develop severe malignant hypertension–their hypertension is milder, thus allowing long-term studies. Accordingly, the *first goal* of our current study was to compare the potentially beneficial effects of a nonselective ET_A/ET_B with a selective ET_A receptor blockade in young heterozygous TGR on a *normal salt intake*. Since our previous study (Opocensky et al., 2004) has shown that chronic treatment of heterozygous TGR on a high-salt diet with the nonselective ET_A/ET_B receptor blocker bosentan substantially improved the survival rate and reduced end-organ damage without altering BP, *the second goal* of our study was to evaluate the influence of *high-salt intake* on the course of hypertension, the degree of end-organ damage, and the survival in young heterozygous TGR, as well as the potential effects of ET receptor blockade.

In our current study, in which the treatment period was half as long as in the previous one (Opocensky et al., 2004), we have first confirmed part of our previous findings, namely that the nephroprotective effects of bosentan were independent of BP changes. We then extended this observation especially to study the effects of a selective ET_A receptor blockade on hypertension and related end-organ damage. With respect to the lack of a BP lowering effect of bosentan, our findings are in accordance with previous observations in young TGR reported by Whitworth et al. (1995) and Rossi et al. (2000). In fact, increasing evidence indicates that the hypertensinogenic and organ damaging effects of ET-1 are mediated via ET_A receptor activation especially in the models of salt-dependent hypertension (Moreau and Schiffrin, 2003). On one hand, it is therefore not surprising that we found a substantial BP lowering effect of ET_A receptor blockade with atrasentan in our young heterozygous TGR. On the other hand, we have no plausible explanation that Rossi et al. (2000) did not find any decrease in BP with selective ET_A receptor blockade except that they used a different ET_A receptor blocker, namely BMS-182874, during a shorter treatment period. However, discrepant results with ETA receptor blockers were also found in other experimental hypertensive models. While no influence on BP was found in one-clip two-kidney hypertension (Ehmke et al., 1999; Saam et al., 2003) and in double renin transgenic rats (Bohlender et al., 2000), a BP lowering effect of ET_A receptor blockade was detected in the Sabra salt-sensitive strain (Rothermund et al., 2003b), in DOCA-salt rats (Allcock et al., 1998), and in stroke-prone SHR (Blezer et al., 1999). However, only in the latter experimental model studies were performed in young animals thus resembling the conditions of our present study. Moreover, our previous experiments in homozygous TGR support the idea of a substantial impact of ET_A receptor blockade on BP at an early age (Vaneckova et al., 2005), while a decreasing effect of this receptor blockade was found with increasing age (Opocensky et al., submitted for publication). The difference in the efficacy of nonselective versus selective blockade is probably due to the fact that the latter inhibits only the vasoconstrictory ET_A receptors and not the vasodilatory ET_B receptors mediating the release of NO and prostaglandins (de Nucci et al., 1988). In addition, the role of ET_B receptors as promotors of natriuresis has been shown in ET_B-deficient rats, which became hypertensive when placed on a high-salt diet (Gariepy et al., 2000). As a cause of hypertension in this model, the lack of normal functioning renal tubular epithelial sodium channels has been suggested (Gariepy et al., 2000). In these animals, selective ET_A blockade decreased systolic BP (Elmarakby et al., 2004). Interestingly, recent studies with renal cross-transplantation have shown that salt sensitivity is related probably to ET_B receptors localized extrarenally (Ohkita et al., 2005).

Regarding the second goal of our study, namely to evaluate the influence of high-salt intake on the course of hypertension, the degree of end-organ damage, and the survival in heterozygous TGR, as well as the potential effects of ET receptor blockade, we found that high-salt diet significantly accelerated the development of hypertension in heterozygous TGR. Moreover, the effect of high-salt intake on the course of hypertension was significant not only in untreated animals but also in bosentan- and atrasentan-treated TGR showing clearly that this strain carries a salt-sensitive component (Callahan et al., 1996). In addition, high-salt diet increased mortality and worsened renal and cardiac damage in this strain. Contrary to our previous finding in heterozygous TGR (Opocensky et al., 2004), in the present study bosentan was only partly effective in opposing these effects. One possible explanation of the smaller efficacy of bosentan might be that in the present study treatment with bosentan lasted only half the time of that in our previous one. In contrast, atrasentan substantially reduced both SBP and MAP, improved survival and had substantial nephro- and cardioprotective effects.

Similar to our previous study on homozygous TGR (Vaneckova et al., 2005), left ventricular ET-1 content, which was substantially lower in our heterozygous than in homozygous TGR, was decreased only partly with bosentan but to a significantly greater extent with atrasentan treatment. This correlates with the findings of Whitworth et al. (1995), who found increased preproET-1 mRNA expression in their animals with severe hypertension. Lower left ventricular ET-1 concentration following atrasentan treatment is in accordance with the findings of Barton et al. (1998) in Dahl salt-sensitive rat, who observed a decreased ET-1 protein content after ET_A receptor blockade, which is probably due to the greater displacement of ET-1 from the ET_A receptors predominating in this tissue.

In line with the recent work of Ortmann et al. (2004), who have shown in aged Wistar rats with podocyte injury a positive effect of selective ET_A receptor blockade on glomerulosclerosis and proteinuria, we also found a similar reduction of renal injury in our heterozygous TGR. Although the exact mechanism is not known, a possible explanation may be derived from the influence of ET receptor blockade on podocytes, which are the

gatekeepers of albumin passage and which are supposed to be affected by ET.

The above-mentioned findings show that the crucial question of the difference between the effectivenes of selective versus nonselective receptor blockers requires further investigations. The unselective blockade suppresses not only vasoconstrictory and proliferative actions mediated by ET_A receptors but concomitantly blocks the vasodilatory actions–mediated through the release of nitric oxide and prostaglandins (de Nucci et al., 1988)–and natriuretic actions mediated by ET_B receptors (Konishi et al., 2002). Even more confusing are the results of a very recent study of Inscho et al. (2005), which supports the idea that both types of ET receptors mediate vasoconstriction of renal afferent arterioles and that there exists a possible interaction between ET_A and ET_B receptors in controlling afferent arteriolar diameter. An interaction of ET_A and ET_B receptors has already been proposed by Just et al. (2004), who–in evaluating the physiological function of the ET system–speculated that not only dual actions of ET_B receptors but also their interaction with ET_A receptors must be taken into account.

5. Conclusions

Taken together, our results strongly support the suggestion that high-salt intake accelerates hypertension and associated end-organ damage in young heterozygous TGR when treated from weaning. They also show that, in contrast to nonselective ET receptor blockade, specific blockade of ET_A receptors exerts substantial positive effects on BP, organ damage and survival on both normal and especially on the high-salt diet. Selective ET_A receptor blockade may provide a new tool for the treatment of salt-sensitive hypertension.

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3.6 Late-onset endothelin receptor blockade in hypertensive heterozygous Ren-2 transgenic rats.

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Late-onset endothelin receptor blockade in hypertensive heterozygous REN-2 transgenic rats

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Abstract

Our previous studies in heterozygous Ren-2 transgenic rats (TGR) have shown that early treatment with selective endothelin (ET)$_A$ receptor blockade is superior to nonselective $ET_{A/B}$ receptor blockade. The aim of this study was to evaluate the role of the ET system in male heterozygous TGR with established hypertension (late-onset treatment). TGR and control Hannover Sprague–Dawley (HanSD) rats were fed a high-salt diet and were treated concomitantly with the nonselective $ET_{A/B}$ receptor blocker bosentan or the selective ET_A receptor blocker atrasentan from day 52 of age on. Survival rate was partly increased by bosentan and fully normalized with atrasentan. Bosentan transiently decreased blood pressure (BP), whereas atrasentan significantly reduced BP as early as one week after the start of the treatment. This effect persisted for the whole experimental period. Atrasentan also substantially reduced cardiac hypertrophy, proteinuria, glomerulosclerosis and left ventricle ET-1 content. Bosentan improved and atrasentan almost restored podocyte architecture and reversed changes in podocyte phenotype represented by the expression of CD 10, desmin and vimentin. Our results demonstrate that selective ET_A receptor blockade has more favorable effects than nonselective $ET_{A/B}$ receptor blockade and, unlike observed in homozygous TGR, ET_A receptor blockade has similar effects in heterozygous rats with established hypertension as in young animals with developing hypertension.
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Keywords: ET_A and ET_B receptors; Heterozygous transgenic Ren-2 rats; Hypertension; End-organ damage; Podocytes

1. Introduction

Endothelin (ET)-1 is a powerful vasoconstrictor (Yanagisawa et al., 1988) and, moreover, has mitogenic properties both in vivo and in vitro (Hirata et al., 1989). ET-1 is the most abundantly produced ET being released by a dual secretory pathway — abluminally by endothelial cells toward underlying smooth muscle cells via the constitutive pathway, and, in contrast, in response to external stimuli from endothelial cell-specific storage granules (Davenport and Maguire, 2006). The action of ET is mediated by activation of two G-protein-coupled receptor subtypes, ET_A and ET_B. While ET_A receptors mediate vasoconstriction and are localized on vascular smooth muscle cells, ET_B receptors are localized mainly on endothelial cells and exert largely vasodilatation in addition to their clearance function mediated by smooth muscle cell ET_B receptors (Brunner et al., 2006). Moreover, they promote renal tubular natriuresis (Ge et al., 2006). However, although ET_B receptors

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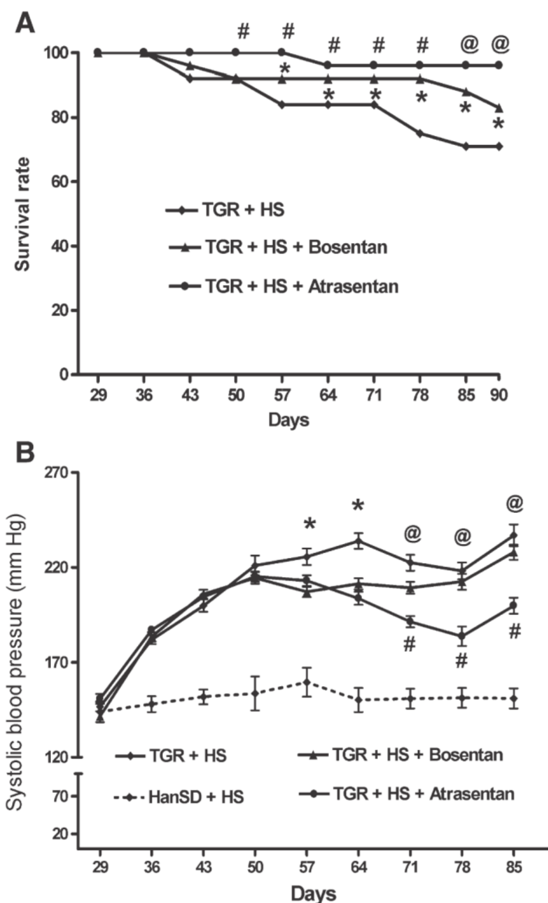

Fig. 1. Survival rates (A) and systolic blood pressure (B) during the course of the experiment in heterozygous male Ren-2 transgenic rats (TGR; +/−) on high-salt intake (HS). $*p<0.05$ bosentan vs. untreated TGR. # $p<0.01$ atrasentan vs. untreated TGR, $p<0.05$ @ atrasentan- vs. bosentan-treated TGR.

were primarily thought to cause vasodilation and natriuresis and thus mouse knockout for collecting duct endothelin B receptors displayed hypertension and sodium retention (Ge et al., 2006), Fink et al (2007) have shown recently that chronic activation of ET_B receptors with sarafotoxin 6c caused hypertension, for which a possible role of sympathetic mediation has been suggested (Pollock et al., 2000).

The detrimental effects of ET-1 in the development of hypertension is ascribed to the activation of ET_A receptors, especially their mitogenic functions (Bouallegue et al., 2007). Therefore, it is not surprising that ET_A receptor blockade results in organ protection (Barton et al., 2006). Moreover, the advantage of selective ET_A receptor blockade is also given by the fact that nonselective receptor blockade inhibits not only the vasoconstrictory effects of ET_A receptors but concomitantly also the beneficial actions of vasodilatory and natriuretic ET_B receptors.

There is a great body of evidence showing that ET production is stimulated by angiotensin II (ANG II) and vice versa and that the ET system plays an important role in the pathogenesis of ANG II-dependent and salt-dependent models of hypertension (Schiffrin, 2005). We (Vaneckova et al., 2006) and others (Pollock and Pollock, 2001) have shown that ET system is significantly activated with increased sodium intake. Although ET_A receptor blockade lowers BP predominantly in salt-dependent models of hypertension, also a substantial impact of ET_B receptors on condition of high-salt intake in promoting higher sodium excretion from the body has been described (Vassileva et al., 2003).

Ren-2 transgenic rats [official strain name TGR(mRen2-27)] represent a model of ANG II-dependent malignant hypertension (given by the insertion of a murine renin gene) with a strong salt-dependent component (Callahan et al., 1996; Vaneckova et al., 2006). Its homozygous line develops severe hypertension with its typical signs of malignant hypertension, i.e. weight loss and impaired kidney and cardiac functions leading to high mortality. Heterozygous animals are therefore more suitable for longterm studies.

In our previous study with late-onset treatment in homozygous TGR, we have found that ultrastructural podocyte damage precedes morphological damage and proteinuria. Therefore we focused in this study on immunohistochemical changes occurring in the early stages of renal hypertensive injury. To assess changes in podocyte phenotype we used desmin (podocyte injury marker), CD 10, α smooth muscle actin (cytoskeletal protein) and intermedial filament protein vimentin.

The approach to treat animals after hypertension has developed is much closer to the clinical situation. Therefore, although it is a well-known fact that young animals are more susceptible to hypertensive stimuli and also to various pharmacological interventions (Kunes and Zicha, 2006), we postponed treatment until the time of established hypertension.

2. Materials and methods

The protocols in the present study were designed in adherence to the "Guide for the Care and Use of Laboratory Animals" and were approved by the Czech Animal Care and Use Committee (protocol 3/2007).

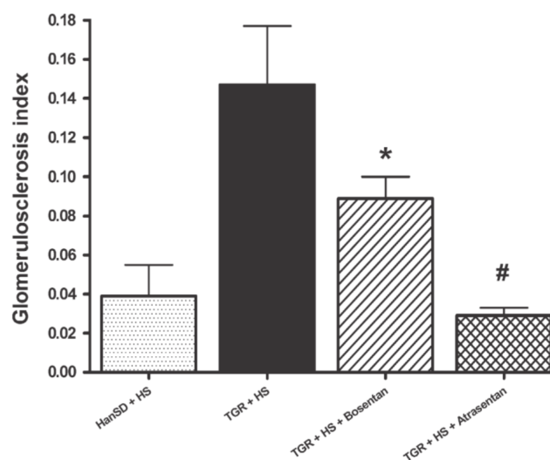

Fig. 2. Glomerulosclerosis index in heterozygous male Ren-2 transgenic rats (TGR; +/−) on high-salt intake (HS) at the end of the experiment. $*p<0.01$ vs. unmarked value, # $p<0.01$ vs. untreated TGR.

2.1. Animals

Heterozygous transgenic rats [TGR; strain name TGR (mRen2)27] and their normotensive Hannover Sprague–Dawley (HanSD) control rats were housed at 25 °C under a 12 h light/dark cycle and had free access to chow and water. All animals used in this study were bred at the Center for Experimental Medicine of the Institute for Clinical and Experimental Medicine from stock animals supplied from Max Delbrück Center for Molecular Medicine, Berlin, Germany.

2.2. Experimental design

Animals were placed on a high-salt diet (HS, 2% NaCl) at the age of 52 days. At the same time, either nonselective ET_A/ET_B receptor blockade by bosentan, or selective ET_A receptor blockade by atrasentan was started. Bosentan (Actelion, Alschwil, Switzerland) was mixed to the high-salt diet in an amount depending on the actual food intake in order to achieve a final consumption of 100 mg kg^{-1} day^{-1} (Roux et al., 1999). The selective ET_A receptor blocker atrasentan (Abbott Laboratories, Abbott Park, IL, USA) was added to the drinking fluid; the dose was adjusted weekly to provide a dose of 5 mg kg^{-1} day^{-1}.

The following experimental groups were investigated:

Male HanSD rats + HS (n = 14)
Male heterozygous TGR + HS (n = 18)
Male heterozygous TGR + HS + bosentan (n = 14)
Male heterozygous TGR + HS + atrasentan (n = 24)

2.3. Blood pressure, proteinuria and organ weights

From day 32 of age on, rats were weighed and systolic blood pressure (SBP) was measured once a week by the tail-cuff method previously validated in our laboratory (Heller and Hellerova, 1998). At the age of 50 and 80 days, animals were individually housed in metabolic cages and measurements of fluid consumption, urine volume as well as proteinuria were monitored over 24 h. Urinary protein determination was performed using the Biuret method (Lachema, Czech Republic).

On termination of the experiment (day 90), animals were weighed, anesthetized with thiopental sodium (50 mg kg^{-1}) and mean arterial pressure (MAP) was monitored directly in the carotid artery using the data acquisition system PowerLab (ADInstruments, Mountain View, California, USA). Kidneys and heart left ventricles were rapidly excised and weighed. Ratios of kidney

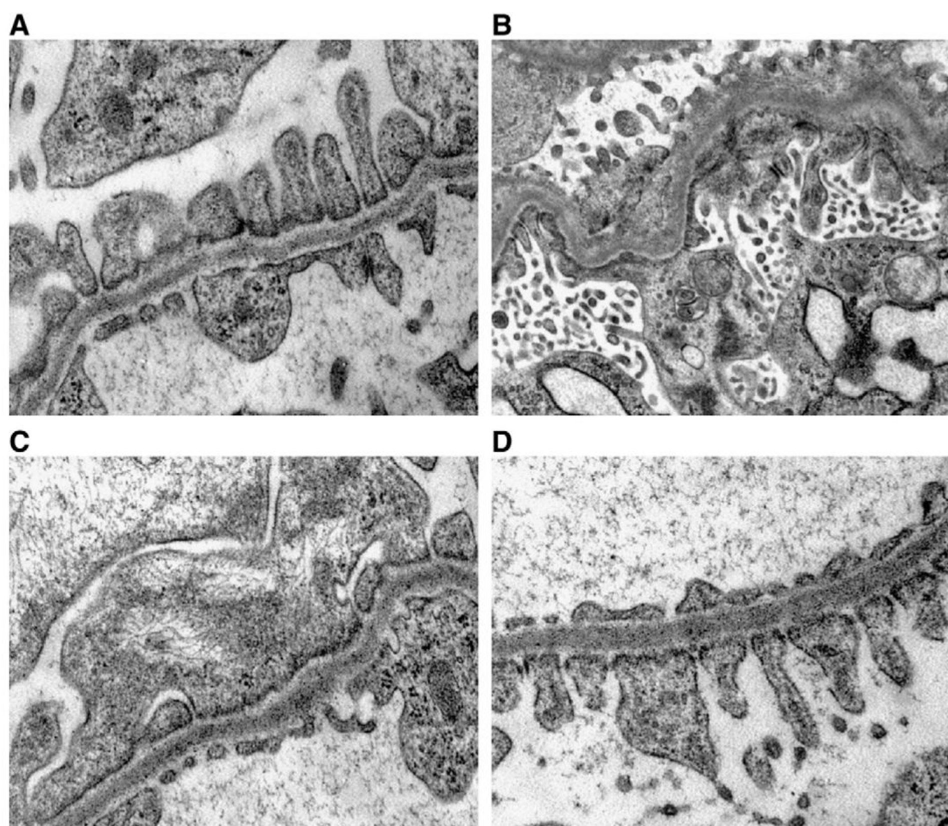

Fig. 3. Transmission electron micrographs of podocytes from treated and untreated male heterozygous TGR. (A) Male HanSD on high-salt diet (HS) with normal glomerular capillary loop ×28 000. (B) untreated male heterozygous TGR on HS with focal foot processes fusion and microvillous transformations of podocytes. ×14 000. (C) Bosentan treated male heterozygous TGR on HS exhibiting only minimal fusion of foot processes. ×28 000. (D) Atrasentan treated male heterozygous TGR on HS with normal podocyte architecture. ×28 000.

weight/body weight (KW/BW) and heart (ventricle) weight/body weight (HW/BW) were used as indices of organ hypertrophy.

2.4. Tissue ET-1 concentrations

Tissues from the right kidney cortex and left ventricles were immediately frozen in liquid nitrogen for ET-1 determination by ELISA (Amersham, Braunschweig, Germany) as previously described (Schiffrin, 2005; Vaneckova et al., 2006; Opocensky et al., 2006).

2.5. Histological examination

The left kidney was quickly removed, fixed in 4% buffered formaldehyde, dehydrated and embedded. Paraffin sections were stained with hematoxylin/eosin and periodic acid-Schiff reaction and examined using Nicon Eclipse E 600 light microscope. Slides were evaluated in a blind fashion. As described previously (Saito et al., 1987), one hundred glomeruli per section were randomly selected and the degree of glomerular damage was evaluated using a semiquantitative scoring method: grade 0: normal glomeruli, grade 1: sclerotic area up to 25% or distinct adhesion present between capillary tuft and Bowman's capsule, grade 2: sclerotic area 25 to 50% glomeruli, grade 3: sclerotic area 50 to 75%, grade 4: sclerotic area 75 to 100%. The glomerulosclerosis index was calculated using the following formula: $GSI=(1\times n_1)+(2\times n_2)+(3\times n_3)+(4\times n_4)/n_1+n_2+n_3+n_4$, where n_x is the number of glomeruli in each grade of glomerulosclerosis.

2.6. Electron microscopy

Small parts of the renal cortex were selectively removed and used for electron microscopy. Two animals from each group were examined. Samples were osmified, dehydrated and embedded in Epon 812. Ultrathin sections stained with uranyl acetate and lead citrate were examined using a Philips EM 286/ Morgagni transmission microscope (FEI company). Evaluations were performed in a blinded fashion.

2.7. Immunohistochemical examination

Five-micron-thick sections cut from formalin-fixed, paraffin-embedded tissue samples were used. Endogenous peroxidase activity was inhibited by 3% H_2O_2 in methanol for 30 min followed by 15 min rinsing in tap water. Nonspecific reactivity was avoided by pretreatment of sections with 1% normal goat serum (Dako Cytomation, Glostrup, Denmark) with 1% bovine fetal albumin for 2 h. The slides were incubated with monoclonal

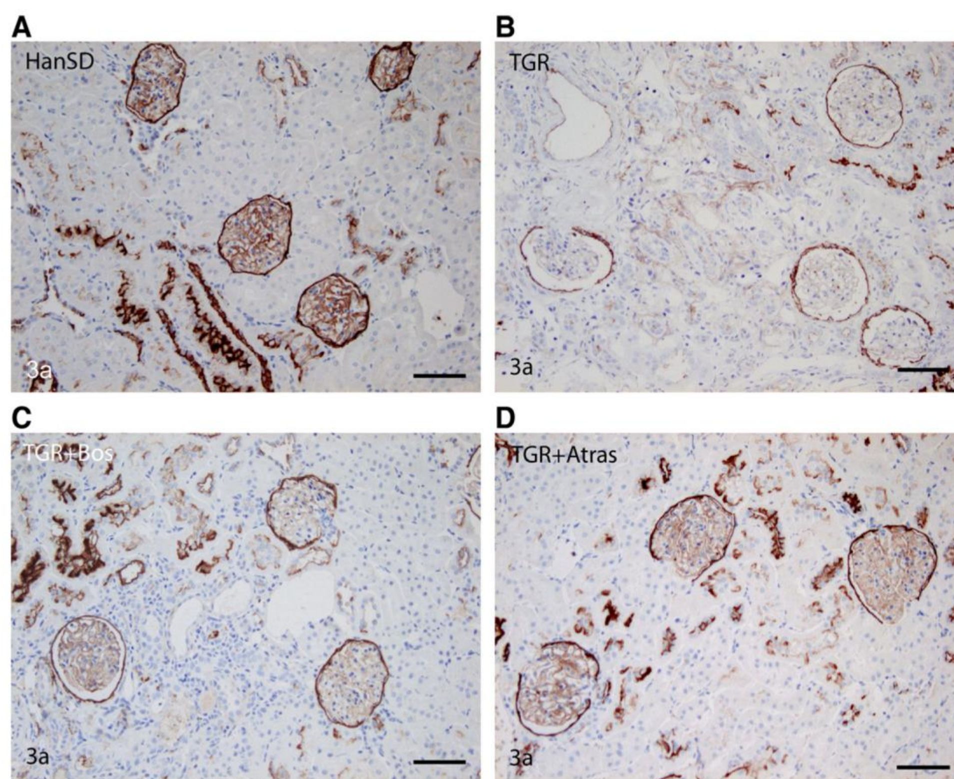

Fig. 4. Podocyte injury in heterozygous TGR. Representative microphotographs of immunostaining for CD 10. (Han SD) Han SD rats fed a high-salt diet (A), (TGR) untreated heterozygous TGR fed a high-salt diet (B), (TGR+Bos) heterozygous TGR fed a high-salt diet and treated with bosentan (C), (TGR+Atras) heterozygous TGR fed a high-salt diet and treated with atrasentan (D). X-fold magnification, scale bars represent 100 μm.

mouse anti-human Actin (smooth muscle) antibody (1A4, 1:50, Dako, Cytomation, Glostrup, Denmark), or monoclonal mouse anti-human CD10 antibody (56C6, 1:20, Novocastra, Newcastle, UK), monoclonal mouse anti-desmin antibody (D33, 1:50 Dako, Cytomation, Glostrup, Denmark), and monoclonal mouse anti-vimentin antibody (V9, 1:50, Dako, Cytomation, Glostrup, Denmark) for 1 h, respectively. The Histofine® kit (Nichirei, Tokyo, Japan) was used to visualize sections incubated with primary antibodies. The chromogen 3, 3-diaminobenzidine (Liquid DAB + Substrate, Dako Cytomation, Glostrup, Denmark) was applied to all sections and counterstaining was performed with Mayer's hematoxylin. Tissue sections incubated without primary antibodies were used as negative controls.

3. Statistical analysis

Statistical analysis of data was performed using Graph-Pad Prism software (Graph-Pad Software, San Diego, California, USA). Group comparisons were determined by two-way ANOVA. Statistical comparisons of the results obtained for heart and kidney weights and for ET-1 concentrations were made by one-way ANOVA. Unless noted, values are expressed as mean ± S.E.M. and *n* represents the number of animals. A *p*-value less than 0.05 was considered significant.

4. Results

4.1. Survival rate

All control HanSD rats survived to the end of the experiment (data not shown). One of untreated and bosentan-treated TGR died at the age of 43 days, i.e. before beginning of the experiment (Fig. 1A). While untreated TGR died throughout the experiment, bosentan treatment stabilized survival until 78 days of age, thereafter it declined gradually. At the end of the experiment, the survival rate of 71 and 83% in untreated and bosentan-treated TGR, respectively, was not significantly different. Atrasentan treatment significantly improved survival to 92% which did not differ from 100% survival in HanSD rats.

4.2. Systolic blood pressure

Systolic blood pressure (SBP) of HanSD rats remained in the normotensive range throughout the course of the experiment (Fig. 1B). On the contrary, SBP of all TGR rose rapidly from day 29 on. At the beginning of the experiment on day 52, SBP did not differ between untreated, bosentan- and atrasentan-treated TGR (221.0 ± 5.2; 214.5 ± 3.1; and 215.2 ± 2.6 mm Hg, respectively). Bosentan transiently decreased SBP between days 57 and 64 of

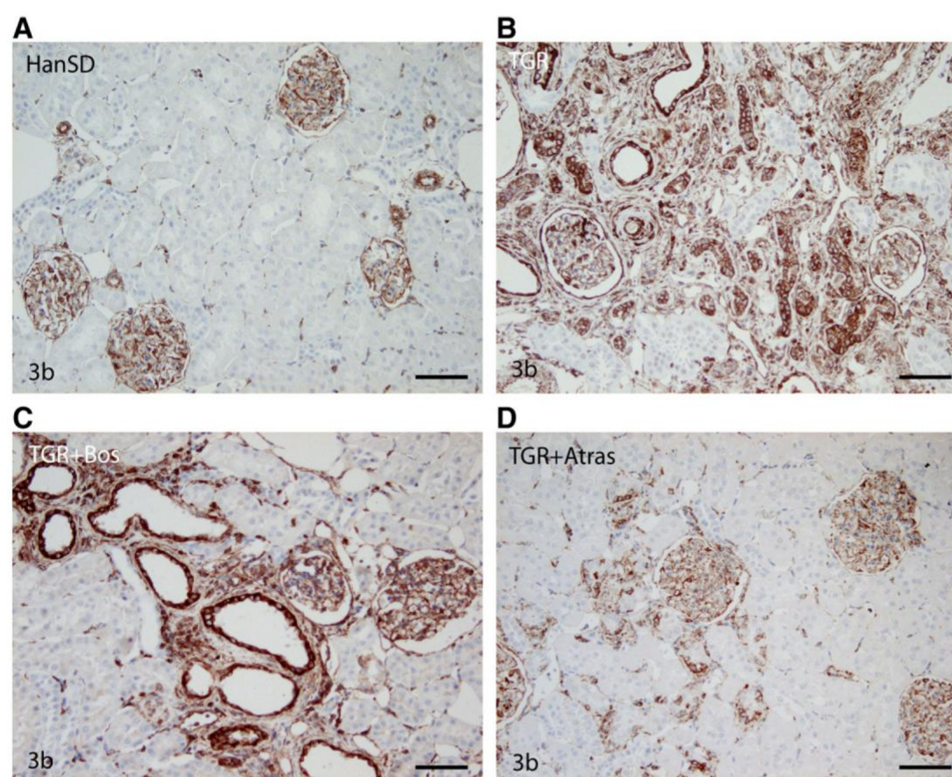

Fig. 5. Hypertensive injury in heterozygous TGR. Representative microphotographs of immunostaining for vimentin. (Han SD) Han SD rats fed a high-salt diet (A), (TGR) untreated heterozygous TGR fed a high-salt diet (B), (TGR + Bos) heterozygous TGR fed a high-salt diet and treated with bosentan (C), (TGR + Atras) heterozygous TGR fed a high-salt diet and treated with atrasentan. X-fold magnification (D), scale bars represent 100 μm.

age but at the end of the experiment it was not different from untreated TGR (228.0 ± 4.0 vs. 236.9 ± 5.7 mm Hg). From day 71 onwards, SBP in atrasentan-treated TGR was significantly lower than in untreated or bosentan-treated TGR.

4.3. Glomerulosclerosis index

Hypertensive glomerular lesions were characterized by mild mesangial expansion and sporadically by segmental sclerosis of the capillary tuft (predominantly grade 1). The most striking changes were found in untreated TGR. Bosentant treatment partially decreased whereas atrasentan fully attenuated morphological hypertensive changes within the glomeruli (Fig. 2). Renal vascular changes were also mild and their extent reflected glomerular changes. On the ultrastructural level only mild regressive changes of podocytes with partial fusion of foot processes were found in untreated TGR. Treatment with bosentan improved (reduced the extent of foot processes effacement) and atrasentan restored normal podocyte architecture (Fig. 3).

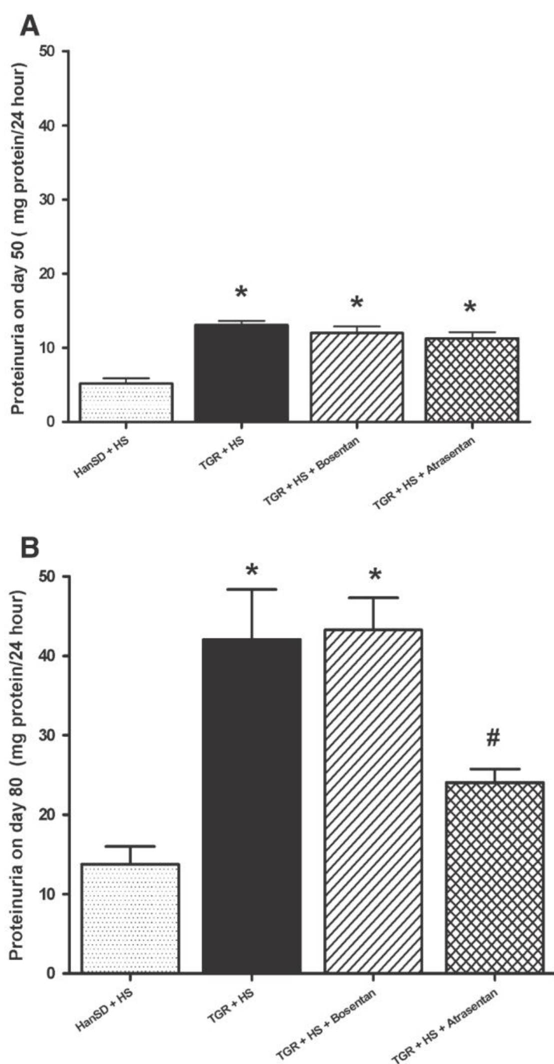

Fig. 6. Proteinuria at the age of 50 days (A) and at the age of 80 days (B) in heterozygous male Ren-2 transgenic rats (TGR; +/−) on high-salt intake (HS). $*p<0.01$ vs. unmarked value, # $p<0.01$ vs. untreated and bosentan-treated TGR.

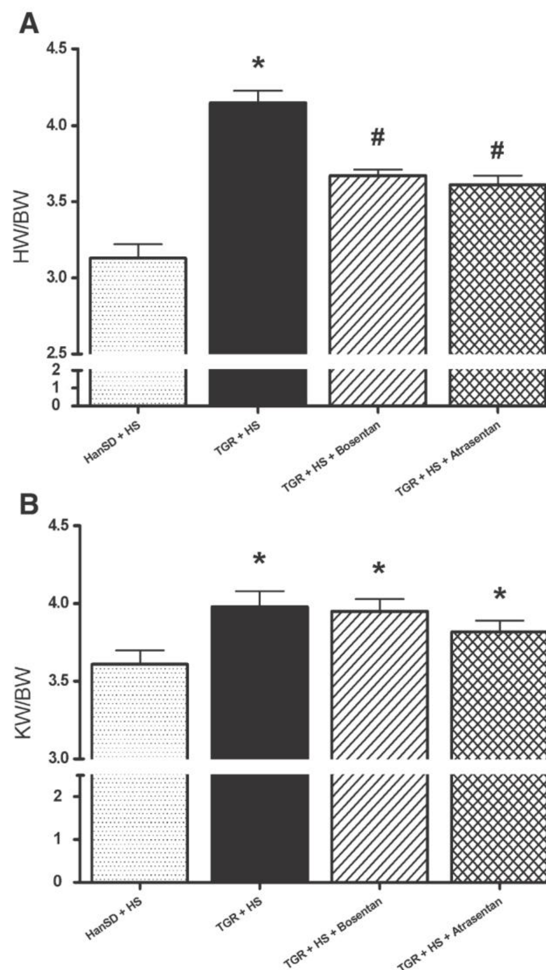

Fig. 7. Indices of cardiac (HW/BW) (A) and kidney hypertrophy (KW/BW) (B) in heterozygous male Ren-2 transgenic rats (TGR; +/−) on high-salt intake (HS). $*p<0.01$ vs. unmarked value, # $p<0.01$ vs. untreated TGR.

4.4. Histochemistry

Histochemical analysis of podocyte injury revealed that desmin, a marker of early podocyte injury, was expressed in a great number of glomeruli of untreated TGR; their positive staining was increased also in the regions of segmental glomerular sclerosis. Treatment with both types of ET receptor blockers markedly reduced induction of desmin in podocytes. Alpha smooth muscle actin was not found in any glomeruli of the examined groups of animals. CD 10 positive staining within the podocytes was attenuated or suppressed in untreated TGR (Fig. 4). Atrasentan treatment almost fully restored CD 10 expression in podocytes in comparison with bosentan which caused only a partial increase in CD10 positive staining. In

control HanSD rats vimentin was detected only in the glomeruli and mainly in the podocytes (Fig. 5). There was a similar staining pattern within the glomeruli in untreated hypertensive TGR but vimentin positive staining appeared also in peritubular capillaries and within the interstitium (in the region of tubular atrophy). Tubular neoexpression of vimentin was also noted in untreated TGR. Abovementioned tubulointerstitial changes in vimentin staining were considerably reduced by bosentan treatment and almost abolished with atrasentan.

4.5. Proteinuria

In all groups of TGR proteinuria at the age of 50 days significantly exceeded that of HanSD rats (Fig. 6A). There were no differences in protein excretion between the different TGR groups on day 50. On day 80 HanSD rats exhibited very small proteinuria of 13.75±2.22 mg protein/day (Fig. 6B). In contrast, proteinuria which was similar in untreated and bosentan-treated TGR significantly exceeded that in HanSD rats (42.07±6.30 and 43.32±4.0 mg protein/day, respectively). Atrasentan treatment substantially reduced proteinuria to 24.06±1.70 mg protein/day ($p<0.01$ vs. untreated TGR).

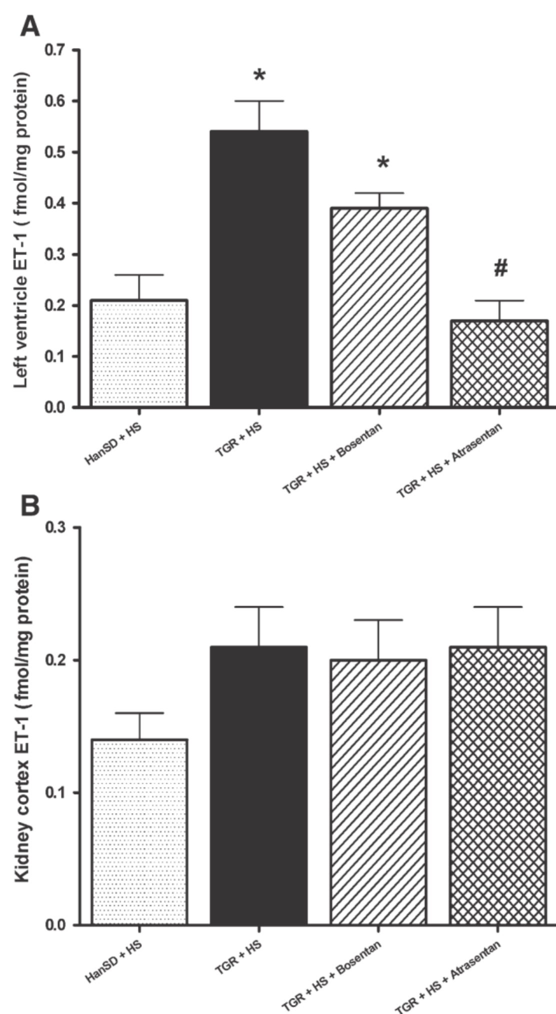

Fig. 8. Endothelin-1 concentrations in the left heart ventricle (A) and in the kidney cortex (B) in heterozygous male Ren-2 transgenic rats (TGR; +/) on high-salt intake (HS). *$p<0.05$ vs. unmarked value, # $p<0.01$ vs. untreated TGR.

4.6. Body and organ weights

There were no differences either in weight gains throughout the course of the experiments or in the final body weights among all examined groups of rats (data not shown). Untreated TGR exhibited progressive cardiac hypertrophy when compared with control HanSD rats (HW/BW 4.15±0.08 vs. 3.13±0.09, $p<0.01$) (Fig. 7A). Both bosentan and atrasentan caused a significant decrease of HW/BW ratio to 3.67±0.04 and to 3.61±0.06, respectively ($p<0.01$ vs. untreated TGR). KW/BW ratios in all TGR groups were significantly higher than in HanSD rats (Fig. 7B).

4.7. ET-1 tissue concentrations

As shown in Fig. 8A, left ventricle ET-1 concentration in untreated TGR exceeded 2.5 fold that in HanSD rats (0.54±0.06 vs. 0.21±0.05 fmol/mg protein, $p<0.05$). Bosentan partly reduced, whereas atrasentan fully normalized this value to 0.39±0.03 and 0.17±0.04 fmol/mg protein, respectively ($p<0.05$). Kidney cortex ET-1 was moderately but insignificantly higher in untreated TGR (0.21±0.03 fmol/mg protein) than in HanSD rats (0.14±0.02 fmol/mg protein). There were no differences between kidney cortex ET-1 levels of untreated and bosentan- (0.20±0.03 fmol/mg protein) or atrasentan-treated (0.21±0.03 fmol/mg protein) TGR (Fig. 8B).

5. Discussion

This study extends our previous studies in homozygous (Opocensky et al., 2006; Vaneckova et al., 2005) and heterozygous (Vaneckova et al., 2006) Ren-2 transgenic rats (TGR). The present data show that, unlike in homozygous TGR, ET receptor blockade in heterozygous TGR has similar effects when applied in rats with established hypertension (regression protocol) as in young animals (prevention protocol). Such difference could be explained by a more activated ET system in homozygous animals, in which severe hypertension develops, as has been shown by Whitworth et al. (1995) or by a lesser effect of dietary sodium on the activation of the ET system in heterozygous animals. It is a well-known fact that dietary sodium causes hypertension — TGR is a strain with a significant salt-sensitive component — and induces endothelial dysfunction through enhanced ET-1 production, which may be stimulated by Ang II. In our previous study we have shown that increased sodium intake does not correspondingly decrease plasma and kidney Ang II levels in conscious male TGR which lead us to the hypothesis that the enhanced plasma and kidney ANG II through increased ET-1 production contributes to the development of hypertension and organ damage in TGRs (Huskova et al., 2006). Moreover, we have shown in heterozygous TGR that high-salt diet significantly accelerated the development of hypertension, increased mortality and

worsened renal and cardiac damage when applied to young animals (Vaneckova et al., 2006). In line with the above-mentioned hypothesis are, on one hand, the similar findings in the present heterozygous and in the previously studied homozygous TGR with early-onset and late-onset ET_A receptor blocker treatment, i.e. these are a decline in BP seen as early as one week after the beginning of the treatment, an almost identical survival rate and similar changes in proteinuria. On the other hand, cardiac and kidney hypertrophy was slightly greater along with higher tissue ET-1 concentrations in our previous study with early-onset ET receptor blockade (prevention protocol). This probably reflects earlier exposure of these rats to high dietary sodium which caused a marked acceleration of blood pressure comparing with regression protocol.

An important finding is that atrasentan treatment lowers BP even in the established phase of hypertension in heterozygous TGR. We have repeatedly confirmed the efficacy of selective ET_A receptor blockade over nonselective blockade in Ren-2 rats (Vaneckova et al., 2006; Opocensky et al., 2006; Vaneckova et al., 2005), which is probably due to the fact that nonselective receptor blockers inhibit concomitantly not only vasoconstrictory ET_A receptors (Kohan, 2006) but also vasodilatory ET_B receptors mediating vasodilation and natriuresis (Ge et al., 2006). The BP lowering effect of ET_A receptor blockers was shown in several other rat strains and was usually accompanied by antiproteinuric actions (Matsumura et al., 1999), which has been explained by the effect on preserving the integrity of podocytes, the filtration barrier of the glomerulus (Pavenstadt et al., 2003). We have found that treatment with atrasentan normalized the thickness of glomerular basement membrane and reduced the extent of podocyte alterations in young homozygous TGR. This was accompanied by a strong correlation between podocyte injury and survival despite severe hypertension (Opocensky et al., 2006). In the present study ultrastructural changes of podocytes in TGR were only mild. This finding, on one hand, may reflect the gradual increase of BP and a possible activation of adaptive mechanisms and, on the other hand, the short duration of the experiment.

Many structural changes observed in hypertensive glomerulopathy are shared with other chronic nephropathies (i.e. diabetic nephropathy, focal segmental glomerulosclerosis). In these renal diseases mesangial cell proliferation/activation, tubular damage/regeneration as well as interstitial fibrosis have been associated with the neoexpression of cytoskeletal proteins and intermedial filaments. The neoexpression of vimentin in some tubular cells of hypertensive rats is not unexpected as this intermediate filament protein has been associated with a wide range of tubular injuries (Nouwen et al., 1994; Sanai et al., 2000). It is possible that these changes reflect an element of dedifferentiation of tubular cells during the injury/repair process (hypertensive or hyperglycaemic). It has been suggested that the expression of vimentin by tubular cells represents their transdifferentiation into fibroblastic lineage (Strutz and Muller, 2000) and in such way these cells could contribute to interstitial fibrosis.

It has been documented that ET-1 through ET_A receptors causes a disruption of podocyte filamentous actin cytoskeleton (Morigi et al., 2006). Since this process seems to be reversible their blockade could be beneficial in a sense that podocytes would promote the growth of endothelial cells. We could not demonstrate smooth muscle actin within the glomeruli. One possible explanation may relate to the short duration of our experiment of only 13 weeks. Remuzzi et al (2006) found actin overexpression in Munich Wistar Fromter rats at 50 and 60 weeks of age. Podocyte damage can be assessed by the expression of various markers. One of them is CD 10 (neprilysin, enkephalinase), a 90–110 kD zinc-dependent metallopeptidase, expressed in the kidney on podocytes, brush border and vascular smooth muscle cells (Debiec et al., 2003). In this study a marked decrease in CD 10 was found in untreated hypertensive rats. Bosentan treatment partially and atrasentan fully restored its positive staining within the podocytes. Desmin, a marker of podocyte injury (Floege et al., 1995), was increased only in damaged podocytes of untreated TGR. ET receptor blockade protected podocytes from more severe injury when considering this marker of injury.

6. Conclusion

In the present study we have found that, unlike in homozygous TGR, late treatment in adult rats with ET receptor blockers had similar effects on hypertension and end-organ damage in heterozygous TGR as had the early treatment, i.e. in young rats. The most probable explanation of this finding is a lesser activation of the ET system in heterozygous animals fed a high-salt diet as was by our previous results obtained in homozygous TGR. However, it seems that podocyte injury plays a crucial role in the renal involvement in heterozygous TGR under these experimental conditions which is substantially attenuated by treatment with ET_A receptor blockers.

Acknowledgements

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4 SHRNUTÍ A ZHODNOCENÍ CÍLŮ PRÁCE

4.1 Účast chemokinového receptoru CCR1 a CCR5 na leukocytární infiltraci a následné fibróze intersticia ledviny u myšího modelu jednostranné obstrukce ureteru (unilateral ureter obstruction = UUO)

Tato práce byla zaměřena na zjištění role chemokinových receptorů CCR1 a CCR5 exprimovaných makrofágy a T lymfocyty v infiltraci intersticiálního kompartmentu ledviny a v rozvoji jeho fibrózy. V práci jsme použili model jednostranné obstrukce ureteru, kde postupně se vyvíjející hydronefróza vede k poškození tubulů a k fibrotizaci intersticia ledviny, s rozvojem zánětlivé infiltrace intersticia leukocyty. V tubulointersticiu se tedy odehrávají procesy simulující vývoj progredujícího chronického onemocnění ledvin. V tomto modelu jde o neimunitní poškození, na jehož rozvoj nemá výrazný vliv možné ovlivnění systémové zánětlivé odpovědi dané blokádou chemokinových receptorů (chybění CCR1 potencuje Th1 imunitní odpověď). Ke studiu efektu receptorů jsme použili CCR1 a CCR5 knock-out myší kmeny a dále blokádu CCR1 nízkomolekulárním antagonistou BX471.

Práce ukázala, že chybění nebo blokáda receptoru CCR1 vede ke snížení počtu makrofágů a T lymfocytů infiltrujících do intersticia UUO ledviny u knock-out nebo BX471 léčených myší v porovnání s divokým kmenem myší C57BL/6. Sledované parametry fibrózy renálního intersticia – počet fibroblastů, objem intersticia, depozice kolagenu a hladina mRNA pro TGF-β1 – byly u CCR1 deficientních myší v poškozené ledvině sníženy. Naproti tomu chybění CCR5 nevedlo ke snížení počtu infiltrujících leukocytů ani k zabránění rozvoje fibrózy UUO ledviny. Použití fluorescenčně značených leukocytů, izolovaných ze sleziny divokého kmene a CCR1 a CCR5 deficientních kmenů myší, injikovaných do myší divokého kmene s jednostrannou hydronefrózou, a následné hodnocení intenzity infiltrace leukocytů do intersticia UUO ledviny těchto zvířat jasně potvrdilo účast CCR1 (ale ne CCR5) na cílené infiltraci makrofágů a T lymfocytů do intersticia poškozené ledviny. Získaná data jsou v souladu s předchozími

pozorováními, kde blokáda CCR1 měla příznivý vliv např. na transplantaci ledviny, srdce, rozvoj plicní fibrózy či experimentální encefalitidy.

Oba receptory, CCR1 i CCR5, jsou exprimovány na povrchu leukocytů infiltrujících intersticium ledviny. Přesto chybění CCR5 nevede ke snížení počtu infiltrujících leukocytů do intersticia ledviny po UUO. U jiných modelových onemocnění, jako například u dextransulfátem indukované kolitidy, nebo u transplantace srdce, chybění CCR5 sníží počet infiltrujících leukocytů. Naopak infiltrace makrofágů v modelu experimentální encefalomyelitidy nezávisí na CCR5. Naše pozorování, že chybění CCR5 nezabrání migraci leukocytů do ledviny po UUO, nelze vysvětlit tím, že chybí ligand, tedy příslušný atrahující chemokin, protože v předchozích pracích bylo prokázáno, že CCL5 je v poškozené ledvině u tohoto modelu výrazně exprimován. Interakce CCL5/CCR5 v ledvině v tomto modelu však může mít jinou roli. Z výsledků lze odvodit, že role CCR5 receptoru při zprostředkování leukocytární infiltrace je u různých modelových onemocnění odlišná, což může být dáno i různou kombinací exprimovaných receptorů.

Podstatným přínosem této práce je zjištění, že chybění receptoru CCR1 vede k redukci fibrózy intersticia po UUO. Infiltrující leukocyty přispívají produkcí prozánětlivých a profibrotických cytokinů k proliferaci fibroblastů, produkci kolagenu a procesu epiteliální - mezenchymální přeměny. Ve studii byla pomocí in situ hybridizace a imunohistochemicky lokalizována produkce TGF-β1 do intersticia ledviny v místě zánětlivého infiltrátu. CCR1 knock-out myši měly sníženou hladinu mRNA pro TGF-β1 v UUO ledvině, spíše díky sníženému počtu infiltrujících leukocytů než díky jeho snížené produkci leukocyty neexprimujícími CCR1. Pozitivní efekt chybění chemokinového receptoru CCR1 na redukci rozvoje fibrózy ledviny je poznatek, který by mohl mít terapeutické využití u pacientů s chronickým onemocněním ledvin.

4.2 Pozdní začátek léčby blokátorem CCR1 receptoru vede k zabránění progrese lupusové nefritidy u MRL (Fas)-lpr myši

V této práci byl zkoumán vliv blokády chemokinového receptoru CCR1 nízkomolekulárním antagonistou BX471 na progresi imunokomplexové

glomerulonefritidy do chronického renálního selhání. Porovnání výsledků histologického vyšetření tkáně ledvin kontrolních a léčených myší ukázalo, že blokáda CCR1 snížila výrazně množství makrofágů a T lymfocytů infiltrujících do tubulointersticiálního kompartmentu MRL$^{lpr/lpr}$ myší léčených BX471, ale neměla vliv na infiltraci makrofágů do glomerulu ani na rozvoj glomerulonefritidy a tíži proteinurie. Blokáda CCR1 vedla k redukci intersticiální fibrózy – jak ukázalo vyhodnocení počtu α-SMA pozitivních myofibroblastů v intersticiu, depozit kolagenu I a vyhodnocení hladin mRNA pro kolagen I v ledvinné tkáni. Jak bylo ukázáno již v předchozí práci, mechanismem redukce fibrózy je nejspíš snížení celkového množství leukocyty produkovaných profibrotických a prozánětlivých cytokinů, což jsme demonstrovali na příkladu nejvýznamnějšího profibrotického cytokinu TGF-β1 na úrovni hladiny mRNA v ledvině pomocí real time RT-PCR, lokálně pak pomocí in situ hybridizace.

Účast CCR1 na infiltraci leukocytů do intersticia ledviny potvrdil i experiment s použitím fluorescenčně značených makrofágů a T lymfocytů inkubovaných s BX471 a nosičem cyklodextrinem nebo samotným nosičem, injikovaných do MRL$^{lpr/lpr}$ myší, kdy leukocyty inkubované s blokátorem CCR1 infiltrovaly intersticium v daleko nižším počtu než leukocyty inkubované s cyklodextrinem.

Sérové hladiny anti-DNA IgG zůstaly při léčbě antagonistou CCR1 nezměněny v porovnání s kontrolní skupinou, blokáda pomocí BX471 tedy nevede k výraznému posunu Th1/Th2 imunitní odpovědi. Toto zjištění je v rozporu s pozorováním na modelu nefrotoxické sérové nefritidy, kde byl patrný posun směrem k Th1 odpovědi. Možným vysvětlením je rozdílný patogenetický mechanismus, u nefrotoxické sérové nefritidy jde o specifickou imunitní odpověď, kde antigenem je protein glomerulární bazální membrány, zatímco u lupusu se jedná o neregulovanou, široce polyklonální protilátkovou odpověď. Navíc v námi použitém modelu byla prováděna blokáda CCR1 v době rozvinutého imunokomplexového poškození tkání a ne v době vzniku onemocnění, jak tomu bylo u nefrotoxické sérové nefritidy.

V porovnání s kontrolními zvířaty, blokáda pomocí BX471 nezabránila infiltraci makrofágů do glomerulu. To je v souladu s předchozími pozorováními na modelech glomerulárního poškození. Chemokinový

receptor CCR1 se zřejmě podílí na infiltraci tubulointersticiálního a ne glomerulárního kompartmentu ledviny, což může být dáno odlišnými adhezními molekulami přítomnými na endotelu cév, které různě modulují chemokiny zprostředkovanou adhezi a transmigraci do tkáně.

4.3 Blokáda CCR1 redukuje intersticiální zánět a fibrózu u myšího modelu adriamycinem indukované nefropatie

Práce zkoumala možnost ovlivnění progrese renálního onemocnění spojeného s těžkou proteinurií blokádou chemokinového receptoru CCR1. Jako modelové onemocnění byla použita adriamycinem indukovaná fokálně segmentální glomeruloskleróza u myšího kmene BALB/c, kde poškození glomerulů spojené s proteinurií a rozvojem nefrotického syndromu s následným poškozením a fibrotizací tubulointersticia modelují průběh chronických glomerulopatií provázených těžkou proteinurií u člověka. V práci jsme nejprve ukázali, že exprese mRNA pro chemokiny CCL3 (MIP-1α) a CCL5 (RANTES) - ligandy CCR1 - je v ledvinách u tohoto modelu několikanásobně zvýšená ve srovnání s kontrolní skupinou zvířat. V pokusech in vitro jsme prokázali, že mRNA pro CCR1 je exprimována makrofágy a T lymfocyty izolovanými ze sleziny, ale není exprimována v buněčné kultuře TGF-β1 stimulovanými renálními fibroblasty ani LPS stimulovanou buněčnou linií buněk epitelu kortikálních tubulů ledviny. Výsledky morfometrické analýzy a imunohistochemického vyšetření tkáně ledvin kontrolní skupiny myší a zvířat léčených nízkomolekulárním antagonistou receptoru CCR1 (BX471) ukázaly, že blokáda receptoru CCR1 výrazně snížila počet makrofágů a T lymfocytů infiltrujících do intersticia ledviny. Léčba BX471 zabránila proliferaci intersticiálních fibroblastů a zpomalila expanzi objemu intersticia a tedy snížila rozsah fibrózy intersticia ledvin. Vzhledem k tomu, že renální fibroblasty ani tubulární epitel neexprimují CCR1, nejde zřejmě o přímý efekt blokády CCR1 receptoru na tyto buňky. Lze odvodit, že snížení počtu infiltrujících leukocytů vede sekundárně ke snížení aktivace a proliferace fibroblastů, patrně mechanismem sníženého množství produkovaných profibrotických cytokinů,

jako je TGF-β1, b-FGF, PDGF a PAF, které zprostředkují proliferaci fibroblastů, syntézu extracelulární matrix a řídí epiteliální-mesenchymální přeměnu (transdiferenciaci). Významný pozitivní efekt blokády receptoru CCR1 vedoucí ke zpomalení rozvoje fibrózy intersticia je velmi zajímavý vzhledem k tomu, že léčbou nedošlo u myší k redukci těžké proteinurie. Těžká proteinurie je významným stimulem pro tubulární epitelové buňky, které na jejím podkladě produkují množství chemokinů a profibrotických cytokinů. Naše nálezy nicméně ukazují, že těžká proteinurie sama o sobě nedokáže vyvolat výraznou intersticiální fibrózu, pokud je zabráněno infiltraci leukocytů do intersticia ledviny ve skupině myší léčených blokátorem BX471.
Léčba BX471 neměla žádný efekt na tíži glomerulárního poškození indukovaného adriamycinem. Předpokládá se, že iniciální poškození glomerulů je u tohoto modelu způsobeno přímým cytotoxickým efektem adriamycinu na podocyty. Progresivní glomeruloskleróza je výsledkem pokračujících změn vyvolaných primárním inzultem. Tyto změny jsou nezávislé na CCR1, proto jeho blokáda nemá na jejich rozvoj žádný vliv. V souladu s tímto jevem se nepodařilo v glomerulárních lézích identifikovat ani makrofágy, ani expresi korespondujících chemokinů CCL2 (MCP-1) a CCL5 (RANTES), ligandů CCR1.

4.4 Pozdní začátek blokády endotelinového receptoru u homozygotních Ren-2 potkanů snižuje podocytární poškození podocytů i přes těžkou hypertenzi

V této práci jsme použili transgenní potkany s inkorporovaným myším genem pro renin - TGR(mRen2)27. Jde o model monogeneticky definované hypertenze. Transgenní homozygotní potkani na vysokoslané dietě byli léčeni neselektivním blokátorem $ET_{A/B}$ bosentanem a selektivním blokátorem ET_A atrasentanem. Léčba byla zahájena 51. den života, aby bylo možné sledovat účinky blokády endotelinových receptorů na dospělé jedince s již rozvinutou hypertenzí.
U kontrolního netransgenního kmene potkanů HanSD zůstávaly hodnoty krevního tlaku během celého experimentu na vysokoslané dietě v rámci

normotenze. U ostatních tří skupin transgenních potkanů krevní tlak stoupal až do 46. dne. Po zahájení podávání vysokoslané diety a současném zahájení léčby (51. den) došlo u potkanů léčených atrasentanem k redukci hypertenze (měřeno 60. den). 81. den byly hodnoty tlaku všech tří skupin transgenních potkanů zvýšené, bez statisticky signifikantních rozdílů. Míra přežití na konci pokusu (90. den) byla u neléčeného divokého kmene HanSD 93% a nelišila se od přežívání atrasentanem léčených transgenních potkanů. U neléčených transgenních potkanů a u transgenních potkanů léčených bosentanem bylo přežívání signifikantně nižší (50 a 64%). Hodnoty proteinurie se u dosud neléčených transgenních potkanů 50. den života signifikantně nelišily. 80. den života měli neléčení TGR potkani na vysokoslané dietě výrazně vyšší proteinurii než HanSD potkani. Potkani léčení oběma typy endotelinových antagonistů měli hodnoty proteinurie obdobné jako kontrolní potkani. Poměr hmotnosti srdce nebo levé komory srdeční k hmotnosti těla u neléčených transgenních potkanů byl signifikantně vyšší než u kontrolních HanSD potkanů a potkanů léčených bosentanem a atrasentanem.

Glomerulosklerotický index se na konci léčby u přežívajících potkanů všech čtyř skupin signifikantně nelišil. V ultrastrukturálním vyšetření u transgenních potkanů na vysokoslané dietě byla GBM nepravidelně ztluštělá, částečně desintegrovaná, a fokálně zvlněná. Fokálně bylo patrné splývání pedicel a regresivní změny podocytů. Léčba bosentanem výrazně zlepšuje morfologii podocytů a podávání atrasentanu ji zcela normalizuje. Tloušťka GBM u neléčených a bosentanem léčených potkanů byla výrazně větší než u kontrolních HanSD potkanů a potkanů léčených atrasentanem. Míra poškození podocytů silně korelovala s mírou přežívání. Koncentrace ET-1 v kůře ledviny byla signifikantně vyšší u neléčených transgenních potkanů než u HanSD potkanů. U bosentanem a atrasentanem léčených potkanů byly korové koncentrace ET-1 podobné jako u HanSD potkanů.

Ze získaných výsledků je zřejmé, že blokáda ET receptorů snižuje orgánové poškození u homozygotních transgenních TGR(mRen2)27 potkanů na vysokoslané dietě. Selektivní blokáda ET_A navíc výrazně zlepšuje přežití a vede k přechodnému snížení krevního tlaku. Na rozdíl od předchozích prací, zabývajících se blokádou ET receptorů u homozygotních TGR potkanů

zahájenou u mladých potkanů, neselektivní blokátor $ET_{A/B}$ bosentan byl mnohem méně efektivní ve snižování mortality a zabránění rozvoje hypertenzního poškození. To je pravděpodobně způsobeno tím, že podávání vysokoslané diety je u tohoto sůl-senzitivního modelu zahájeno v experimentu poměrně pozdě, a tím jsou i hladiny ET-1 ve tkáni nižší. Výraznější efekt selektivní ET_A blokády na zlepšení přežívání je dán spíše jeho antiproliferačními než antivazokonstrikčními účinky.

4.5 Časný začátek blokády endotelinového receptoru u hypertenzních heterozygotních Ren-2 potkanů

Práce zkoumala roli ET-1 při rozvoji hypertenze u heterozygotních Ren-2 potkanů v preventivním protokolu, s důrazem na zjištění možného rozdílu mezi neselektivní $ET_{A/B}$ a selektivní ET_A blokádou endotelinových receptorů. Experimenty byly prováděny u mladých potkanů na normoslané a vysokoslané dietě.

U potkanů na normoslané dietě přežili do závěru experimentu všichni potkani. U neléčených transgenních potkanů došlo k rozvoji hypertenze, vzniku hypertrofie srdeční komory a v ledvinách k rozvoji glomerulosklerózy s proteinurií. ET-1 koncentrace v levé komoře byla zhruba o jednu třetinu, v kůře ledviny pak více než dvakrát vyšší ve srovnání s kontrolní skupinou netransgenních potkanů. U transgenních potkanů léčených atrasentanem, selektivním blokátorem ET_A receptoru, došlo v porovnání s kontrolní skupinou neléčených transgenních potkanů k výraznému zpomalení vzestupu systolického i středního krevního tlaku, s méně výraznou hypertrofií srdeční komory, s normálními hodnotami glomerulosklerotického indexu, s nízkými hodnotami proteinurie a s nižšími tkáňovými koncentracemi ET-1. U potkanů léčených neselektivním blokátorem $ET_{A/B}$ bosentanem nedošlo naproti tomu k redukci hypertenze, ostatní měřené hodnoty byly taktéž sníženy, avšak signifikantně méně, s výjimkou ET-1 koncentrace v koře ledviny, kde byl pokles proti blokádě atrasentanem výraznější.

Na vysokoslané dietě bylo přežití na konci experimentu u neléčených potkanů 70%, u potkanů léčených neselektivním blokátorem bosentanem

83%. Transgenní potkani na vysokoslané dietě léčení atrasentanem přežili všichni. Systolický a střední krevní tlak rostl u potkanů na vysokoslané dietě významně u všech skupin, včetně kontrolní skupiny netransgenních zvířat, nicméně u této skupiny byl nižší než u transgenních potkanů a stále zůstával na hodnotách považovaných za normální. Na konci experimentu byly systolický a střední krevní tlak u neléčených zvířat a u zvířat léčených bosentanem signifikantně vyšší v porovnání s hodnotami tlaku zvířat léčených atrasentanem. U neléčených zvířat a zvířat léčených bosentanem došlo také k výraznému zvýšení glomerulosklerotického indexu, poškození glomerulů u zvířat léčených bosentanem bylo mírnější. Léčba atrasentanem vedla k zabránění rozvoje glomerulosklerózy. Proteinurie byla výrazně zvýšená u neléčených zvířat, při léčbě bosentanem byla jen nevýznamně nižší, naopak atrasentan způsobil podstatné snížení proteinurie až k normálním hodnotám. U všech skupin zvířat došlo k srdeční hypertrofii a ke zvýšení tkáňových koncentrací endotelinu ET-1. Tkáňové koncentrace ET-1 byly v levé komoře i v kůře ledviny výrazně nižší u potkanů léčených atrasentanem, v kůře ledviny byl pokles vyšší při léčbě bosentanem, který koncentraci ET-1 v levé komoře redukoval méně.

Výsledky naší práce ukázaly, že vysokoslaná dieta výrazně akceleruje rozvoj hypertenze u heterozygotních transgenních potkanů a zvyšuje mortalitu a orgánové poškození u tohoto kmene. Práce potvrdila předchozí výsledky, kde nefroprotektivní efekt neselektivní blokády bosentanem byl nezávislý na změnách krevního tlaku. Ukázali jsme, že působení ET-1 na rozvoj hypertenze a orgánové poškození je zprostředkováno ET_A receptorem, zejména u sůl-senzitivních modelů hypertenze. Selektivní blokáda ET_A má výrazný vliv na snížení krevního tlaku a orgánového poškození a na míru přežití. Bosentan nemá vliv na snižování krevního tlaku, neselektivní blokáda ET zřejmě potlačí nejen vazokonstrikční a proliferační účinky zprostředkované ET_A, ale zároveň i vazodilatační a natriuretický efekt zprostředkovaný ET_B.

4.6 Pozdní začátek blokády endotelinového receptoru u hypertenzních heterozygotních Ren-2 transgenních potkanů

Cílem této práce bylo zjistit úlohu endotelinového systému u samčích heterozygotních transgenních TGR(mRen2)27 potkanů s již rozvinutou hypertenzí. Transgenní a kontrolní HanSD potkani byli od 52. dne života krmeni vysokoslanou dietou. Od stejného dne jim byly podávány buď neselektivní $ET_{A/B}$ (bosentan) nebo selektivní ET_A (atrasentan) blokátory. Neléčení transgenní potkani umírali během celé doby experimentu, s mírou přežívání 71% na konci experimentu (90. den). Při podávání bosentanu přežívali všichni potkani až do 78. dne, s mírou přežití 83% na konci experimentu. Atrasentan výrazně zlepšoval míru přežívání na 92%. Systolický tlak všech tří skupin transgenních potkanů stoupal od 29 dne. Na konci experimentu se krevní tlak potkanů léčených bosentanem nelišil od tlaku neléčených potkanů, systolický krevní tlak potkanů léčených atrasentanem byl výrazně nižší. U neléčených transgenních potkanů se vyvinula hypertrofie levé srdeční komory, podávání bosentanu a zejména atrasentanu vedlo k její redukci. Tkáňová koncentrace endotelinu ET-1 v levé komoře srdeční u neléčených transgenních potkanů byla 2,5x vyšší než u kontrolních HanSD potkanů. U potkanů léčených bosentanem došlo k částečné redukci koncentrace ET-1, léčba atrasentanem normalizovala tkáňovou hladinu ET-1 v levé komoře srdeční. Proteinurie byla 50. den experimentu u všech transgenních potkanů signifikantně vyšší v porovnání s HanSD potkany. Osmdesátý den byla proteinurie u neléčených potkanů a u potkanů léčených bosentanem signifikantně vyšší než v kontrolní skupině potkanů, zatímco léčba atrasentanem proteinurii výrazně redukovala. Hypertenzní poškození glomerulů v morfologickém vyšetření ledvin zahrnovalo mírné rozšíření mezangia a segmentální sklerózu kapilárního trsu. V elektronovém mikroskopu byla patrna částečná fúze pedicel. Nejvýraznější změny byly patrné u neléčených TGR potkanů, méně výrazné u potkanů léčených bosentanem, a potkani léčení atrasentanem nevykazovali žádné změny glomerulů na úrovni světelné mikroskopie ani na úrovni ultrastrukturální. Imunohistochemicky byla v četných glomerulech u neléčených potkanů patrna pozitivita desminu, markeru podocytárního

poškození. U potkanů léčených bosentanem i atrasentanem byla indukce pozitivity desminu v podocytech výrazně nižší. Podocytární exprese CD10 (neprilysin) byla u neléčených potkanů výrazně redukována, léčba bosentanem vedla jen k částečnému zvýšení exprese, léčba atrasentanem prakticky úplně restituovala expresi CD10 u podocytů.

Výsledky této práce ukázaly, že selektivní blokáda ET_A má výraznější efekt na snížení krevního tlaku a orgánového poškození než neselektivní $ET_{A/B}$ blokáda. Ve srovnání se studiemi provedenými na homozygotních TGR potkanech se ukazuje, že blokáda ET receptorů má u heterozygotních TGR potkanů podobný efekt, když je zahájena u potkanů s již rozvinutou hypertenzí (regresní protokol), jako u mladých jedinců (preventivní protokol). Na rozdíl od studií provedených na homozygotních transgenních potkanech, u heterozygotních potkanů je neselektivní blokáda $ET_{A/B}$ bosentanem daleko méně účinná ve snižování mortality než selektivní ET_A. Rozdíl je pravděpodobně dán vyšší aktivací ET systému u homozygotních potkanů.

4.7 Závěr

Předložená práce přispěla ke zjištění role některých chemokinových receptorů u vybraných modelů zánětlivých onemocnění ledvin. Práce zejména ukázala pozitivní efekt blokády chemokinového receptoru CCR1 exprimovaného lymfocyty a makrofágy infiltrujícími tubulointersticiální kompartment na redukci rozvoje fibrózy ledviny, poznatek, který by eventuelně mohl mít terapeutické využití u pacientů s chronickým onemocněním ledvin.

Dalším přínosem práce je zpřesnění úlohy endotelinu ET-1 a jeho receptorů ET_A a ET_B v rozvoji hypertenze a orgánového poškození. Ukazuje se, že na snížení krevního tlaku, zmenšení rozsahu orgánového poškození a následné mortality má výrazný vliv zejména selektivní blokáda ET_A. Klíčovou roli v poškození ledvin u studovaných modelů hypertenze hraje poškození podocytů, blokáda ET_A vede k výraznému potlačení rozvoje tohoto poškození.

5 SUMMARY

Irrespective of the primary lesion, glomerulosclerosis and tubulointerstitial fibrosis are the final common pathways of progression seen in most chronic renal diseases. Once renal damage reaches a certain threshold, the progression of renal disease is irreversible and independent of the initial insult. Impairment of the renal functions is better correlated with the degree of tubulointerstitial damage than with that of glomerular one. This finding has led to the recognition, that the final common pathway of kidney failure occures principally in the tubulointerstitium. Renal tubulointerstitial fibrosis is an iterative result of hyperfiltration, persistent proteinuria, hypoxia, cellular inflammation and the local release of subset of cytokines that disturb structural and functional relationships among anatomical structures of the kidney. Migration of macrophages and lymphocytes into the tubulointerstitium mediates chronic interstitial inflammation and subsequent renal interstitial fibrosis leading to the end-stage renal disease.

Chemokines, a family of small chemotactic cytokines, play a major role in attracting circulating leukocytes into inflamed renal tissue in a variety of kidney diseases. Thus, specific chemokine receptor antagonists may represent an attractive therapeutic option for progressive renal disorders and renal fibrosis.

Studies on progressive renal fibrosis in the mouse have demonstrated that chemokines receptor CCR1 and CCR5 were expressed on infiltrating macrophages and T lymphocytes at sites of tubular damage and interstitial fibrosis. We therefore hypothesized that both CCR1 and CCR5 might be involved in macrophage and T cell infiltration and the development of renal fibrosis in mice. Using model of unilateral ureteral obstruction (UUO) in genetically generated CCR1-deficient and CCR5-deficient mice, their respective leukocytes for transfer, and the CCR1 antagonist BX471, we have demonstrated that lack or blockade of CCR1 effectively reduced the infiltration of macrophages and T cells into the UUO kidney and subsequent renal fibrosis, whereas lack of CCR5 had no effect.

The model of immune complex glomerulonephritis in autoimmune MRL lpr/lpr mice was used to evaluate whether late onset of chemokine receptor CCR1

blockade can affect progression to renal failure. Mice were treated with subcutaneous injections of BX471, a small molecule CCR1 antagonist. We have clearly demonstrated that blockade of CCR1 receptor reduced the amount of macrophages, T lymphocytes, apoptotic and proliferating cells in the renal interstitium but not in glomeruli.

Using murine model of adriamycin-induced focal segmental glomerulosclerosis with nephrotic syndrome and subsequent progressive interstitial inflammation and fibrosis we showed that CCR1 blockade significantly reduced the amount of macrophages and CD3 lymphocytes in interstitial lesions. Markers of renal fibrosis such as amount of interstitial fibroblasts and interstitial volume were significantly reduced by BX471 treatment. In contrast, the extent of proteinuria and glomerular sclerosis was not affected by BX471 treatment. We conclude that CCR1 may be a valuable target for therapeutic intervention for chronic nephropathies accompanied by leukocyte-mediated progressive interstitial fibrosis.

Endothelin-1 (ET-1) is a potent vasoconstrictor with proinflammatory, mitogenic and profibrotic effects involved in both normal renal physiology and pathology. The action of ET is mediated by the activation of two G protein-coupled receptor subtypes ET_A and ET_B. ET_A receptors localized on vascular smooth muscle cells cause vasoconstriction, while ET_B receptors on endothelial cells exert largely vasodilatation. In pathology, ET-1 mediates vasoconstriction, mesangial cell proliferation, extracellular matrix production and inflammation, effects that are primarily conveyed by ET_A receptors. Renal ET-1 expression correlates with disease severity and renal function impairment.

Numerous studies have shown that ET system plays an important role in the pathogenesis of high blood pressure and associated end-organ damage in salt-sensitive rat models of hypertension. The hypertensive rat strain transgenic for the mouse Ren-2 renin gene ([TGR] strain name TGR[mRen2]27) is a valuable monogenetic model of renin-dependent and thus angiotensin II (Ang II) – dependent hypertension, which exhibits typical signs of fulminant hypertension, that is, reduced glomerular filtration rate and proteinuria associated with glomerulosclerosis. This model carries a salt-sensitive component. Our first study was performed to determine whether

onset of ET receptor blockade in animals with established hypertension will have similar protective effects as does early-onset therapy. Male homozygous TGRs and normotensive Hannover Sprague-Dawley (HanSD) rats were fed a high-salt diet between days 51 and 90 of age. TGRs received vehicle (untreated), the selective ET_A receptor blocker atrasentan, or the nonselective $ET_{A/B}$ receptor blocker bosentan. Survival rates in untreated and bosentan-treated TGRs were 50% and 64%, respectively, whereas with atrasentan, survival rate of TGR was 96%, thus, similar to 93% in HanSD rats. Systolic blood pressure in atrasentan-treated TGRs was transiently lower than in untreated or bosentan-treated TGRs. Glomerular podocyte injury was substantially reduced with atrasentan treatment independent of severe hypertension and strongly correlated with survival. Our data indicate that in homozygous TGR ET receptors play an important role also in established hypertension. Selective ET_A receptor blockade not only reduces podocyte injury and end-organ damage but also improves survival rates independently of hypertension.

Heterozygous TGR provide a suitable model of hypertension, since – in contrast to homozygous TGR who develop severe malignant hypertension – their hypertension is milder, thus allowing long-term studies. The aim of our subsequent projects was to evaluate the role of ET-1 in the onset and maintenance of hypertension in heterozygous Ren-2 transgenic rats (early onset treatment) and to evaluate the role of the ET system in male heterozygous TGR with established hypertension (late-onset treatment). At early age (from day 29 on), male heterozygous Ren-2 transgenic rats and nontransgenic HanSD rats fed a normal or high-salt diet were either untreated or treated with the nonselective $ET_{A/B}$ receptor blocker bosentan or the selective ET_A receptor blocker atrasentan. High-salt diet significantly accelerated the development of hypertension in heterozygous TGR. Nonselective $ET_{A/B}$ receptor blocker did not significantly influence the course of hypertension in transgenic rats, whereas selective ET_A receptor blocker significantly decreased blood presure on both diets. Atrasentan substantially reduced glomerulosclerosis, proteinuria and cardiac hypertrophy on both diets. Survival rate was partly increased by bosentan and fully normalized by atrasentan. Our data indicate that selective ET_A receptor blockade has

substantial positive effects on blood presure, end-organ damage and survival on both normal and especially on the high-salt diet.

Because antihypertensive therapy in general is known to be more efficient when started at early age, our last study was performed to determine whether onset of ET receptor blockade at a later age in animals with established hypertension will have similar protective effects as does early-onset therapy. TGR and control HanSD rats were fed a high-salt diet and were treated concomitantly with the nonselective $ET_{A/B}$ receptor blocker bosentan or the selective ET_A receptor blocker atrasentan from day 52 of age on. Survival rate was partly increased by bosentan and fully normalized with atrasentan. Bosentan transiently decreased blood pressure (BP), whereas atrasentan significantly reduced BP as early as one week after the start of the treatment. This effect persisted for the whole experimental period. Atrasentan also substantially reduced cardiac hypertrophy, proteinuria and glomerulosclerosis. Bosentan improved and atrasentan almost restored podocyte architecture and reversed changes in podocyte phenotype represented by the expression of CD 10, desmin and vimentin. Our results demonstrate that selective ET_A receptor blockade has more favorable effects than nonselective $ET_{A/B}$ receptor blockade and, unlike observed in homozygous TGR, ET_A receptor blockade has similar effects in heterozygous rats with established hypertension as in young animals with developing hypertension.

6 PŘEHLED PUBLIKAČNÍ A ODBORNÉ AKTIVITY

Publikované originální práce vztahující se k tématu disertační práce v časopisech s impact factorem

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- Pokroky v biologii a patologii buňky, LGE ÚEM AV a 1.LF UK, Praha
- Základy práce s laboratorními zvířaty
- Minikurzy v patologii 2006, Nádory hlavy a krku, Patologie zažívacího traktu, Nádory mammy, Nádory kůže, Nádory urologického traktu, Vybrané problémy v chirurgické patologii, Plzeň
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