UNIVERZITA KARLOVA V PRAZE

1. LÉKAŘSKÁ FAKULTA

Anatomický ústav 1.LF UK
Klinika otorinolaryngologie a chirurgie hlavy a krku 1.LF UK, FN v Motole

DISERTAČNÍ PRÁCE

GLYKOBIOLOGIE NÁDORŮ HLAVY A KRKU

MUDr. ZDENĚK ČADA

OBOROVÁ RADA: EXPERIMENTÁLNÍ CHIRURGIE

HLAVNÍ ŠKOLITEL: Prof. MUDr. Karel Smetana Jr., DrSc.
ŠKOLITEL KONZULTANT: As. MUDr. Jan Plzák, Ph.D.

PRAHA **2009**

Prohlašuji, že dizertační práci jsem vypracoval samostatně a použil jsem pouze prameny, které cituji a uvádím v seznamu literatury.

V Praze 15. 6. 2009

.....
MUDr. Zdeněk Čada

Tato PhD disertační práce byla vypracována v rámci prezenčního studia postgraduálního studia biomedicíny na Anatomickém ústavu a klinice ORL a chirurgie hlavy a krku 1. lékařské fakulty Univerzity Karlovy v Praze.

Doktorský studijní program v biomedicíně: Experimentální chirurgie

Předseda oborové rady: Prof. MUDr. Jaroslav Živný, DrSc.

Uchazeč: MUDr. Zdeněk Čada

Školitel: Prof. MUDr. Karel Smetana Jr., DrSc.

Školitel konzultant: As. MUDr. Jan Plzák, Ph.D.

Datum zahájení studia: 1.10.2005

PODĚKOVÁNÍ

V prvé řadě bych rád poděkoval za vynikající vedení a podporu v průběhu celého mého postgraduálního studia panu Prof. MUDr. Karlu Smetanovi Jr., DrSc.
Dále bych rád poděkoval panu Prof. MUDr. Janu Betkovi, DrSc., přednostovi Kliniky otorinolaryngologie a chirurgie hlavy a krku 1. LF UK Fakultní nemocnice v Motole za vyčerpávající podporu v mém studiu a klinické práci.
Nemalé díky patří as. MUDr. Janu Plzákovi, Ph.D. a as. MUDr. Martinu Chovancovi, Ph.D. za jejich podporu a cenné připomínky, které našly své uplatnění nejen v sepsání této disertační práci.

Rád bych také poděkoval svým nejbližším kolegům z Anatomického ústavu 1. LF UK, především RNDr. Barboře Dvořánkové, Ph.D. a MUDr. Lukáši Lacinovi, Ph.D. za velmi cenné rady z oblasti práce s tkáňovými kulturami, dále Evě Vancové, Ivě Burdové a Vítu Hajduchovi za skvělou technickou asistenci.

Velké díky patří německému vědeckému týmu vedenému Profesorem Hansem-Joachimem Gabiusem z Univerzity Ludwiga Maxmiliana v Mnichově za poskytnutí konzultací při korekci publikací a částečnou materiální pomoc.

OBSAH

1. Úvod

1.A. **Dlaždicové karcinomy hlavy a krku**

Dlaždicové karcinomy hlavy a krku představují kolem 5% všech tumorů. Naprostou většinu z nich (90%) tvoří dlaždicové karcinomy vycházející ze sliznic horních cest dýchacích a polykacích. Z klinického hlediska se dělí především dle lokalizace na karcinomy dutiny ústní, orofaryngu, epifaryngu, hypofaryngu, dutiny nosní, hrtanu a slinných žláz. Jedním z nejvíce rizikových faktorů pro vznik těchto nádorů je kouření. Více než 80% nádorů hlavy a krku je spojeno s expozicí tabákovému kouři (Myers et al., 2003). Mezi další rizikové faktory dále patří především alkohol, lidský papiloma virus (HPV, sérotypy 2, 6, 11, 16 a další), virus Epsteina a Barrové (EBV), dietní faktory (nedostatek β-karotenů, vitamínu A) (Nomura et al., 1997), faryngolaryngeální reflux, genetická predispozice (genetický polymorfismus genů enzymů, jež se podílejí na neutralizaci kancerogenů, např. CYP1A1, GSTM1 a další) (Fronhoffs et al. 2001), vlivy zevního prostředí (azbest, chrom, dřevný prach, prach v kožedělném průmyslu). Dlaždicové karcinomy hlavy a krku se nejčastěji vyskytující v orofaryngu a laryngu a jsou charakterizovány lokálním agresivním chováním a časným metastazováním do regionálních uzlin.

Systémové metastázy jsou především v plicích a játrech. Terapie je chirurgická, onkologická nebo kombinace obou modalit. Cílem terapie je zajistit radikální odstranění nádoru a dosažení uspokojivé kvality života (Parkin et al., 1988, Boring et al., 1992).

Navzdory diagnostickým i terapeutickým pokrokům zůstává stále prognóza pacientů s karcinomy hlavy a krku vážná. Při léčbě je nutné zachování dostatečné radikality a zároveň ochrana pacientů před zbytečně agresivními postupy, které zhoršují funkční výsledky (Chiesa et al., 1999, Ogawa et al., 1999).

Prognostické informace jsou nezbytné pro zhodnocení a výběr optimální léčebné modality s cílem dosáhnout co nejlepší kvality života a nejdelšího přežití. Na **prognostické znaky** je možno nahlížet z různého hlediska a podle toho je též klasifikovat:

a) Rizikové faktory (věk, kouření, konzumace alkoholu, atd.) stanovují riziko výskytu nádoru v populaci exponované tomuto faktoru ve srovnání s celou populací. Jejich stanovení má význam především v prevenci.

b) Diagnostické faktory (SCC Ag, Cyfra 21-1, atd.) napomáhají k detekci nových nádorů nebo k průkazu relapsu onemocnění v preklinickém stádiu. Jejich stanovení má význam především diagnostický. Záchyt změny hladiny těchto znaků nás vede k indikaci dalších vyšetření a eventuální včasné terapeutické intervenci.

c) Prognostické a prediktivní znaky v užším slova smyslu slouží k efektivnějšímu definování biologických vlastností nádoru (TMN klasifikace, histologická klasifikace, atd.). Jejich význam spočívá v určení správného léčebného postupu kdy napomáhají k předpovědi účinnosti terapeutického postupu a tudíž k volbě nejoptimálnějších léčebných modalit.

Je celá řada studovaných znaků na molekulární úrovni ve vztahu k patofyziologii dlaždicových karcinomů hlavy a krku, které by mohly být použity jako prognostické znaky. Obecně by se tyto znaky daly rozdělit podle mnohastupňových fenotypových alterací, které vedou k maligní transformaci na:

1. Získání autonomní proliferační signalizace (EGFR-receptor pro epidermální růstový faktor, HGFR-receptor pro hepatocytární růstový faktor atd.).
2. Inhibice růst tlumících signálů (alterace cyklinu D1, p27, p16, Rb proteinu, p53, atd.).
3. Únik mechanismům programované buněčné smrti (alterace bcl proteinů).
4. Imortalizace (zvýšený výskyt enzymu telomerázy v některých karcinomech).
5. Získání dostatečného cévního zásobení (iniciace neoangiogeneze: např. zvýšenou expresí receptorů pro vaskulární růstové faktory - VGFR, zvýšenou produkcí růstových angiogenních faktorů, např. hepatocytárního růstového faktoru, HGF atd.).
6. Získání invazivního a metastatického fenotypu (abnormality v expresi integrinů, lamininů atd.).

Zhodnocením lokálního nálezu pacienta celkového statutu s výše zmiňovanými je cesta k nejlepšímu výběru terapeutického schématu a další dispenzarizace pacienta. Dalšími molekulami, které by se mohly stát nadějnými prognostickými znaky, jsou členové rodiny endogenních lektinů-galektiny a jejich ligandy (Smetana et al., 2008, Gabius et al., 1997).

1.B. **Lektiny**

Lektiny jsou proteiny, které nemají charakter enzymů či protilátek a jsou schopné specificky rozpoznat sacharidové struktury (Barondes et al., 1988, Kocourek et al., 1981). S lektiny se setkáváme u všech živých organismů od virů, bakterií a rostlin až po živočichy. Nejdůležitější strukturní součástí molekuly každého lektinu je **doména rozpoznávající sacharidy** (Carbohydrate Recognition Domain, **CRD**).

Rostlinné lektiny byly popsány před více jak sto lety v souvislosti jejich schopností shlukovat erytrocyty a některé jiné buňky. Ačkoliv se lektiny vyskytují v celé rostlinné říši, největší množství jich bylo izolováno z luštěnin. Biologický význam rostlinných lektinů není zcela jasný, zdá se však, že se podílejí na ochraně rostlin před patogenními mikroorganismy a herbivorními živočichy. Význam rostlinných lektinů pro glykobiologii spočívá v možnosti užití jejich specifické vazby na vybraný cukerný motiv, čehož lze využít v chemii, biologii i diagnostice.

Živočišné (endogenní) **lektiny** dělíme na základě strukturního uspořádání na pět tříd (Tab. 1).

Tab. 1 Klasifikace živočišných lektinů

Rodina	Charakteristika	Sacharidové ligandy
C-lektiny	konzervativní CRD, pro vazbu se sacharidem potřebují divalentní kationty jako kofaktor	různé (manosa, galaktosa, fukosa, heparinový tetrasacharid)
I-lektiny	vykazují strukturální homologii s imunoglobuliny	různé (hyaluronová kyselina, α2,3/α2,6-sialyllaktosa, manosa-6N-acetylglukosa)
Galektiny (dříve S-lektiny)	konzervativní CRD, postrádají transmembránové hydofobní úseky, pro vazbu nepotřebují divalentní kationy	β-galaktosidy
Pentraxiny	pentamerové uspořádání	4,6 cyklický acetal β-galaktosy, galaktosa, sulfonylované a fosforylované monosacharidy
P-lektiny	konzervativní CRD	Manoso-6-fosfát

Zkratky: CRD karbohydráty-rozpoznávající doména

Galektiny patří mezi endogenní lektiny dříve nazývané S-lektiny, které jsou charakterizovány vysoce konservativní CRD a afinitou k ß-galaktosidům. Jsou aktivní i bez přítomnosti divalentních kationů. Nacházejí se především v cytoplasmě, v buněčném jádře a extracelulární matrix a mohou se vázat i na buněčnou membránu. Postrádají však hydrofobní transmembránový motiv. Doposud bylo popsáno minimálně 14 zástupců rodiny galektinů.

Dle struktury se dají rozdělit do 3 skupin.

1. „Prototype" typy-jsou složeny z peptidového řetězce s jednou CRD (galectin -1,-2, -5, -7, -10, -11, -13, -14)
2. „Tandem repeat" typy- dvě CRD spojené krátkým peptidovým řetězcem (galektin -4, -6, -8, -9, -12)
3. „Chimera typ"-CRD je lokalizovaná na C-konci molekuly, v oblasti N-konce je přítomna nelektinová doména bohatá na prolin, glycin a tyrozin (galektin-3) viz tab. 2

Tab. 2 Klasifikace galektinů dle struktury

Typ galektinu	Struktura	Zástupci
Prototyp – nekovalentní homo-dimery, obě části mají stejnou CRD se specifitou pro stejný oligosacharid		**galektin-1, -2, -5, -7, -10, -11, -13, -14**
Chimera typ – obsahuje CRD na C konci aminokyselinového řetězce, N konec oligosacharid neváže		**galektin-3,**
Tandem-repeat typ – obsahuje dvě kovalentně vázané CRD s různou specifitou		**galektin-4 -6, -8, -9, -12**

Karbohydráta rozpoznávající doména

Galektiny se uplatňují v široké škále biologických dějů, kde se podílejí na regulaci proliferace, diferenciace, apoptózy a modulaci mezibuněčné interakce a interakce s extracelulární matrix a to jak v normě, tak i za patologických stavů. V kancerogenezi

se uplatňují především galektin-1, -3, -7 (Plzák et al., 2001, Chiariotti et al., 2004, Hughes et al., 2001).

K nejvíce prozkoumaným galektinům ve vztahu ke kancerogenezi patří galektin-1, galektin-3 a galektin-7, který byl centrem zájmu této disertační práce.

Galektin-1 (molekulární hmotnost 14,5 kDa) se vyskytuje v mnoha tkáních (kostní, svalová, srdeční, placenta, lymfatická). Jeho funkce lze shrnout do následujících bodů:

1. buněčná adheze a mezibuněčné interakce
2. imunomodulace, zánětlivé procesy
3. regulace buněčného růstu
4. apoptóza
5. sestřih pre-mRNA.

Galektin-1 vykazuje jak pozitivní, tak negativní efekt na buněčnou adhezi. Příkladem takového antagonistického chování je zesílení adheze u buněk melanomové linie, buněk čichového epitelu či rabdomyosarkomu ve srovnání s normálními myoblasty kde adhezi inhibuje (Cooper et al., 1991). Galektin-1 je popisován jako významně proapoptotický lektin, který má zřejmě důležitou úlohu při selekci a vyzrávání T lymfocytů (Perillo et al., 1997). Je zvýšeně exprimován v imunologicky privilegovaných orgánech jako je placenta a oko. Pravděpodobně se uplatňuje jako protektivní faktor autoimunitních chorob právě pro jeho indukční vlastnosti apoptózy u aktivovaných autoagresivních klonů T lymfocytů (Levi et al., 1983, Offner et al., 1990). Protichůdné je působení galektinu-1 na buněčnou proliferaci. Jeho exprese stimuluje proliferaci endotelových buněk (Sanford et al., 1990). Rovněž přidání nízkých dávek exogenního lektinu jejich proliferaci stimuluje. Naopak vysoká ji inhibuje (Adams et al., 1996). Galektin-1 je asociován s ribonukleproteiny buněčného jádra (RNP), které jsou součástí sestřihových komplexů a podílejí se na vzniku definitivní podoby mRNA (Dagher et al., 1995, Vyakarman et al., 1997). Jeho zvýšená exprese byla nalezena ve většině transformovaných buněčných linií a tumorů (Plzák et al., 2004), při chronické pankreatitidě a u parazitárních onemocněních.

Karcinomy hlavy a krku vykazují heterogenní expresi galektinu-1 (Gillenwater et al., 1996). Exprese galektinu-1 je v literatuře popisována u karcinomů s výrazně maligním fenotypem a zvýšenou tendencí k metastazování především v karcinomech jazyka. V karcinomech laryngu a hypofaryngu se galektin-1 vyskytuje heterogenně, kde je jeho výskyt ovlivněn hypoxií v nádoru. Tyto nálezy u nádorů je možno dát do souvislosti s výskytem galektinu-1 u kmenových buněk dlaždicových epitelů (Purkrábková et al., 2003), neboť se zdá, že se kmenové buňky podílejí na vzniku nádorů vycházejících z dlaždicových epitelů (Motlík et al., 2007). Vysoký výskyt galektinu-1 ve stromatu dlaždicových karcinomů hlavy a krku je charakteristický (Lacina et al., 2007) a může se podílet na indukci apoptózy lymfocytů infiltrujících oblast nádoru (viz výše).

Galektin-3 se podobně jako galektin-1 vyskytuje v buňkách (jádro/cytoplasma) a v mezibuněčné hmotě. Podílí se rovněž se na adhezi buněk i intercelulárních interakcích, regulaci dělení a apoptózy a sestřihu pre-mRNA (Dumic et al., 2006). V dlaždicových epitelech je typická jeho přítomnost v suprabazálních vrstvách. Kromě toho je přítomen v makrofázích a Langerhansových buňkách (Smetana et al., 1999). Zatímco jeho přítomnost je typická pro karcinomy prostaty a štítné žlázy, v adenomech štítné žlázy zaznamenána nebyla (Sawangareetrakul et al., 2008). Exprese galektinu-3 je má proproliferační a antiapoptotický účinek (Polyak et al., 1997). Karcinomy hlavy a krku vykazují rozdílný výskyt galektinu-3 v závislosti na oblasti, z níž tumor pochází. Rovněž subcelulární lokalizace galektinu (jádro/cytoplasma/membrána) může přinést cenné informace o biologickém chování nádoru a prognóze (Honjo et al., 2000, Piantelli et al., 2002). Podobný význam má i průkaz vazebných míst pro galektin-3, která se nacházejí zejména v mezibuněčných kontaktech buněk dobře diferencovaných dlaždicových karcinomů. Naopak snížená vazba galektinu je typická pro méně diferencované karcinomy a metastázy do uzlin. Tyto nálezy se odrazily v nižším metastatickém potenciálu nádorových buněk a ve zvýšeném přežití pacientů s vysokou expresí vazebných míst pro galektin-3 (Plzák et al., 2004).

Galektin-7 představuje endogenní lektin prototypního typu exprimovaný ve všech vrstvách dlaždicového epitelu. Za fyziologických podmínek se uplatňuje v procesech regulace proliferace, apoptózy a stratifikace dlaždicových epitelů. Předpokládá se, že hraje důležitou roli v embryonálním vývoji vrstevnatých epitelů (Magnaldo et al., 1998, Timmons et al., 1999). Tyto výsledky naznačují, že galektin-7 by mohl být dobrým markerem normální stratifikace dlaždicových epitelů. Velice zřídka je detekován v bazocelulárních karcinomech (Chovanec et al., 2005). Zvýšená exprese mRNA byla zaznamenána u linie keratinocytů po expozici UVB záření a po aplikaci prodiferenciačních činidel (Bernerd et al., 1999). Je proto popisován jako p53 inducibilní gen 1 a jeho podíl na spuštění apoptózy, zejména u buněk s poškozenou DNA je zřejmý. Exprese tohoto lektinu v dlaždicových karcinomech je popisována s rozdílnými výsledky a prognostickými výhledy pro pacienta (Saussez et al., 2006).

1.C. **Epidermová kmenová buňka, nukleostemin**

V poslední době vzrostl velký zájem o studium tzv. kmenových buněk. Především pro jejich možné efektní a cílené použití v rámci ztrátových (tkáňových) patologických stavů. Kmenová buňka by se dala definovat jako buňka, která má schopnost se neomezeně dělit v buňky opět kmenové a v buňky, které vstupují do procesu časově omezené diferenciace. Kmenové buňky se obecně dělí na embryonální a tkáňové, které se nacházejí v tkáních dospělého jedince. Jiné všeobecně uznávané dělení kmenových buněk je na tzv. totipotentní, pluripotentní, multipotentní a monopotentní. Totipotentní kmenové buňky jsou takové kmenové buňky, které obsahují plnou genetickou informaci, jsou přítomné v oplozeném vajíčku a ve velmi časném embryu. Pluripotentní kmenové buňky jsou představovány embryonálními kmenovými buňkami, jenž se nacházejí embryoblastu blastocysty. Multipotentní kmenové buňky jsou přítomné v dospělém jedinci a dávají vznik omezenému počtu buněčných linií. Monopotentní kmenové buňky, které se také nacházejí v dospělém jedinci, dávají vznik pouze jedné buněčné linii. Existence kmenové buňky je závislá na prostředí ve kterém se nachází. Toto prostředí, ve kterém dochází k interakcím jak na subbuněčné tak buněčné úrovni, se nazývá Niche (Lanza et al., 2004). Vztah stromatu

v netransformovaných a transformovaných tkáních je probírán v kapitole epitelo-mezenchymální transformace a nádorové stroma-viz níže.

Epidermová kmenová buňka

Epidermis je vrstevnatý dlaždicový epitel, původem z ektodermu, který se skládá z keratinocytů, buněk obsahující intermediální filamenta - keratiny.

Důležitou úlohu v procesu stratifikace mají bázální buňky, které jsou v kontaktu s bazální membránou, jenž jsou schopné se dělit a od nichž začíná proces stratifikace a diferenciace. Kromě nich se epidermis skládá ze stratum spinosum, granulare, lucidum a korneum. Epidermální multipotentní kmenové buňky jsou uloženy v místě zevní pochvy vlasového folikulu zvaného „bulge" v blízkosti mazové žlázy a pravděpodobně i ve stratum basale. Z epidermálních kmenových buněk vznikají dělením nové multipotentní kmenové buňky s neomezeným potenciálem dělení a tzv. transit amplifying cells (TA cells), které mají omezený počet mitóz před terminální diferenciací (Morasso et al., 2005).

Centrem zájmu řady výzkumných skupin je najít specifické znaky nebo jejich kombinace charakterizující epidermální kmenové buňky využít je pro separaci těchto elementů.

Jedním z takových znaků je β1-inetgrin a α6β4 integrin. V epidermis zajišťují díky vysoké afinitě k fibronektinu adhezi buněk k bazální lamině. Zároveň se podílejí na přenosu signálu z extracelulárního prostředí do nitra buňky, tímto se uplatňují v procesech zajišťující organizaci cytoskeletu, proliferaci, apoptóze a diferenciaci (Mainiero et al., 1996). Někteří autoři popisují kombinaci vysoké exprese α6 integrinu a nízké exprese transferinového receptoru jako znaky epidermální kmenové buňky (Morasso et al., 2005, Li et al., 2004). Kromě β1-integrinu, se za znaky epidermální kmenové buňky popisují protein p63, β-katenin a také galektin-1.

β1 integrin je schopen udržovat buňky v nediferencovaném stavu a s přibývající diferenciací buněk exprese β1 integrinu klesá. U knock-out myši K14cre pro β1 integrin byl zaznamenán defekt v proliferaci (Morasso et al., 2005, Raghavan et al., 2000).

P63 je protein z rodiny p53 transkripčních faktorů. Je představován dvěma isoformami proteinů: TA p63 nebo ΔNp63. Oba proteiny se vyskytují ve 3 isoformách α, β, γ. Protein se uplatňuje především v regulaci kontroly buněčného cyklu, v signální transdukci dějů uplatňující se v morfogenezi. Dominantní

isorformou v transformovaných/netransformovaných dlaždicových epitelech je ΔNp63, která společně s izoformou TAp63 je schopna blokovat aktivity proteinu p53 a tím inhibovat apoptózu. P63 je popisován v literatuře jako protein, který se podílí v procesech diferenciace a přežití kmenových buněk v mnoha epitelech včetně dlaždicového (Morasso et al., 2005).

β-katenin je multifunkční protein, uplatňující se během embryonálního a neoplastického vývoje. Normálně se vyskytuje v membráně buněk dlaždicového epitelu, kde kooperuje s kadheriny. Jeho posun do oblasti cytoplasmy a jádra je pozorován v období embryogeneze a při maligních transformacích epitelů. Přítomnost tohoto proteinu v jádře je považována některými autory za známku kmenových buněk (Ridanpaa et al., 2001).

Galektin-1 vazebná místa (galectin-1 binding sites, Gal-1-BS), byla exprimovaná v jádrech buněk, které pocházely z oblasti „bulge" zevní pochvy vlasového folikulu a zároveň byly pozitivní na keratin-19 (Klíma et al., 2007). Mezi další znaky epidermálních kmenových buněk považují někteří autoři Ki-67, keratin-8 a CD 71 (Kaur a Li 2000).

Nukleostemin je jaderný a jadérkový protein vyskytující se v kmenových buňkách stromatu kostní dřeně, v nervových a hematopoetických kmenových buňkách (Lacina et al., 2006, Tsai et al., 2002, Yaghoobi et al., 2005). Proto se tento protein stává zájmem u studia kmenových buněk, včetně epidermálních kmenových buněk.

Exprese nukleosteminu je typická pro ranná stádia proliferace multipotentních buněk a klesá s postupnou diferenciací buněk. Nukleostemin se zřejmě uplatňuje v procesech řízení embryonálních, proliferačních, regeneračních dějů řízení apoptózy (Beekman et al., 2006). Přesný mechanismus působení nukleosteminu není znám, ale udává se, že váže GTP a kooperuje jako regulační faktor s proteinem p53. Jeho exprese byla zjištěna v některých nádorových buněčných linií, např. v SGC-7901, HepG2, HeLa, OS-732, MMK-7, HEK-293 (Liu et al., 2004, Sijín et al., 2004). V dlaždicových epitelech byla exprese nukleosteminu pozorována v bazálních i suprabazálních postmitoticky aktivních buňkách, pozitivní na keratin-10, který je znakem diferencovaných buněk. Nicméně zvýšená exprese m-RNA byla zaznamenána microarray technologií v místě bulge, tedy v místě uložení epidermálních kmenových buněk (Tumbar et al., 2004). V *in vitro* pokusech byla

zjištěna exprese nukleostemin pozitivních jadérek v kultuře keratinocytů z vlasového folikulu kokultivovaných s feederem z mesenchymálních nenádorových buněk, nebyla prokázáná v kultuře z interfolikulárních buněk (Lacina et al., 2006). Zajímavé je, že nebyla prokázána závislost mezi expresí nukleosteminu a keratinu-19, jenž je považován za jeden ze znaků epidermálních kmenových buněk (Lacina et al., 2006).

1.D. Nádor a nádorové stroma, epitelomezenchymová transformace

Klasicky se dají nádory rozdělit na benigní a maligní. Maligní nádor v plném slova smyslu se dá definovat jako soubor populace buněk s přechodně neomezeným růstem a schopností růstu v nejméně ve třech kompartmentech: v původním kompartmentu, ze kterého tumor vyrůstá, v mezenchymu tkáně, do které tumor vrůstá (tzv. invazivní fronta nádoru) a do tzv. vzdáleného kompartmentu, který umožňuje regionální či vzdálený rozsev-metastázy.

Zatímco některé nádory se vyskytují disperzně v tkáních v podobě „suspenze", příkladem je možné uvést leukemie, jiné tumory tvoří solidní tkáňové masy-tzv. solidní nádory. Solidní nádory se skládají ze dvou základních komponent, z vlastních nádorových buněk-parenchymu a z nádorového stromatu. V případě epitelových nádorů, které jsou centrem našeho zájmu, je stromální komponenta zpravidla oddělená od vlastních nádorových buněk bazální laminou, která bývá velmi často neúplná či chybějící.

Obecně patologové rozdělují tumory na ty, které obsahují málo stromatu, příkladem je možné uvést medulární karcinomy prsu nebo lymfomy a tumory označované jako desmoplastické, jenž obsahují velké množství stromatu. Do této skupiny patří většina nádorů žaludku, pankreatu aj. Udává se, že v těchto nádorech je až 90% stromální komponenty (Dvorak et al., 1991).

Stromální komponenta je přítomna již při velikosti tumoru kolem 1-2 mm. Nádorové stroma obsahuje řadu buněk. Nejvíce zastoupenou skupinou buněk představují fibroblasty, žírné buňky, endotelové buňky, adipocyty, makrofágy a buňky imunitního systému. Kromě buněčných elementů se zde nacházejí i cévy, produkty rozpadu buněk, plasmatické proteiny, proteoglykany, glykosaminoglykany, fibrin,

kolagen (především typ I, III), fibronektin, fibroblasty aj. Ačkoliv většina těchto buněk a složek extracelulární matrix je původem z nemaligních tkání, jejich vzájemné interakce mezi sebou a vlastním nádorem vedou k udržování funkčního maligního fenotypu s přítomnými interakcemi buňka-buňka, buňka-extracelulární matrix a produkty tansformovaných a netransformovaných buněk, mezi které patří různé typy chemokinů, cytokinů a růstových faktorů (Kulbe et al., 2004).

V minulosti byl epitel rozlišován od mezenchymálních buněk pouze na podkladě morfologické struktury, kdy epitel tvoří víceméně uniformní a kompaktní celek, charakterizovaný svojí polaritou a těsnými mezibuněčnými kontakty (Shook and Keller 2003), který v 3-D prostoru tvoří jasně definovaný celek s určitou funkcí a morfologickou charakteristikou. Mezenchymální buňky jsou jakoby opakem pravidelnosti a uniformity epiteliálních buněk. Netvoří jasně definované celky, jenž jsou ve vzájemném kontaktu, ale disperzně uložené buňky či shluky buněk, jejichž protáhlý tvar a menší vazby mezi buňkami a extracelulární matrix nedovolují vytvořit kompaktní systém. Na druhou stranu umožňují snazší migraci buněk v tomto prostoru.

Nejpočetnější buněčnou populací v nádorovém stromatu představují fibroblasty, jejichž jednotlivé zastoupení v nádorech se liší. Obecně se tyto fibroblasty označují tumory asociující fibroblasty (CAFs-cancer associated fibroblasts), pro které je charakteristické vedle vřetenovitého tvaru exprese α-SMA. Rozdíl mezi těmito a normálními fibroblasty je, že CAFs jsou trvale aktivovány, nekonvertují zpět na normální fibroblasty a nepodléhají klasické apoptóze (Li et al., 2007). Původ tumor asociujících fibroblastů, není stále jasný. Je několik teorií, které se snaží vysvětlit jejich původ. Jedna z nich uvažuje o fůzi nádorových buněk s pozičně blízkými rezidentními fibroblasty (Duelli a Lazebnik 2003), jiná teorie zvažuje jejich původ z CAFs prekurzorů buněk aktivované kostní dřeně, přičemž je stále nejasné zdali buňky derivované z kostní dřeně se stávají aktivní teprve v interakci s mikroprostředím tumoru a nebo představují již fenotypově předurčenou skupinu buněk v kostní dřeni (Ganss et al., 2006), a konečně se velmi intenzivně studuje teorie epitelomezenchymální transformace (EMT).

EMT bychom mohli definovat jako změnu epiteliálních buněk v jejich morfologii a architektuře, provázené změnou adheze, zvýšením migrační aktivity a rezistencí k anoikis/apoptóze. Jiná definice EMT, která zahrnuje fakt, že EMT je zaznamenávána v mnoha buněčných linií v *in vitro* studií, tak v *in vivo* studiích,

například u karcinomu prsu se vyskytuje pouze v 18%, definuje EMT jako přechod epiteliální komponenty do stavu fenotypově odpovídající mesenchymu s paralerní expresí vimentinu a ztrátou exprese E-cadherinu. Petersen (Petersen et al., 2003) definuje EMT u karcinomu prsu jako expresi mesenchymálních znaků-vimentinu, tenascinu a stromelysinu, u variabilního počtu nádorových buněk.

Jak již bylo výše zmíněno EMT může být objektivizována změnou a expresí molekulárních markerů. Obecně uznávanými markery EMT je zvýšená exprese N-kadherinu, vimentinu, jadérková lokalizace β-kateninu, zvýšená produkce transkripčních faktorů jako Snail 1(Snail), Snail 2(Slug), EF1/ZEB1, SIP1/ZEB2, E47, který inhibuje produkci E-cadherinu. Molekulární základ EMT prochází řadou intenzivních studií již od roku 1985, kde byl centrem zájmu hepatocytární růstový faktor (HGF), jenž byl označován „dispersing" faktor pro epiteliální buňky působením na c-met receptor s tyrosinkinázovou aktivitou (Thiery et al., 2003). Kromě HGF se studují další růstové faktory a proteiny, především TGF-β, PDGF, LEF, integriny β4 a α5, Snail a Slug (Tse a Kalluri et al., 2007). Poslední dva zmiňované se uplatňují především v „rozpuštění" mezibuněčných kontaktů (Shi and Massagué 2003). Působí především svým aktivním C-koncem na promotory DNA a tím ovlivňují transkripční aktivitu buňky (Huber et al., 2004).

Popisuje se celá řada molekulárních kaskád, které při aktivaci vedou k epitelomezenchymální transformaci. Jednou z nich je aktivace tyrosinkinásových receptorů, spuštěním kaskády Ras/Raf/MAPK, končící aktivací transkripčních faktorů Snail/Slug. Jiný příklad kaskády iniciovanou aktivací tyrosinkinázového receptoru je Ras/PI3K/AKT. Netyrosinkinásové kaskády jsou představovány transkripčními faktory SHIP-1, Crk, Rhoa, Smad2, Notch, Hedgehog, NF-κB (Thiery 2003).

Oblasti epitelomezenchymálního přechodu je možné dle některých prací definovat jako přítomnost buněk s koexpresí vimentinu a cytokeratinů s paralerní expresí transkripčního faktoru Snail (Huber et al., 2004, Petersen et al., 2003). Jiné studie poukazují na místo EMT se zvýšenou expresí Snail a sníženou expresi E-cadherinu (Yokoyama et al., 2003).

Důležitou otázkou je, zdali vzniklé mezenchymální buňky-(CAFs) jsou schopné nějakým způsobem ovlivňovat biologickou aktivitu vlastní transformované či netransformované tkáně. Příkladem můžeme uvést výrazně rychlejší růst karcinomových linií po přidání separovaných nádorových stromálních buněk. Na druhou stranu je nutné konstatovat, že aplikace těchto buněk do netransformované

tkáně, i přes stejné genetické mutace, nevyvolalo tumorigenní chování (Petersen et al., 2003).

V procesu kancerogenese hraje EMT dle některých literárních údajů velkou roli, neboť umožňuje zatím neagresivním benigním nádorovým buňkám (neinvazivní a neschopné metastazovat) stát se invazivními, tedy se schopností prorůstat do okolní tkáně a zakládat vzdálené metastázy (Li et al., 2007).

Na závěr je nutné říct, že proces EMT je velmi zajímavou oblastí ve studiu kancerogenese, ale je nutné na něj pohlížet jako na dílčí důležitou podjednotku velkého a složitého celku biologie tumorů. Kromě toho se zdá, že by se tento mechanismus mohl uplatňovat i při vzniku biologicky aktivních stromálních fibroblastů.

2. Cíle disertační práce:

- Glykobiologická charakterizace dlaždicových karcinomů hlavy a krku zejména z hlediska exprese vybraných galektinů ve srovnání s normálnimi tkáněmi v korelaci s výskytem funkčně významných jaderných a cytoplasmatických proteinů.

- Exprese nukleosteminu v dlaždicových karcinomech hlavy a krku a jeho korelace s expresí znaků charakterizující epidermální kmenové buňky.

- Objasnění funkce, vzniku a původu nádorově asociovaných stromálních fibroblastů stromatu jako niche pro nádorovou kmenovou buňku.

3. Materiál a metodika

3.1. **Použitý biologický materiál**

Všechny vzorky byly odebírány s příslušným informovaným souhlasem pacienta. Vzorky normální epidermis, bazocelulárních karcinomů a dlaždicových karcinomů pocházely převážně z Dermatovenerologické kliniky 1.LF UK a VFN v Praze 2, z kliniky ORL a chirurgie hlavy a krku 1.LF UK a FN v Motole, Praha 5 a z Kliniky plastické chirurgie 3. LF UK a FN Královské Vinohrady .

3.2. **Kultivace buněk**

Vzorky normální kůže a nádorů byly odebrány přímo na operačním sále a uloženy do transportního kutivačního média s přídavkem antibiotik (penicilin, streptomycin- Sigma Aldrich, Praha, ČR) a antimykotik (amfotericin B- Sigma-Aldrich, Praha, ČR) a převezeny do laboratoře na Anatomickém ústavu, kde vzorky byly enzymaticky rozvolněny roztokem trypsinu (Sigma-Aldrich, Praha, ČR) a ethylendiamintetraoctové kyseliny-EDTA (Sigma-Aldrich, Praha, ČR). Z epidermis byla získaná primární kultura interfolikulárních keratinocytů, z dermis kultura folikulárních keratinocytů a fibroblastů. Ze vzorků bazocelulárních a dlaždicových karcinomů byly připraveny primokultury stromálních fibroblastů a keratinocytů. Dále jsme použili linie myších embryonálních fibroblastů 3T3, linie FaDu-původně izolovaná z dlaždicového karcinomu hypofaryngu a buněčné linie TC-1 původně získané transformací epiteliální myší linie C57BL/6 pomocí HPV 16 E6/E7 a aktivovaného H-ras proonkogenů. Linie 3T3 buněk jsme použili jako podpůrnou půdu pro kultivaci keratinocytů. Před nasazením 3T3 buněk ve vhodné denzitě ke kokultivaci s keratinocyty jsme použili Mytomycin k zastavení jejich proliferační aktivity. Buňky byly ko-kultivovány v médiu HMEM
(Sevapharma, Praha, ČR) s 10% bovinním sérem (ZVOS, Hustopeč, ČR) a se zvýšenou tenzí CO_2 (3,3%). Vedle 3T3 linií jsme jako podpůrné fibroblastové buňky použily linie dermálních lidských fibroblastů, fibroblasty izolované z karcinomů a TC1 buněk, jenž byly kultivované v DMEM médiu (Biochrom, Berlín, NSR) s 10% fetálním bovinním sérem (Biochrom, Berlín, SRN) při 37 °C a 5% tenzi CO_2. Výše zmiňovaná linie Fadu byla kultivována ve EMEM médiu (Biochrom, Berlín, SRN) s 10 % fetálním bovinním sérem při 37 °C a 5% tenzi CO_2.

Jednotlivé buňky (mezenchymové buňky/keratinocyty) byly studovány jak ve 2D prostoru po nasazení na krycí skla nebo ve 3D rostoru po nasazení do Matrigelu (BD, Biosciences Erembodegen, Belgie). Jednotlivé interakce epitelových a mezenchymových buněk byla studována buď v přímém vzájemném kontaktu nebo v systému Insert (BD-Falcon, Franklin Falls, USA), který umožňuje za pomocí mikroporózní membrány studovat vzájemnou interakci dvou buněčných populací pomocí solubilních faktorů, pronikající přes mikroporózní membránu, aniž by došlo k jejich vzájemnému fyzickému kontaktu.

3.3. **Imunohistochemie**

Vzorky normálních i nádorových byly po odběru ihned ještě na operačním sále upraveny na vhodnou velikost cca 5x5x5mm a ponořeny do zmražovacího média Tissue-Tek (Sakkura,Zoeterwoude, Nizozemí). Po 60 minutách, kdy byl vzorek uložen při teplotě +4°C, bylo provedeno rychlé zmražení v tekutém dusíku a dále byl vzniklý bloček uchován při teplotě -80°C do definitivního zpracování. Zmražená tkáň byla následně nakrájena na kryostatu Cryocut-E (Reichert-Jung, Vídeň, Rakousko) na řezy o síle 7 μm. Tyto byly přeneseny na skla s povrchem modifikovaným poly-L-lysinem (Sigma-Aldrich, Praha, ČR).

Získané kultury rostoucí na krycích sklech byly po opakovaném opláchnutí v pufrovaném fyziologickém roztoku (PBS) rychle usušeny v laminárně proudícím vzduchu a uchovávány do definitivního zpracovaní v mrazicím boxu při teplotě -20°C. Kultury v Matrigelu byly po odsátí kultivačního média bleskově zmraženy v tekutém dusíku a tovněž uchovávány do definitivního zpracování při teplotě -80°C, kdy byly nakrájeny na kryostatu. Před vlastním imunohisto- a cytochemickým zpracováním byly vzorky krátce fixovány v paraformaldehydu (2 % /w/v/ paraformaldehydu v PBS /pH 7.3/) a permeabilizovány za použití Triton X-100 (Sigma-Aldrich, Praha, ČR). Bylo použito metody vícenásobného značení na úrovni jedné buňky. Ředění protilátek použitých ve studiích respektovalo pokyny uvedené výrobci jednotlivých protilátek. Nespecifická vazba protilátek druhého kroku byla blokována pomocí prasečího séra (DAKO, Brno, ČR). Po obarvení byl vzorek zamontován do média Vectashield (Vector Laboratories, Burlingame, CA, USA) a hodnocení vzorků a měření bylo prováděno na fluorescenčním mikroskopu Optiphot-2 a později Nikon Eclipse 90i (Nikon, Praha, ČR) vybaveném specifickými filtry (FITC = fluoresceinisothiokyanat, TRITC = tetrametylrodamin

isothiokyanat, DAPI= 4',6'-diamidino-2-phenylindole dilactate) a chlazenou CCD kamerou o vysokém rozlišení Cool-1300Q (Vosskühler, Osnabrick, NSR). Analýza obrazu a měření fluorescenčních profilů bylo prováděno pomocí softwarového systému Lucia 3.2 respektive 5.1 (Laboratory Imaging, Praha, ČR). Výsledky byly hodnoceny Studentovým t- testem. Specificita imunohistochemické reakce byla ověřena nahražením protilátky prvního kroku jinou v dané tkáni se nevyskytující protilátkou. Barvení jaderné DNA bylo univerzálně prováděno pomocí DAPI (Sigma-Aldrich, Praha, ČR).

3.4. **Lektinová histochemie**

K detekci vazebných míst pro jednotlivé galektiny byly použity biotinylované galektiny, které připravil připravil H.-J. Gabius a S. André (Univerzita Ludwiga-Maximiliana, Mnichov), jako značení druhého kroku byl použit ExtrAvidin-TRITC (Sigma-Aldrich, Praha, ČR). Jako test specifické reakce při lektinové histochemii byl buď vypuštěn z protokolu biotinylovaný galektin, popřípadě byla provedena inhibice laktózou.

3.5. **Analýza na FACS**

Měření byla prováděna na přístroji FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA,USA) v suspenzi enzymaticky odvolněných buněk po neutralizaci trypsinu sérovými proteiny. Analýza výsledků byla provedena softwarem Summit V3.3. Build 1024 (DakoCytomation, Fort Collins, CO, USA).

3.6. **Cytogenetická analýza**

Cytogenetická analýza SCCF (mezenchymální buňky izolované ze spinocelulárního karcinomu) kultivovaných 4 hodiny v médiu s obsahem demekolcemidu (Sigma Aldrich, Praha, ČR) po dobu 4 hodin, které byly následně roztokem trypsinu a EDTA odvolněny, ošetřeny v hypotonickém roztoku KCl a fixovány v kyselém methanolu. Metafazické chromozómy byly obarveny metodou G-/R-banding a analyzovány systémem Ikaros version 5 (MetaSystems, Altlussheim, NSR). Takto bylo analyzováno 50 buněk ve všech vzorcích.

Podrobně jsou všechny postupy a podmínky experimentů popsány v publikacích, které jsou uvedené v příslušné kapitole.

4. Výsledky a diskuze

4.1. **Galektin-7** (publikace č. 4)

Zjistili jsme, že výskyt galektinu-7 v dlaždicových karcinomech nebyl uniformní. Dle rozložení a intenzity signálu pro galektin-7 byly rozlišeny 4 modelové situace v tumorech: **A:** silná intenzita signálu s homogenním rozložením signálu, **B:** silná intenzita signálu s heterogenním rozložením signálu, **C**: slabá intenzita signálu s homogenním rozložením signálu, **D**: žádná intenzita signálu.

Statisticky nejvýznamnější korelace (P=0,0105) silné exprese galektinu-7 bez závislosti na homogenitě či heterogenitě signálu byla zaznamenána ve vztahu ke keratinizaci, kde silná exprese galektinu-7 byla detekována v rohových perlách tumoru s nejčastější lokalizací v centrálních partiích nádorů. Statisticky významná korelace (P=0,0024) byla rovněž zaznamenána mezi silnou homogenní expresí galektinu-7 a přítomností kontinuálně formované bazální laminy. Další statistická významnost byla potvrzena u silně homogenní exprese galektinu-7 a diferenciací nádoru (P=0,0009), kde tumory se silnou intenzitou signálu exprese galektinu-7 vykazovaly dobrou diferenciaci (Grading-1 a 2). Tento nález koreluje s výsledky některých publikací (Bernerd et al., 1999). Vzhledem k některým publikovaným pracím (Saussez et al., 2006), které poukazují například na špatnou prognózu u pacientů s karcinomem hypofaryngu stádia IV s paralerně nízkou expresí galektinu-7, nebyla v našem souboru tato závislost zjištěna. Zajímavým nálezem byla detekce galektinu-7 v jádrech buněk a to především v tumorech se silnou a homogenní expresí, což by mohlo souviset s určitou rolí galektinu-7 při sestřihu pre-mRNA, jako je tomu u galektinu-1 a 3 (Wang et al., 2004). Bohužel nebyla zaznamenána statistická závislost mezi expresí galektinu-7 a přežitím.

Exprese galektinu-7 nebyla detekována v bazocelulárním karcinomu (n=10) (Chovanec et al., 1995).

Závěrem je možné konstatovat, že z výsledků této práce není možné v tuto chvíli považovat galektin-7 za suverénní diagnostický a prognostický marker.

4.2. **Nukleostemin** (publikace č. 1)

Zjistili jsme, že jadérková exprese nukleosteminu byla přítomna v kontrolních normálních sliznicích laryngu a orofaryngu, a to jak v bazálních tak suprabazálních

vrstvách (Smetana et al., 2006), přičemž velikost jadérek byla v obou kompartmentech srovnatelná. Průměrná velikost plochy jadérek byla v rozmezí 6-10 μm^2.

V dlaždicových karcinomech hrtanu, jazyka, orofaryngu, FaDu buňkách a tumorech transplantovaných nu/nu myší jsme zaznamenali silnou expresi nukleosteminu v jadércích, jejichž velikosti nebyly tak uniformní ve srovnání s normálním epitelem, ale na druhou stranu byly daleko větší. Zjistili jsme jadérka i o velikosti plochy do 35 μm^2. Současně s expresí nukleosteminu v jadércích jsme detekovali expresi β-kateninu, jehož jaderná exprese je popisována jako znak epidermální kmenové buňky (Smetana et al., 2006, Lacina et al., 2007). Zjistili jsme, že velikost jadérek v nukleostemin pozitivních buňkách s jadernou či cytoplasmatickou expresí β-kateninu byla ve srovnání s expresí β-kateninu na buněčné membráně vyšší. Zrovna tak jadérka keratin-10 negativních a nukleostemin pozitivních buněk byla větší ve srovnání s buňkami pozitivní na keratin-10. Na druhou stranu je třeba dodat, že jsme ojediněle nalezli buňky s velkou plochou jader, které byly cytokeratin-10 pozitivní a současně se u nich vyskytovala membránová exprese β-kateninu. Tento stav by mohl být přirovnán k popisovanému výskytu disproporce mezi diferenciací a maturací v dlaždicových karcinomů objektivizovaný současnou expresí Ki-67 s markery charakterizující terminálně diferenciační stav buňky (Chovanec et al., 2005).

Expresi galektinu-1, který je považován některými autory znakem epidermálních kmenových buněk (Klíma et al., 2005), jsme detekovali pouze ve stromatu tumoru. Tento nález je obdobný s popisovanou expresí tohoto endogenního lektinu ve stromatu bazocelulárního karcinomu, psoriázy a dermis (Lacina et al., 2006).

Ačkoliv je řadou autorů nukleostemin považován za marker kmenové buňky (Tsai et al. 2002, Yaghoobi et al., 2005) výsledky naší práce tuto hypotézu zatím nepodporují.

4.3. **Interakce stromálních a epitelových buněk *in situ* a *in vitro*, nádorové stroma, epitelomezenchymová transformace**

(publikace č. 2, 3, 5, 6)

Zjistili jsme, že izolované SCCF (fibroblasty připravené z dlaždicového karcinomu) vykazovaly znaky typické pro mezenchymové buňky. Byly vimentin pozitivní a keratin negativní. Ve 30 % exprimovaly Ki67 a byla zjištěna jádra s nukleostemin pozitivními jadérky koexprimující Ki-67 i jádra s nukleosteminem bez současné

přítomnosti proliferačního markeru Ki-67. 20 % buněk obsahovalo galektin-1. Tyto buňky rovněž produkovaly extracelulární matrix obsahující tento galektin. Tento nález je obdobný s popisovaným výskytem galektinu-1 ve stromatu bazocelulárního karcinomu (Lacina et al., 2007).

Zajímavý byl rozdílný fenotyp kolonií interfolikulárních epitelových buněk kokultivovaných s dermálními fibroblasty či s 3T3 buňkami a keratinocyty kokultivovanými s SCCF. Zatímco keratinocyty kokultivované s nenádorovými fibroblasty tvořily klasické ploché oválné kolonie, byly kolonie keratinocytů kokultivované se SCCF nepravidelné a s výskytem keratinocytů spíše podobných i fibroblastům. Vedle morfologických rozdílů jsme u těchto buněk zaznamenali expresi keratinu-8. Tento keratin je u nádorových keratinocytů znakem agresivity a v mnoha případech signalizuje špatnou prognózu pacienta (Casanova et al., 2004, Raul et al., 2004). Je zároveň i znakem prekurzorů epidermové kmenové buňky (Troy a Turksen 2005). Kromě toho jsme pozorovali i expresi keratinu-19 a přesun β-kateninu z membránové lokalizace do cytoplasmy. Současná exprese keratinu-19, který je považován za znak kmenových buněk epidermis a přesmyku β-kateninu do cytoplasmy jsou popisovány u velmi agresivních tumorů (Smetana et al., 2005, Morasso et al., 2005). Velmi zajímavým nálezem, který současně podporuje teorii vznik nádorového stromatu epitelomezenchymovým přechodem, bylo zjištění koexprese vimentinu a keratinu v četných keratinocytech kokultivovaných se SCCF. Tato skutečnost byla podpořena i expresí transkripčního faktoru Snail, jenž tento proces řídí a je považován za znak přechodu epitelových buněk do fibroblastů (Thiery et al., 2003, Thiery a Sleeman 2006). Nutno však podotknout, že současnou expresi vimentinu a keratinu jsme ve velmi omezeném množství zaznamenali i v normálních keratinocytech kokultivovaných s dermálními a 3T3 fibroblasty.

Tyto vzájemné interakce mezi SCCF a interfolikulárními epidermálními keratinocyty mohou být vysvětleny dvěma mechanismy, a to interakcí vzájemných kontaktů buňka-buňka a/nebo parakrinním působením růstových faktorů/cytokinů produkovanými SCCF. Proto jsme dále kultivovali keratinocyty oddělené mikroporózní membránou od buněk SCCF. V tomto modelu normální keratinocyty opět exprimovaly keratin-8 včetně koexprese keratinu a vimentinu-tedy znaky epitelomezenchymového přechodu.

Ve snaze o vytvoření modelu, který by se co nejvíce podobal podmínkám *in vivo*, jsme kultivovali normální interfolikulární keratinocyty se SCCF ve 3D systému v matrigelu. I za těchto podmínek keratinocyty exprimovaly keratin-8 a vimentin.

Dalším příkladem interakce mezenchymálních a epitelových buněk je možné uvést na na příkladu fibroblastů izolovaných z benigního fibrózního histiocytomu (FBFH) a normálních keratinocytů. Toto pozorování ukázalo, že interakce nádorového epitelu a okolního mezenchymu hraje roli i u benigních nádorů a může vysvětlit hyperproliferaci keratinocytů nad ložiskem tohoto typu nádoru.

K dalšímu důkazu vzájemné interakce stromálních a epiteliálních buněk (normální folikulární-NHF, interfolikulární keratinocyty-NIF) jsme použili geneticky upravenou buněčnou linii TC-1, která byla připravena transfekcí myších plicních epitelových buněk geny *E6/E7* lidského papilomaviru HPV16a genem pro *H-ras*. Tyto buňky lze považovat za model buněk, které vznikly epitelověmezenchymovou transformací buněk nádorových a hypoteticky se mohou podílet na vytváření bioaktivního stromatu. Kontrolu představovaly 3T3 myší fibroblasty kokultivované s výše zmiňovanými keratinocyty.

Rozdíly jsme opět zaznamenali v morfologii kolonií. NHF a NIF kokultivované s 3T3 buňkami tvořily klasické ploché oválné kolonie. Stejné keratinocyty kokultivované s TC-1 buňkami tvořily malé, místy až papilomatózně vzhlížející kolonie. Po separaci TC-1 buněk mikroporózní membránou NIF keratinocyty tvořily velké kolonie, zatímco kolonie NHF keratinocytů byly opět malé. Je nutné konstatovat, že hranice buněk v koloniích byly obtížně patrné, ale bylo jasné, že buňky jsou extrémně malé. Měřili jsme proto plochu jádra (DAPI). Velikost jader keratinocytů kokultivovaných s TC-1 buňkami byla velmi malá. Podobala se velikosti jader rychle adherujících keratinocytů, které jsou rovněž velmi malé a jsou považovány za epidermovou kmenovou buňku (Barrandon et al., 1985; Klíma et al., 2007).

Všechny keratinocyty kokultivované s TC-1 buňkami exprimovaly keratin-8 a 19, tedy keratiny, jež charakterizují méně diferencované až kmenové epidermální buňky a buňky nádorové (Michel et al., 1996, Commo et al., 2000). TC-1 rovněž indukovaly expresi Ki-67, vimentinu a nukleosteminu.

Zároveň je možné konstatovat, že ačkoliv byly použité myší modelové linie (3T3, TC-1) v interakci s lidskými keratinocyty, je proces vzájemného působení mezi stromálními a epiteliálními buňkami druhově nespecifický. Uvedené nálezy rovněž naznačují na podíl mezenchymových buněk vzniklých epitelověmezenchymovou

transformací na vzniku bioaktivních stromálních elementů. Ukazují i na možnou úlohu kmenových buněk v ontogenezi nádorů vycházejících z dlaždicových epitelů.

5. Souhrn výsledků

Nejdůležitější výsledky lze shrnout do těchto bodů.

- Exprese galektinu-7 v karcinomech hlavy a krku je heterogenní, s rozdílným rozložením a intenzitou signálu. Statisticky nejvýznamější korelace byla zaznamenána mezi silnou expresí galektinu-7, keratinizací, gradingem a přítomností dobře formované bazální membrány. Nebyla zaznamenána statistická závislost mezi expresí galektinu-7 a přežitím.

- Prokázali jsme, že exprese nukleosteminu není závislá na proliferačním stavu buňky, a to jak v normální-netransformované tkáni, tak v tkáni transformované. Dále jsme zaznamenali určitou korelaci mezi velikostí jadérek a nucleostemin-pozitivními nádorovými buňkami. Ačkoliv je nukleostemin považován řadou autorů za znak některých kmenových buněk (např. hematologických, nervových), z našich poznatků nelze tento protein považovat za znak kmenovosti v epitelových buňkách.

- Zjistili jsme, že izolované nádorové fibroblasty jsou schopné změnit fenotyp netransformovaných/normálních keratinocytů do podoby nádorových buněk. Zároveň jsme prokázali, že při vzájemných interakcích těchto buněk se v keratinocytech exprimují znaky, které jsou typické pro méně diferencované až kmenové buňky. Rovněž jsme nastínili možnost vzniku nádorového stromatu procesem epitelomezenchymové transformace.

6. Summary

Cancers of head and neck represents about 5% of all tumors. 80 to 90% of these tumors are constituted of squamous cell carcinomas. Despite a rapid progress in diagnostics and therapy the overall 5-year survival of this type of cancer is among the lowest of the major cancer types. This unfavourable situation needs the extensive research to found new markers to better characterize biological behavior of tumors as a rational background for more sophisticated therapeutic modalities. One of the most promising markers are endogenous lectins called galectins and their ligands. Especially galectin-1, -3 and -7 play a key role in pathology of squamous cell carcinomas. Galectin-7 is described in literature as a protein which has anti and pro-malignant features in different *in vitro* models. We studied tissue sections immunohistochemically and disclosed a correlation to increased status of differentiation and keratinization in head and neck squamous cell carcinomas.

Other marker which could better characterizes the tumors is nucleolar protein nucleostemin. We proved that presence of nucleostemin was documented in head and neck cancer, and its detection, together with the size properties of positive nucleoli, may relate to tumor cell features. Although nucleostemin is described as a marker of stem cells (e.g. neural or hematopoietic stem cells), we cannot consider this protein as realible marker of epidermal stem cells, becouse it is expressed by suprabasal, terminally differentiated keratinocytes.

The fibroblasts prepared from stroma of squamous cell carcinoma influence the phenotype of normal human epidermal keratinocytes to be similar to epidermal stem cell. These fibroblasts can participate in the control of biological properties of this type of cancer.

7. Použitá literatura

- Adams L, Scott,G K, Weinberg C: Biphasic modulation of cell growth by recombinant human galectin-1. Biochem Biophys Acta, 1996, 1312, s. 137-144.

- Barondes S H: Functional properties of lectins: Lectin redefined. Trends Biochem Sci., 1988, 13, s. 480-482.

- Barrandon J, Green H: Cell size as a determinant of the clone-forming ability of human keratinocytes. Proc Natl Acad Sci USA, 1985, 82, s. 5390-5394.

- Beekman C, Nichane M, De Clerq S, Maetens M, Floss T,Wurst W Bellefroid E and Marine JC: Evolutionarily conservedrole of nucleostemin: controlling proliferation of stem/progenitor cells during early vertebrate development. Mol Cell Biol., 2006, 26, s. 9291-9301.

- Bernerd F, Sarasin A, Magnoldo T: Galectin-7 overexpression is associated with the apoptotic process in UVB-induced sunburn keratinocytes. Cell Biology, 1999, 96, s. 11329–11334.

- Boring, C C, Squires, T S, Ton, T: Cancer statistics, 1992. CA Cancer J Clin., 1992, 42, s. 19-38.

- Casanova M L, Bravo A, Martinez-Palacio J, Fernandez-Acenero MJ, Villanueva C, Larcher F, Conti CJ, Jorcano JL: Epidermal abnormalities and increased malignancy of skin tumors in human epidermal keratin 8-expressing transgenic mice. FASEB J., 2004, 18, s. 1556–1558.

- Commo S, Gaillard O, Bernard BA: The human hair follicle contains two distinct K19 positive compartments in outer root sheath: a unifying hypothesis for stem cell reservoir? Differentiation, 2000, 66, s.157-164.

- Cooper, D N W, Massa, S M, Barondes, S H: Endogenous muscle lectin inhibit myoblast adhesion to laminin. J Cell Biol., 1991, 115, s. 1437-1448.

- Dagher S F, Wang J L, Patterson R J: Identification of galectin-3 as a factor in pre-mRNA splicing. Proc Natl Acad Sci USA, 1995, 92, s. 1213-1217.

- Duelli D, Lazebnik Y. Cell fusion: a hidden enemy ? Cancer Cell. 2003, 3, s. 445-8.

- Dumic J, Dabelic S, Flögel M: Galectin-3: An open ended story. Biochim. Biophys. Acta 2006, 1760, s. 616-635.

- Dvorak H F, Nagy J A, Dvorak A M: Structure of solid tumors and their vasculature: implications for therapy with monoclonal antibodies Cancer Cells 1991. 3, s. 77-85.

- Fronhoffs S, Bruning T, Ortiz-Pallardo E, Brode P, Koch B, Harth V, Sachinidis A, Bolt H M, Herberhold C, Vetter H, Ko Y: Real-time PCR analysis of the N-acetyltransferase NAT1 allele *3, *4, *10, *11, *14 and *17 polymorphism in squamous cell cancer of head and neck. Carcinogenesis., 2001, 22, s. 1405-1412.

- Gabius H-J: Animal lectins. Eur J Biochem., 1997, 243, s. 543-576.

- Ganss R: Tumor stroma fosters neovascularization by recruitment of progenitor cells into the tumor bed. J Cell Mol Med. 2006, 10, s. 857-865.

- Gillenwater A, Xiao-Chun X, El-Naggar A K et al.: Expression of galectins in head and neck squamous cell carcinoma. Head Neck, 1996, 19, s. 422-432.

- Gonacci-Sorrell M, Zhurinsky J, Ben Ze´ev A: The cadherin-catenin adhesion system in signalling and cancer. J. Clin Invest., 2000, 109, s. 987-991.

- Hay E D.: The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Developmental Dynamics, 2005, 233, s. 706-720.

- Honjo Y, Inohara H, Akahani S et al.: Expression of cytoplasmic galectin-3 as a prognostic marker in tongue carcinoma. Clin Cancer Res., 2000, 6, s. 4635–4640.

- Huber M A, Azoitei N, Baumann B, Grünert S, Sommer A, Pehamberger H, Kraut N, Beug H, Wirth T: NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest., 2004, 114, s. 569-581.

- Hughes R C: Galectins as modulators of cell adhesion. Biochemie, 2001, 83, s. 667-676.

- Chiariotti L, Salvatore P, Frunzio R, Bruni B C: Galectin genes: Regulation of expression. Glycoconjugate Journal, 2004, 19, s. 441-449.

- Chovanec M, Smetana K Jr, Betka J, Plzák J, Brabec J, Moya Álvarez V André S, Kodet R and Gabius H-J: Correlation of expression of nuclear protein pKi67 and p63 with lectin histochemical features in head and neck squamous cell cancer. Int J Oncol., 2005, 27, s. 409-415.

- Chovanec M, Smetana K Jr., Plzák J, Betka J, Plzáková Z, Štork J, Hrdličková E, Kuwabara I, Dvořánková B, Liu F-T, Kaltner H, André S, Gabius H-J: Detection of new diagnostic markers in pathology by focus on growth-regulatory endogenous lectins. The case study of galectin-7 in squamous epithelia. Prague Med Rep., 2005, 106, s. 209-216.

- Kaur P, Li A: Adhesive properties of human epidermal cells: an analysis of keratinocyte stem cells. J Invest Dermatol., 2000, 114, s. 413–420.

- Klíma J, Motlík J, Gabius H-J, Smetana K Jr: Phenotypic characterization of porcine interfollicular keratinocytes separated by elutriation: a technical note. Folia Biol., 2007, 53, s. 33-36.

- Klíma J, Smetana K Jr, Motlík J, Plzáková Z, Liu F-T, Stork J, Kaltner H, Chovanec M, Dvoránková B, André S and Gabius H-J: Comparative phenotypic characterization of keratinocytes originating from hair follicles. J Mol Histol., 2005, 36, s. 89-96.

- Kocourek J, Hořejší V: Defining a lectin. Nature, 1981, 290, s.188.

- Kong W, Li S, Liu C, Bari A S, Longaker M, Lorenz H P: Epithelial-mesenchymal transition occurs after epidermal development in mouse skin. Exp Cell Res., 2006, 312, s. 3959-3968.

- Kulbe H, Levinson N R, Balkwill F, Wilson J L. The chemokine network in cancer--much more than directing cell movement. Int J Dev Biol. 2004, 48, s. 489-496.

- Lacina L, Plzáková Z, Smetana K Jr, Stork J, Kaltner H and André S: Glycophenotype of psoriatic skin. Folia Biol., 2006, 52, s. 10-15.

- Lacina L, Smetana K Jr, Dvoránková B, Pytlík R, Kideryová L, Kucerová L, Plzáková Z, Stork J, Gabius H-J and André S:Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. Brit J Dermatol., 2007, 156, s. 819-829.

- Lacina L, Smetana K Jr., Dvořánková B, Štork J, Plzáková Z, Gabius H-J, Immunocyto- and histochemical profiling of nucleostemin expression: Marker of epidermal stem cells?, Journal of Dermatological Science. 2006, 44, s.73-80.

- Lanza R, Gearhart J, Hogan B, Melton D, Pedersen R, Thomson J, West M: Handbook of stem cells, Elsevier, 2004, 19, s. 433-440.

- Lee J M, Dedhar S, Kalluri R, Thompson E W: The epithelial–mesenchymal transition: new insights in signaling, development, and disease. The Journal Cells of Biology., 2006, 30, s. 973-981.

- Levi G, Tarrab-Hazdi R., Teichberg VI.: Prevention and therapy with electrolectin of experimental autoimmune myasthenia gravis in rabbits. Eur J Immunol., 1983, 13, s. 500-507.

- Li A, Normand P, Redvers R, Kaur P: Extensive tissue-regenerative capacity of neonatal human keratinocyte stem cells and their progeny. J. Clin. Invest., 2004, 113, s. 390-400.

- Li H, Fan X, Houghton J: Tumor microenvironment: the role of the tumor stroma in cancer. J Cell Biochem., 2007, 101, s. 805-815.
- Liu S J, Cai Z W, Liu Y J, Dong M Y, Sun L Q, Hu G F, et al.: Role of nucleostemin in growth regulation of gastric cancer, liver cancer and other malignancies. World J Gastroenterol., 2004, 10, s. 1246-1249.
- Magnaldo T, Bernerd F, Darmon M: Galectin-7, a human 14-kDa S-lectin, specifically expressed in keratinocytes and sensitive to retinoic acid. Dev Biol., 1995, 168, s. 259-271.
- Magnaldo T, Fowlis D, Darmon M: Galectin-7, a marker of all types of stratified epithelia. Differentiation, 1998, 63, s. 159-168.
- Mainiero F, Pepe A, Yeon M, Ren Y, Giancotti F.G.: The intracellular functions of α6β4 integrin are regulated by EGF. J. Cell Biol., 1996, 134, s. 241-253.
- Michel M, Török N, Godbout M-J, Lussier M, Gaudreau P, Royal A and Germain L: Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially located in function of anatomic sites, and their number varies with donor age and culture stage. J Cell Sci., 1996, 109, s. 1017-1028.
- Morasso M I, Marjana Tomic –Canic: Epidermal stem cells: the cradle of epidermal determination, differentiation and wound healing. Biol. Cell 2005, 97, s. 173-183.
- Motlík J, Klíma J., Dvořánková B, Smetana K Jr.: Porcine epidermal stem cells as a biomedical model for wound healing and normal/malignant epithelial cell propagation. Theriogenology 2007, 67, s. 105-111.
- Myers E N, Suen J Y, Myers J N, Hanna E Y N, Cancer of the head and neck, Saunders, 2003.
- Nomáda A M, Ziegler R G, Stemmermann G N, Chyou P H, Craft N E: Serum micronutrients and upper aerodigestive tract cancer. Cancer Epidemiol Biomarkers Prev., 1997, 6, s. 407-412.
- Nomura A M, Ziegler RG, Stemmermann GN, Chyou PH, Craft NE. Serum micronutrients and upper aerodigestive tract cancer. Cancer Epidemiol Biomarkers Prev., 1997, 6, s. 407-412.
- Offner H, Celnik B, Bringman T S, et al.: Recombinant human β-galactoside binding lectin suppresses clinical and histological signs of experimental autoimmune encephalomyelitis. J Neuroimmunology, 1990, 28, s. 177-184.

- Ogawa T, Tsurusako Y, Kimura N, et al.: Comparison of tumor markers in patients with squamous cell carcinoma of the head and neck. Acta Otolaryngol (Stockh) Suppl., 1999, 540, s. 72-76.

- Parkin D M, Laara E, Muir C S: Estimates of the worldwide frequency of sixteen major cancers in 1980. Int J Cancer., 1988, 41, s. 184-197.

- Perillo N L, Uittenbogaart C H, Nguyen J T, et al.: Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes. J Exp Med., 1997, 185, s. 1851-1858.

- Petersen O W, Lind Nielsen H, Gudjonsson T, Villadsen R, Rønnov-Jessen L, Bissell M J: The plasticity of human breast carcinoma cells is more than epithelial to mesenchymal conversion. Breast Cancer Res., 2001, 3, s. 213-217.

- Petersen O W, Nielsen H L, Gudjonsson T, Villadsen R, Rank F, Niebuhr E, Bissell M J, Rønnov-Jessen L: Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. Am J Pathol., 2003, 162, s. 391-402.

- Piantelli M, Iacobelli S, Almadori G, et al.: Lack of expression of galectin-3 is associated with a poor outcome in node-negative patients with laryngeal squamous-cell carcinoma. J Clin Oncol., 2002, 20, s. 3850–3856.

- Plzák J, Betka J, Smetana K Jr., Chovanec M, Kaltner H, Andre´S, Kodet R, Gabius H.-J: Galectin-3 – an emerging prognostic indicator in advanced head and neck carcinoma, European Journal of Cancer, 2004, 40, s. 2324–2330.

- Plzák J, Smetana K Jr., Hrdličková E, Kodet R, Holíková Z, Liu F T, Dvořánková B, Kaltner H, Betka J, Gabius H J: Expression of galectin-3-reactive glycoligands in squamous cell cancer and normal epithelial cells as a marker of differentiation. Int J Oncol, 2001, 19, s. 59-64.

- Plzák J, Smetana K Jr., Chovanec M, Betka J: Glycobiology of head and neck squamous epithelia and carcinomas. ORL, 2005, 67, s. 61-69.

- Polyak K, Xia Y, Zweier J L, Kinzler K W, Vogelstein B: A model for p53-induced apoptosis. Nature, 1997, 389, s. 300-305.

- Purkrábková T, Smetana K, Jr., Dvořánková B, Holíková Z, Böck,C, Lensch M, André S, Pytlík R, Liu F-T, Klíma J, Smetana K, Motlík J, Gabius H-J: New aspects of galectin functionality in nuclei of cultured bone marrow stromal and epidermal cells: biotinylated galectins as tool to detect specific binding sites. Biol Cell, 2003, 95, s. 535-545.

- Raghavan S, Bauer C, Mundschau G, Li Q, Fuchs E: Conditional ablation of β1 integrin in the skin: severe defects in epidermal proliferation, basement membrane formation, and hair follicle invagination. J. Cell Biol., 2000, 150, s. 1149-1169.

- Raul U, Sawant S, Dange P, Kalraiya R, Ingle A, Vaidya M: Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: induction of transformed phenotype. Int J Cancer, 2004, 111, s. 662–668.

- Ridanpaa M, Fodde R, Kielman M: Dynamic expression and nuclear accumulation of b-catenin during the development of hair follicle-derived structures. Mech. Dev., 2001, s. 173–181.

- Sanford G L, Harris-Hooker S: Stimulation of vascular cell proliferation by β–galactoside-binding lectins. FASEB J., 1990, 4, s. 2912-2918.

- Saussez S, Cucu D R, Decaestecker C, Chevalier D, Kaltner H, Andre S, Wacreniez A, Toubeau G, Camby I, Gabius H-J, Kiss R: Galectin 7 (p53-induced gene 1): a new prognostic predictor of recurrence and survival in stage IV hypopharyngeal cancer. Ann Surg Oncol., 2006, 13, s. 999-1009.

- Sawangareetrakul P, Srisomsap C, Chokchaichamnankit D, Svasti J.: Galectin-3 expression in human papillary thyroid carcinoma. Cancer Genomics Proteomics. 2008, 5, s.117-122.

- Shi Y, Massagué J: Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell, 2003, 113, s. 685-700.

- Shook D, Keller R: Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev. 2003, 120, s.1351-1383.

- Sijin L, Ziwei C, Yajun L, Meiyu D, Hongwei Z, Guofa H, et al. The effect of knocking-down nucleostemin gene expression on the in vitro proliferation and in vivo tumorigenesis of HeLa cells. J Exp Clin Cancer Res., 2004, 23, s. 529-538.

- Smetana K, Jr., André S: Mammalian lectin as tool in glycochemistry and histochemistry with relevance for diagniostic procedure. In McMahn, R.J. (Ed.) Avidin-Biotin Interactions, Methods and Applications. Humana Press, Totowa, NJ, USA, 2008, s. 171-185.

- Smetana K Jr., Dvořánková B, Chovanec M, Bouček J, Klíma J, Motlík J, Lensch M, Kaltner H, André S, Gabius J-H: Nuclear presence of adhesion-/growth-regulatory galectins in normal /malignant cells of squamous epithelial origin. Histochem Cell Biol., 2006, 125, s.171-182.

- Smetana K Jr., Holíková Z, Klubal R, et al.: Coexpression of binding sites for A(B) histo-blood group trisaccharides with galectin-3 and Lag antigen in human Langerhans cells. J Leukocyte Biol., 1999, 66, s. 644-649.

- Thiery J P, Sleeman J P: Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol., 2006, 7, s. 131-142.

- Thiery J P. Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol. 2003, 15, s. 740-746.

- Timmons M P, Colnot C, Cail I, Poirier F, Magnaldo T: Expression of galectin-7 during epithelial development coincides with the onset of stratification. Int. J. Dev. Biol., 1999, 43, s. 229-235.

- Troy T C, Turksen K: Commitment of embryonic stem cells to an epidermal cell fate and differentiation in vitro. Developmental Dynamics, 2005, 232, s. 293-300.

- Tsai R Y L, McKay R D G: A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Develop, 2002, 16, s. 2991-3003.

- Tse J C, Kalluri R: Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. J Cell Biochem. 2007, 101, s. 816-829.

- Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E: Defining the epithelial stem cell niche in the skin. Science. 2004, 303, s. 359-363.

- Vyakarman A, Daggher S F, Wang J L, et al.: Evidence for a role for galectin-1 in pre-mRNA splicing. Mol Cell Biol., 1997, 17, s. 4730-4737.

- Wang J L, Gray R M, Haudek K C, Patterson R J. Nucleocytoplasmic lectins. Biochim Biophys Acta. 2004, 1673, s.75-93.

- Yaghoobi M M, Mowla S, Jand Tiraihi T: Nucleostemin, a coordinator of self-renewal, is expressed in rat marrow stroma cells and turns off after induction of neural differentiation. Neurosci Lett., 2005, 390, s.81-86.

- Yokoyama K, Kamata N, Fujimoto R, Tsutsumi S, Tomonari M, Taki M, Hosokawa H, Nagayama M. Increased invasion and matrix metalloproteinase-2 expression by Snail-induced mesenchymal transition in squamous cell carcinomas. Int J Oncol., 2003, 22, s. 891-898.

8. Vlastní publikace vztahující se k tématu disertační práce

Publikace jsou řazeny v chronologickém sledu.

1. **Čada Z**, Bouček J, Dvořánková B, Chovanec M, Plzák J, Kodet R, Betka J, Pinot G L, Gabius H-J, Smetana K Jr. Nucleostemin expression in squamous cell carcinoma of the head and neck, Anticancer Research, 2007, 27, s. 3279-3284. **(IF 1.604)**

2. Lacina L, Dvořánkova B, Smetana K Jr, Chovanec M, Plzák J, Tachezy R, Kideryová L, Kučerová L, **Čada Z**, Bouček J, Kodet R, André S, Gabius HJ: Marker profiling of normal keratinocytes identifies the stroma from squamous cell carcinoma of the oral cavity as a modulatory microenvironment in co-culture. Int J Radiation Biol., 2007, 83, s. 837-848. **(IF 1.468)**

3. Smetana K Jr, Dvořánková B, Lacina L, **Čada Z**, Vonka V. Human hair follicle and interfollicular keratinocyte reactivity to mouse HPV16-transformed cells: An in vitro study. Oncol Rep., 2008, 20, s. 75-80. **(IF 1.597)**

4. **Čada Z**, Plzák J, Chovanec M, Dvořánková B, Lacina L, Szabó P, Smetana K., Jr., Betka J: Galektiny v dlaždicových karcinomech hlavy a krku. Časopis lékařů českých, 2008, č. 11, s. 559-563.

5. **Čada Z**, Chovanec M, Smetana K Jr., Betka J, Lacina L, Plzák J, Kodet R, Štork J, Lensch M, Kaltner H, André S, Gabius HJ. Galectin-7: Will the lectin´s activity establish clinical correlations in head and neck squamous and basal cell carcinomas? Histol Histopathol., 2009, 24, s. 41-48. **(IF 2.007)**

6. Kideryová L, Lacina L, Dvořánková B, Štork J, **Čada Z**, Szabo P, Andre S, Kaltner H, Gabius HJ, Smetana K Jr: Phenotypic characterization of human keratinocytes in coculture reveals differential effects of fibroblasts from benign fibrous histiocytoma (dermatofibroma) as compared to cells from its malignant form its malignant form and to normal fibroblasts. J Dermatol Sci., 2009, in press **(IF 2.515)**

7. **Čada Z**, Smetana K Jr., Lacina L, Plzáková Z, Štork J, Kaltner H, Russwurm R, Lensch M, André S, Gabius HJ. Immunohistochemical fingerprinting of the network of seven adhe-

sion/growth-regulatory lectins in human skin and detection of distinct tumor-associated alterations. Folia Biologica, 2009, in press.

9. Příloha

ANTICANCER RESEARCH *27*: xxx-xxx (2007)

No: **8071-C**
Please mark the appropriate section for this paper
☐ Experimental
☐ Clinical
☐ Epidemiological

Nucleostemin Expression in Squamous Cell Carcinoma of the Head and Neck

ZDENEK CADA[1,2], JAN BOUCEK[2,3], BARBORA DVORANOVA[1,4], MARTIN CHOVANEC[1,2,4], JAN PLZAK[1,2,4], ROMAN KODET[5], JAN BETKA[2], GIAN L. PINOT[1], HANS-JOACHIM GABIUS[6] and KAREL SMETANA Jr.[1,4]

[1]Institute of Anatomy and [2]Department of Otorhinolaryngology, Head and Neck Surgery, First Faculty of Medicine and [4]Center of Cell Therapy and Tissue Repair and [5]Institute of Pathology and Molecular Medicine, Second Faculty of Medicine, [3]Institute of Microbiology, Academy of Science of the Czech Republic, Prague, Czech Republic; [6]Institute of Physiological Chemistry Ludwig-Maximilians-University, Faculty of Veterinary Medicine, Munich, Germany

Abstract. *Background: This study presents initial data on presence of nucleostemin – a nucleolar protein typical for stem cells in the normal squamous epithelium of the oropharynx and larynx – in squamous cell carcinoma originating from these epithelia. Materials and Methods: Differentiation and proliferation markers such as keratins, β-catenin, galectin-1, and Ki67 were studied in parallel with nucleostemin for defining cell characteristics. Results: Nucleostemin was detected in nucleoli of both proliferating basal cells and terminally differentiated suprabasal cells of normal epithelium and in tumor cells. Importantly, malignant transformation was connected with a significant enlargement of nucleostemin-positive nucleoli in these cell types. Conclusion: Therefore, the pattern of nucleostemin presence deserves as new marker for evaluation of tumor differentiation and biology.*

Head and neck cancers represent about 6% of malignant tumor cases worldwide; at least 90% of these tumors are squamous cell carcinomas. Despite rapid progress in diagnosis and therapy the overall 5-year survival rate for this malignancy is among the lowest of the major cancer types (1). This unfavorable situation calls for research activities to aim at finding new markers to better characterize the biological behavior of tumors in order to serve as a rational guideline to improve therapeutic modalities (2-5). Respective candidates may originate from applying the stem cell concept to this tumor class.

Adult tissue stem cells have several similarities with cancer cells, and the idea of stem cells as a source of solid cancer was put forward recently (6, 7). As a consequence, potential roles of epidermal stem cells in cancer, especially in squamous cell carcinoma, have been proposed (8). Fitting this concept, characteristics of the epidermal stem cell phenotype could be detected in *in vitro* propagated cells from cancer lines of squamous cell epithelial origin (9, 10). Moreover, cells of a very low differentiation level, akin to epidermal stem cells, have been observed on the periphery of tumor lesions in the so-called "aggressive front" of carcinomas. Tumors abundantly populated by these cells exhibit a highly anaplastic aggressive phenotype (11). At present, no single specific marker of adult tissue stem cells (including stem cells of squamous epithelia) has yet been discovered. These cells are currently identified by the detection of a combination of markers. In this situation, the systematic study of individual proteins will help characterize the phenotype of these cells thoroughly. This rationale prompts the study of nucleostemin, a nuclear/nucleolar protein present in neural and bone marrow stem cells and their related malignancies (12, 13). Nucleostemin, of note, participates in the control of proliferation in these cells and also in early embryonic development (14) and tissue regeneration (15), explaining why monitoring of its presence in cancer is warranted. In the human epidermis, this protein is not exclusively expressed by cells of the stem cell pool, and even nucleoli of terminally differentiated suprabasal cells reveal the presence of nucleostemin (16). However, nucleostemin expression is up-regulated in follicular bulge epidermal stem cells when measured by microarray technology at the mRNA level (17); *in vitro* only those cells cocultured with non-tumor feeder cells contain nucleoli positive for nucleostemin expression (16).

This study demonstrates the expression of nucleostemin in nucleoli of cells of normal squamous cell epithelium (namely of the larynx and oropharynx) and in squamous cell carcinomas originating from these epithelia. The scope of these results was extended to nucleostemin presence in FaDu cells, a model line of human squamous cell carcinoma

Correspondence to: Karel Smetana Jr., Charles University, First Faculty of Medicine, Institute of Anatomy, U nemocnice 3, 128 00 Prague 2, Czech Republic. Tel: +420 2 24965873, Fax: +420 2 24965770, e-mail: karel.smetana@lf1.cuni.cz

Key Words: Basal cell carcinoma, β-catenin, epidermis, keratin, lectin, nucleolus, nucleostemin.

from the hypopharynx, *in vitro* and *in vivo* after tumor development in nu/nu mice. To relate nucleostemin presence to other cellular characteristics, the presence of the following well-established markers was determined: Ki-67, β-catenin, and keratin 10. Proliferating cells were detected by the nuclear expression of Ki67 (18). β-Catenin is usually a membrane-associated protein in the majority of cells of the squamous cell epithelium; its shift to the cytoplasm and nucleus is related to with tumor progression (19). Keratin 10 expression is associated with terminal differentiation in cells of squamous epithelia under physiological conditions and in cancer (11, 20). In addition, the presence of a key member of the adhesion/growth-regulatory galectins, galectin-1 was determined. These endogenous lectins can interact with distinct glycan epitopes and proteins at different sites of the cell to trigger efficient signaling leading to diverse cell responses (21-23). In this context it is noteworthy that nuclear presence of galectin-1 has been observed in cells of the bulge region of the hair follicle which are phenotypically similar to epidermal stem cells (24).

Materials and Methods

Clinical material. Five specimens of laryngeal squamous cell carcinoma, three specimens of squamous cell carcinoma of the tongue and four specimens of oropharyngeal squamous cell carcinoma at stage T3 and without previous therapy as well as five control samples of normal laryngeal mucosa and three control samples of oropharyngeal mucosa (control samples were obtained from tumor-free organs as verified by histology) were taken. All samples were donated with the informed consent of the patients. The tissue donors had not undergone previous cytostatic (chemo)therapy. The samples were frozen in liquid nitrogen using Tissue-Tek (Christine Gröpl, Tulln, Austria) as a cryoprotective medium and stored at -85°C until further processing.

Tissue culture and animal experiments. The human hypopharyngeal squamous cell carcinoma line FaDu (HTB-43) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, antibiotics (100 units/mL of penicillin, 100 μg/mL of streptomycin; Sigma, St. Louis, MO, USA), 1.5 g/L $NaHCO_3$, 0.11 g/L sodium pyruvate, 0.292 g/L *L*-glutamine, and 10 mM *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES). FaDu cells were also cultured on coverslips as described previously (10, 16). The cells were cultured under standard conditions *i.e.* under 5% CO_2 tension at 37°C. Three independent experimental series were immunohistochemically evaluated. For xenotransplantation, two female nu/nu CD-1 mice, aged 8-12 weeks, were purchased from the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic. The mice were housed in accordance with approved guidelines and provided food and water *ad libitum*. A total of $1x10^6$ FaDu cells from tissue culture were resuspended in 100 μl of phosphate buffered saline (PBS) and mixed with 50 μl of BD Matrigel™ (BD Biosciences, Erembodegen, Belgium) according to supplier instruction. The resulted suspension was then subcutaneously injected into each nu/nu CD-1 female mice.

The animals were sacrificed after 49 days and the specimens were frozen as described above.

Immunohistochemistry. Frozen sections, 7 μm each, were prepared using Cryocut E (Reichert-Jung, Vienna, Austria): The tumor sections and the FaDu cells grown on coverslips were washed with PBS, briefly fixed with 4% paraformaldehyde in PBS (pH 7.3) at room temperature, and then washed once with PBS. Diluted porcine serum (1%) (DAKO, Brno, Czech Republic) was used as a blocking solution to prevent the nonspecific binding of first and second step antibodies. Nucleostemin was detected by goat polyclonal antibody (Neuromics, Bloomington, MN, USA). Ki-67, a pankeratin, and keratin 10 were visualized by commercial mouse monoclonal antibodies (DAKO, Brno, Czech Republic) and β-catenin by a rabbit polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA). Rabbit polyclonal antibody against galectin-1 (non-crossreactive with other galectins) was employed to visualize this antigen (25, 26). TRITC-labeled donkey anti-goat (Jackson Laboratories, West Grove, PA, USA) along with FITC-labeled swine anti-mouse (SwAM-FITC, AlSeVa, Prague, Czech Republic) and FITC-labeled swine anti-rabbit (SwAR-FITC, AlSeVa, Prague, Czech Republic) were used as second-step reagents. All commercial antibodies were diluted according to supplier recommendations. Five sections from the each tumor samples were employed for the each antibody combinations. Sections and cultured cells were stained at room temperature for 60 minutes. Specificity controls were performed by omitting the first-step antibody or by replacing it with monoclonal/polyclonal antibodies against thyroglobulin (not expressed in the studied tissues; DAKO, Brno, Czech Republic) to exclude any interaction of an antibody with sections of the studied tissues *via* Fc receptor. The nuclei were then counterstained with DAPI (4',6'-diamidino-2-phenylindole dilactate) (Sigma-Aldrich, Prague, Czech Republic). The specimens were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent the UV bleaching of fluorochromes. A Nikon Eclipse-90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with filter blocks specific for DAPI, FITC and TRITC, a cooled CCD Vosskühler Cool-1300Q camera (Vosskühler, Osnabrick, Germany) and a computer-assisted image analyzer LUCIA 5.1 (Laboratory Imaging, Prague, Czech Republic) were used for imaging. The image analyzer was also used for measuring the size of nucleolar area positive for nucleostemin. A total of 300-500 cells were analyzed in each specimen. The results were statistically processed using Student's unpaired *t*-test.

Results

Normal oropharyngeal and laryngeal epithelium exhibited nucleostemin-positive nucleoli in both basal and suprabasal cells (Figure 1A). This observation is in accordance with a previous study of normal epidermis, hereby serving as internal quality control (16). The size of nucleostemin-positive nucleoli was identical in both compartments (Figure 2A). In order to support this notion the presence of Ki-67was measured and found to be restricted to cells of the basal layer in samples of normal epithelium (not shown).

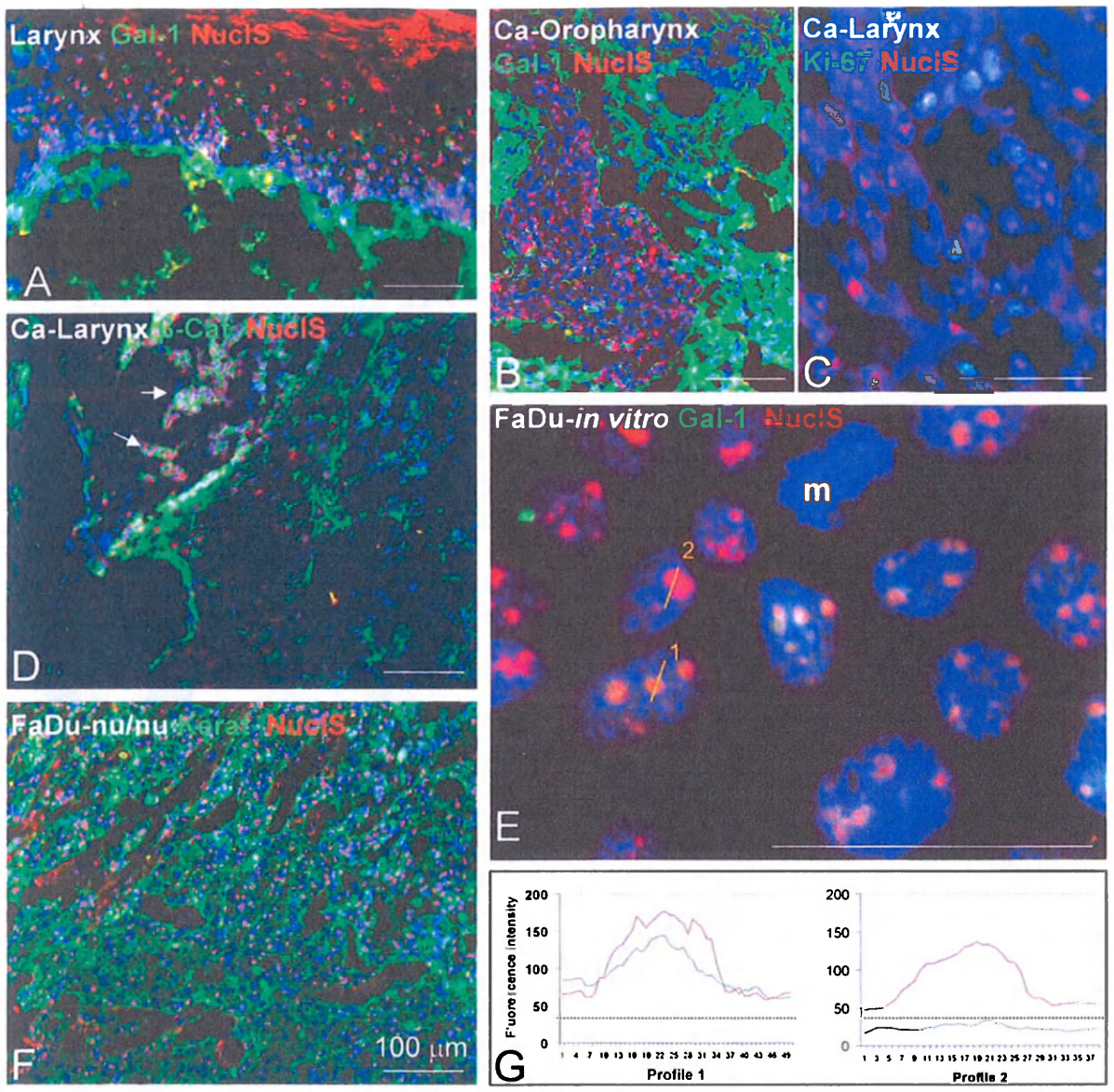


Figure 1. *Detection of nucleostemin (NuclS, red) (A-E), galectin-1 (Gal-1, green) (A, B, E), Ki-67 (green) (C), β-catenin (β-cat, green) (D) and of a pankeratin (Kerat, green) (F) in normal laryngeal epithelium (A), squamous cell carcinoma of the oropharynx (B), squamous cell carcinoma of the larynx (C, D),cultured FaDu cells (E) and in FaDu cells grown in nu/nu mice (F). All nuclei are counterstained with DAPI. Arrows indicate cells with cytoplasmic/nuclear expression of β-catenin. Mitotic cells are marked by "m". Fluorescence intensity profiles were measured for FaDu cell nucleoli marked 1 and 2 (G).*

The nuclei of cells from squamous cell carcinomas contained nucleoli which gave a strong nucleostemin signal (Figure 1B-D). Similar findings were also obtained in cultured FaDu cells (Figure 1E) and in tumors from FaDu cells grafted into mice (Figure 1F). In addition to the signal intensity the size of nucleostemin-positive nucleoli was significantly larger in cells of squamous cell carcinomas than in cells of normal epithelia (Figure 2A). This property was also detectable in FaDu cells grown both *in vivo* and *in vitro*, although it was not statistically verified (Figure 2A). Analyzing the distribution of nucleoli according to their size, the nucleolar area in normal epithelium was rather uniform with a high incidence of nucleoli in the range of 6 to 10 μm^2 (Figure 2B). In contrast, the size distribution of nucleoli in both laryngeal and oropharyngeal squamous cell carcinomas was broad, with occurrence of very large nucleoli up to 35 μm^2 (Figure

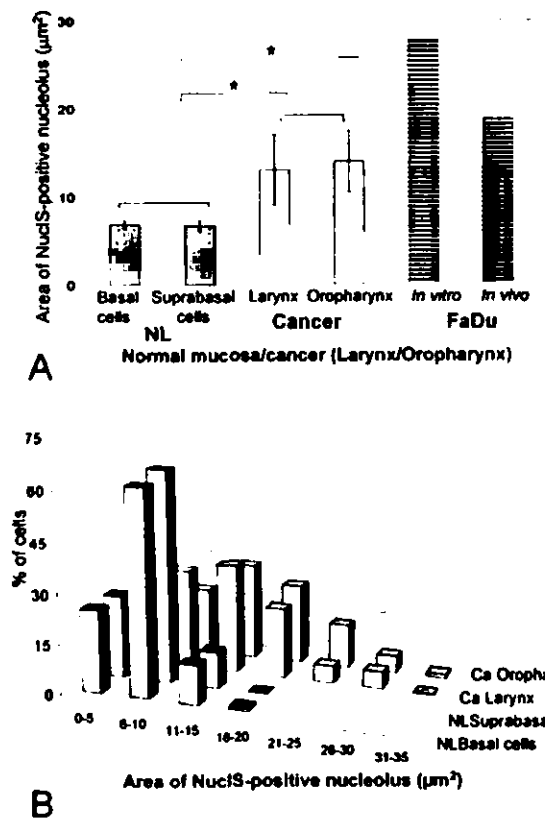


Figure 2. *A) Size of nucleostemin-positive nucleoli in basal layer cells (NL -Basal cells) of normal laryngeal epithelium, in suprabasal layer cells (NL -Suprabasal cells) of normal laryngeal epithelium, in laryngeal (Ca -Larynx) and in oropharyngeal (Ca -Oropharynx) cancer cells, in cultured FaDu cells (FaDu-in vitro), and in cells of tumors formed by grafting FaDu cells in vivo. Statistically significant differences are marked by asterisks; p=0.05. B) Size-dependent distribution of nucleostemin-positive nucleoli in basal and suprabasal layer cells, as well as in carcinoma cells of the oropharynx and larynx.*

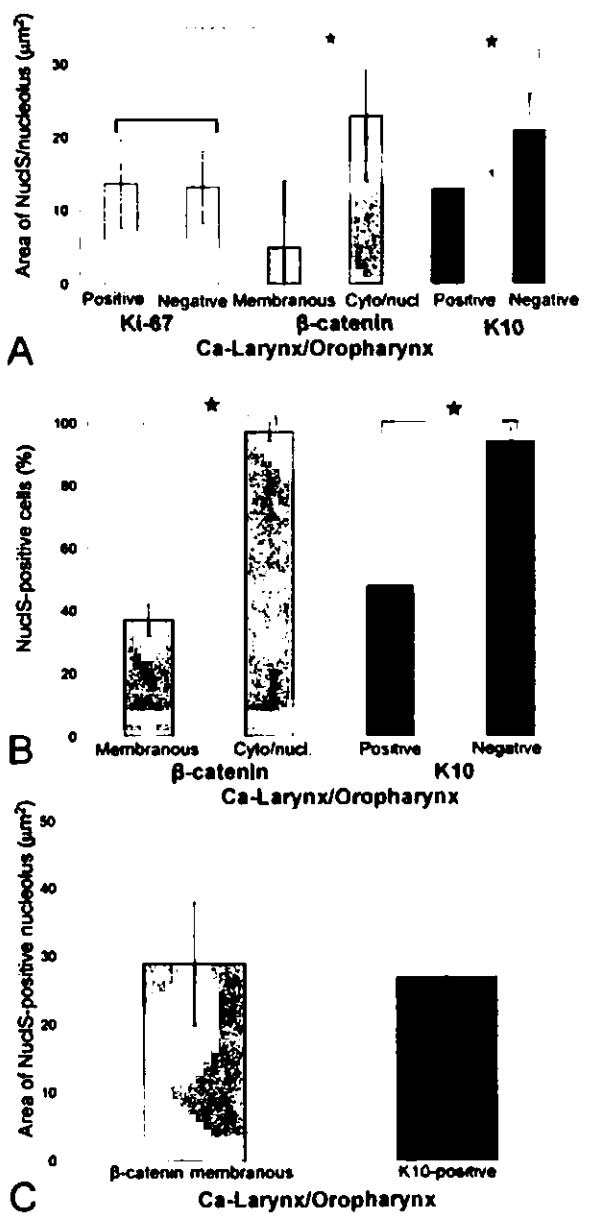


Figure 3. *A) Size of nucleostemin positivity per nucleolus in cancer cells, in relation to the expression of the proliferation marker Ki-67, the expression pattern of β-catenin (membranousscytoplasmic/nuclear) and the expression of keratin 10. B) Incidence of cancer cells according to their phenotype. C) Comparison of the size of the area expressing nucleostemin in cells positive for this marker and presenting a membrane-associated signal for β-catenin, and for keratin 10. The difference is statistically nonsignificant; p=0.05.*

2B). Having first focused on features of nucleostemin presence, we next set the immunohistochemical data in relation to proliferation and other cellular markers.The studied tumors contained groups of cells with membrane-associated signals for β-catenin with the cytoplasmic and nuclear presence of this protein (Figure 1D) that is associated with tumor progression. The mean size of the nucleostemin-positive area per nucleolus was smaller in cells with membrane-associated positivity for β-catenin than in cells with positivity in the cytoplasm/nucleus (Figure 3A). However, this result should be be considered cautiously due to the rather low degree of nucleostemin positivity in cells with membrane-associated β-catenin (Figure 1D), where approximately one half of the cells contained nucleostemin-positive nucleoli (Figure 3B). When the signal for nucleostemin was evaluated based on positive cells only, these were found to exhibit large nucleostemin-positive nucleoli (Figure 3C). Interestingly, the same phenomenon was observed for keratin 10-positive cells (Figure 3A-C).

Nuclear/nucleolar expression of galectin-1, known to be expressed in cells sharing features with epidermal stem cells was detected in cultured FaDu cells (Figure 1E). No signal for the expression of this endogenous lectin was found in the nuclei of cells from normal epithelia or carcinomas (Figure 1A, B), or in tumors from FaDu cells grown in nu/nu mice (not shown). Of note when examining the tumor sections was the abundant presence of galectin-1 in the tumor stroma (Figure 1B); its level was significantly higher than in the connective tissue of the normal mucosa (Figure 1A).

Discussion

Evidently, expression of nucleostemin is not dependent on the proliferation status of cells in squamous epithelia of either ectodermal (epidermis) or endodermal (larynx) origin, knowing that only basal cells are able to proliferate (4, 27). Similarly, the proliferation status of tumor cells has no influence on the expression of nucleostemin in their nucleoli. However, the nucleostemin-positive nucleoli are larger than these in the normal epithelia. Surprisingly, nucleostemin-positive nucleoli of a very large area were found in cancer cells exhibiting membrane attached β-catenin and keratin 10, markers indicating differentiated phenotype in the normal cells (11, 19). This finding is similar to our observation in a previous study comparing the expression of keratins, ligands for galectin and Ki-67 where difference between expression of markers of the terminal differentiation and Ki-67 can be explained by the disparity between cell maturation and differentiation in cells of squamous cell carcinomas of the head and neck (11). Galectin-1 expression in the cell nucleus and/or nucleolus was observed in cells sharing features of epidermal stem cells (24) and it was also observed in FaDu cells (10). While nucleostemin was expressed in all cultured FaDu cells nucleoli, galectin-1 was detected in one half of studied cells where the good agreement of both proteins localization was present.

Extensive expression of galectin-1 in the tumor stroma represents one of dominant features of all the studied carcinomas. Increased presence of galectin-1 in the stroma has been observed, for example, in basal cell carcinomas (28) and the dermis of psoriatic skin (29).

The presented results document the presence of nucleostemin in squamous cell carcinoma of the head and neck. A high level of expression of this nuclear protein has also been observed in brain tumors (12), basal cell carcinomas (16), stomach and liver cancers (30) and cancer of the kidney (31). By immunohistochemical means it is not possible to determine whether this high level is an inherent property of tumor cells or is induced by a crosstalk between the cancer epithelium and tumor stromal cells (28). Looking at functional aspects, nucleostemin is likely not involved in the production of rRNA (32), but it may exert other regulatory functions during malignant transformation (33). One proposed function of nucleostemin is the control of proliferation and the inhibition of senescence, a potential means by which tumor cells avoid restrictions to their growth potential also related to galectins (34-36).

Conclusion

The presence of nucleostemin was documented in head and neck cancer here, and its detection, together with the size properties of positive nucleoli, may relate to tumor cell features (37).

Acknowledgements

This study was supported by grant NR 9049-3 from the Grant Agency of the Ministry of Health of the Czech Republic, grants MSM0021620806 and 1M0021620803 from the Ministry of Education, Youth, and Sport of the Czech Republic, and grant MRTN-CT-2005-019561 from an EC Marie Curie Research Training Network. The authors are grateful to Eva Vancova, Iva Burdova, and Vit Hajduch for their excellent technical assistance.

References

1 Hoffman HT, Karnell LH, Funk GF, Robinson RA and Menck HR: The national cancer data base report on cancer of the head and neck. Arch Otolaryngol Head Neck Surg *124*: 951-962, 1998.

2 Chiesa F, Mauri S, Tradati N, Calabrese L, Giugliano G, Ansarin M, Andrle J, Zurrida S, Orecchia R and Scully C:. Surfing prognostic factors in head and neck cancer at the millenium. Oral Oncol *35*: 590-596, 1999.

3 Hardisson D: Molecular pathogenesis of head and neck squamous cell carcinoma. Eur Arch Otorhinolaryngol *260*: 502-508, 2003.

4 Plzak J, Smetana K Jr, Chovanec M and Betka J: Glycobiology of head and neck squamous epithelia and carcinomas. ORL *67*: 61-69, 2005.

5 Chung CH, Parker JS, Ely K, Carter J, Yi Y, Murphy BA, Murphy BA, Ang KK, El-Naggar AK, Zanation AM, Cmelak AJ, Levy S, Slebos RJ and Yarbrough WG: Gene expression profiles identify epithelial-to-mesenchymal transition and activation of nuclear factor-Î B signaling as characteristics of a high-risk head and neck squamous cell carcinoma. Cancer Res *66*: 8210-8218, 2006.

6 Reya T, Morrison SJ, Clake MF and Weissman IL: Stem cells, cancer and cancer stem cells. Nature *414*: 105-111, 2001.

7 Sell S: Stem cell origin of cancer and differentiation therapy. Crit Rev Oncol Hematol *51*: 1-28, 2004.

8 Motlik J, Klima J, Dvorankova B and Smetana K Jr: Porcine epidermal stem cells as biomedical model for wound healing and normal/malignant epithelial cell propagation. Theriogenology *67*: 105-111, 2007.

9 Mackenzie IC: Retention of stem cell patterns in malignant cell lines. Cell Prolif *38*: 347-355, 2005.

10 Smetana K Jr, Dvorankova B, Chovanec M, Boucek J, Klima J, Mothk J, Lensch M, Kaltner H, André S and Gabius H-J: Nuclear presence of adhesion/growth-regulatory galectins in

normal/malignant cells of squamous epithelial origin. Histochem Cell Biol *125*: 172-181, 2006.

11 Chovanec M, Smetana K Jr, Betka J, Plzak J, Brabec J, Moya-Alvarez V André S, Kodet R and Gabius H-J: Correlation of expression of nuclear protein pKi67 and p63 with lectin histochemical features in head and neck squamous cell cancer. Int J Oncol *27*: 409-415, 2005.

12 Tsai RYLand McKay RDG: A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Develop 16: 2991-3003, 2002.

13 Yaghoobi MM, Mowla SJand Tiraihi T: Nucleostemin, a coordinator of self-renewal, is expressed in rat marrow stroma cells and turns off after induction of neural differentiation. Neurosci Lett *390*: 81-86, 2005.

14 Beekman C, Nichane M, De Clerq S, Maetens M, Floss T, Wurst W Bellefroid E and Marine JC: Evolutionarily conserved role of nucleostemin: controlling proliferation of stem/progenitor cells during early vertebrate development. Mol Cell Biol *26*: 9291-9301, 2006.

15 Maki N, Takechi K, Sano S, Tarui H, Sasai Y and Agata K. Rapid accumulation of nucleostemin in nucleolus during newt regeneration. Dev Dyn in press, 2007.

16 Lacina L, Smetana K Jr, Dvorankova B, Stork J, Plzakova Z and Gabius H-J: Immunocyto- and histochemical profiling of nucleostemin expression: marker of epidermal stem cells? J Dermatol Sci *44*: 73-80, 2006.

17 Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M and Fuchs E: Defining the epithelial stem cell niche in the skin. Science *303*: 359-363, 2004.

18 Brown DC and Gatter KC: Ki67 protein: the immaculate deception. Histopathology *40*: 2-11, 2002.

19 Gonacci-Sorrell M, Zhurinsky J and Ben Ze'ev A: The cadherin-catenin adhesion system in signalling and cancer. J Clin Invest *109*: 987-991, 2002.

20 Plzak J, Holikova Z, Smetana K Jr, Dvorankova B, Hercogova Z, Kaltner H and Motlik J, Gabius H-J: Differentiation-dependent glycosylation of cells in squamous cell epithelia detected by a mammalian lectin. Cells Tissues Organs *171*: 135-144, 2002.

21 Gabius H-J: Animal lectins. Eur J Biochem *243*: 543-576, 1997.

22 Gabius H-J: Cell surface glycans: the why and how of their functionality as biochemical signals in lectin-mediated information transfer. Crit Rev Immunol *26*: 43-79,2006.

23 Villalobo A, Nogales-Gonzalez A and Gabius H-J: A guide to signaling pathways connecting protein-glycan interaction with the emerging versatile effector functionality of mammalian lectins. Trends Glycosci Glycotechnol *18*: 1-37, 2006.

24 Klima J, Smetana K Jr, Motlik J, Plzakova Z, Liu F-T, Stork J, Kaltner H, Chovanec M, Dvorankova B, André S and Gabius H-J: Comparative phenotypic characterization of keratinocytes originating from hair follicles. J Mol Histol *36*: 89-96, 2005.

25 Kayser K, Hoeft D, Hufnagl P, Caselitz J, Zick Y, André S, Kaltner H and Gabius H-J: Combined analysis of tumor growth pattern and expression of endogenous lectins as a prognostic tool in primary testicular cancer and its lung metastases. Histol Histopathol *19*: 771-779, 2003.

26 André S, Kaltner H, Furuike T, Nishimura S-I and Gabius H-J: Persubstituted cyclodextrin-based glycoclusters as inhibitors of protein-carbohydrate recognition using purified plant and mammalian lectins and wild-type and lectin-gene-transfected tumor cells as targets. Bioconjugate Chem *15*: 87-98, 2004.

27 Seery JP: Stem cells of the oesophageal epithelium. J Cell Sci *115*: 1783-1789, 2002.

28 Lacina L, Smetana K Jr, Dvorankova B, Pytlik R, Kideryova L, Kucerova L, Plzakova Z, Stork J, Gabius H-J and André S: Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. Brit J Dermatol *156*: 819-829, 2007.

29 Lacina L, Plzakova Z, Smetana K Jr, Stork J, Kaltner H and André S: Glycophenotype of psoriatic skin. Folia Biol *52*: 10-15, 2006.

30 Liu S-J, Cai Z-W, Liu Y-J, Dong M-Y, Sun L-Q, Hu G-F, Wei Y-Y and Lao W-D: Role of nucleostemin in growth regulation of gastric cancer, liver cancer and other malignancies. World J Gastroenterol *10*: 1246-1249, 2004.

31 Fan Y, Liu Z, Zhao S, Lou F, Nilsson S, Ekman P, Xu D and Fang X: Nucleostemin mRNA is expressed in both normal and malignant renal tissues. Br J Cancer *94*: 1658-1662, 2006.

32 Ritland Politz JC, Polena E, Trask I, Bazett-Jones DP and Pederson T: A nonribosomal landscape in the nucleolus revealed by the stem cell protein nucleostemin. Mol Biol Cell *16*: 3401-3410, 2005.

33 Maggi LB and Weber JD: Nucleolar adaptation in human cancer. Cancer Invest *23*: 599-608, 2005.

34 Chovanec M, Smetana K Jr, Dvorankova B, Plzakova Z, André S and Gabius H-J: Decrease of nuclear reactivity to growth-regulatory galectin-1 in senescent human keratinocytes and detection of non-uniform staining profile alterations upon prolonged culture for galectins-1 and -3. Anat Histol Embryol *33*: 348-354, 2004.

35 Rotblat B, Niv H, André S, Kaltner H, Gabius H.-J and Kloog Y: Galectin-1 (L11A) predicted from a computed galectin-1 farnesyl-binding pocket selectivity inhibits Ras-GTP. Cancer Res *64*: 3112-3118, 2004.

36 Zhu Q, Yasumoto H and Tsai RY: Nucleostemin delays cellular senescence and negatively regulates TRF1 protein stability. Mol Cell Biol *26*: 9279-9290, 2006.

37 Smetana K: Structural features of nucleoli in blood, leukemic, lymphoma and myeloma cells. Eur J Histochem *46*: 125-132, 2002.

Received May 14, 2007
Revised July 19, 2007
Accepted August 1, 2007

Int. J. Radiat. Biol., Vol. 83, Nos. 11 - 12, November/December 2007, pp. 837 - 848

informa
healthcare

Marker profiling of normal keratinocytes identifies the stroma from squamous cell carcinoma of the oral cavity as a modulatory microenvironment in co-culture

LUKAS LACINA[1,2], BARBORA DVOŘÁNKOVA[1,3], KAREL SMETANA Jr[1,3], MARTIN CHOVANEC[1,3,4], JAN PLZÁK[1,3,4], RUTH TACHEZY[5], LINDA KIDERYOVÁ[6], L. KUČEROVÁ[7], ZDENEK ČADA[1,4], JAN BOUČEK[1,8], R. KODET[9], S. ANDRÉ[10], & HANS-JOACHIM GABIUS[10]

[1]*First Faculty of Medicine, Institute of Anatomy,* [2]*First Faculty of Medicine, Department of Dermatovenerology,* [3]*Second Faculty of Medicine, Center of Cell Therapy and Tissue Repair,* [4]*First Faculty of Medicine, Department of Otorhinolaryngology, Head and Neck Surgery, all from Charles University in Prague,* [5]*Institute of Hematology and Blood Transfusion, Experimental Virology, Prague,* [6]*Charles University in Prague, First Faculty of Medicine, 1st Department of Internal Medicine,* [7]*Department of Molecular Genetics, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic,* [8]*Institute of Microbiology, Academy of Science of the Czech Republic, Prague,* [9]*Charles University in Prague, Second Faculty of Medicine, Institute of Pathology and Molecular Medicine, and* [10]*Ludwig-Maximilians-University Munich, Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Munich, Germany*

(Received 22 June 2007; revised 13 September 2007; accepted 20 September 2007)

Abstract

Purpose: The microenvironment established by stromal cells may or may not influence phenotypic aspects of epithelial cells and may be relevant for tumor and stem cell biology. We address this issue for keratinocytes using tumor-derived stromal cells in a co-culture system.

Materials and methods: We isolated stromal cells from human squamous cell carcinoma tissue and studied their effect on phenotypic characteristics of normal human interfollicular keratinocytes *in vitro*.

Results: Stromal fibroblasts significantly influence immuno- and lectin cytochemical properties of co-cultured normal keratinocytes. Expression of keratins 8 and 19, the nucleolar protein nucleostemin, parameters related to adhesion/growth-regulatory galectins and the epithelial-mesenchymal transition were altered. This biological activity of tumor-derived stromal cells, which did not require cell contact, appeared to be stable, because it was maintained during passaging of keratinocytes in the absence of cancer cells.

Conclusions: Tumor-derived stromal fibroblasts acquire distinct properties to shape a microenvironment conducive to altering the phenotypic characteristics of normal epithelial cells *in vitro*.

Keywords: *Cell biology, skin, stem cells, tumour physiology*

Introduction

Advances in stem cell research are likely to broaden the clinical potential of regenerative medicine, cell therapy and tissue engineering. Equally important is our understanding of stem cell biology in adult tissues, currently linked to the origin of solid tumors, which can notably factor into development of new therapeutic concepts (Reya et al. 2001, Sell 2004, Keith 2006). From this point of view, epidermal stem cells are believed to participate in the development of basal cell and squamous cell carcinomas (Owens & Watt 2003, Costea et al. 2006, Motlík et al. 2007). This hypothesis is based on functional aspects shared by normal adult stem cells and cancer cells as well as on the similarity of their phenotypes observed either under *in vitro* conditions or in biopsies. (Chovanec et al. 2005a, Dvořánkova et al. 2005, Mackenzie 2005, Smetana et al. 2006). In order to maintain their unique properties tissue stem cells require a special

Correspondence: Dr Karel Smetana, Charles University in Prague, 1st and 2nd Faculty of Medicine, Institute of Anatomy and Center of Cell Therapy and Tissue Repair, Prague, Czech Republic. E-mail: Karel.Smetana@lf1.cuni.cz

ISSN 0955-3002 print/ISSN 1362-3095 online © 2007 Informa UK Ltd.
DOI: 10.1080/09553000701694343

microenvironment, whose essential biochemical components are not clearly defined as yet (Watt & Hoggan 2000). The stem cell compartment in skin is being intensively studied to address this issue (Tumbar et al. 2004). Assuming a lineage from adult stem cells to tumor cells, it is reasonable to consider the analysis of the cellular microenvironment in tumors to identify spot clues to be followed in stem cell research. Focusing on fibroblasts in the tumor stroma, an intriguing question arises as whether they contribute to the establishment of niche-like properties (Kenny & Bissell 2003, Bissell & LaBarge 2005). Of relevance are studies of the establishment of malignancy in a prostatic epithelium cell line and tumor spread after inoculation into mice, in which stroma fibroblasts appeared to favor transformation (Hayward et al. 2001). Such reports are a paradigm for the potential of tumor stromal fibroblasts to act as modulators of other cell types. This potential for modulation is the main aim of our report.

We presume that these cells are recruited from the fibroblast pool of the local mesenchyme responding to growth factors/cytokines produced by the malignant epithelium. They can also originate from the malignant epithelium by epithelial-mesenchymal transition (Petersen et al. 2003, Weber et al. 2006). A fusion of cancer epithelial cells with stroma fibroblasts was also proposed to create bioactive stroma cells (Jacobsen et al. 2006). In a previous study, we initiated monitoring of the properties of normal human keratinocytes exposed to fibroblasts derived from a human basal cell carcinoma in a co-culture system (Lacina et al. 2007). Notable changes, for example, concerned expression characteristics of keratin 19 and nucleostemin and spurred our interest to extend this line of investigation to epidermal keratinocytes under similar experimental conditions. A pertinent issue was to test stroma derived from a different tumor type, i.e., squamous cell carcinoma. Consequently, the present study focuses on monitoring phenotypic properties of normal human epidermal keratinocytes under the influence of human squamous cell carcinoma.

The stromal fibroblasts were first characterized by karyotyping, profiling of cell surface markers and immunocytochemical analysis. Keratinocytes co-cultured with the tumor-derived stromal cells, using human dermal fibroblasts and 3T3 cells as controls in parallel, were subjected to monitoring of various features relevant for differentiation and growth. Among keratins, we looked especially at keratin 8, because this type of keratin is not normally expressed in postnatal normal squamous epithelia including cultured cells and its presence in squamous cell carcinoma is an indicator of poor prognosis of patients (Gires et al. 2004). We also included keratin 19 present in epidermal stem cells, which is not normally expressed in interfollicular epidermis (Michel et al. 1996, Dvořánková et al. 2005). Keratin 19 expression can be induced in the interfollicular keratinocytes by a suspension regimen and readhesion or by co-cultivation of these cells with fibroblasts prepared from basal cell carcinoma (Dvořánková et al. 2005, Lacina et al. 2007). Although it has not been proven a reliable marker of epidermal stem cells, we added nucleostemin to our panel because this marker is expressed in keratinocytes at an early stage of differentiation under *in vitro* conditions (Lacina et al. 2006, 2007). The region of epithelial-mesenchymal transition was defined by the level of coexpression of keratins and vimentin and also by detection of snail, the transcription factor involved in control of this process (Huber et al. 2005). Due to the association of β-catenin with the course of embryogenesis and also to cancer progression the extent of its translocation from the cell membrane to either the cytoplasm or nucleus was assessed (Conacci-Sorrell et al. 2002). The same, and even in more general terms, holds true for glycan epitopes of cellular glycoconjugates acting as biochemical signals in the interplay with endogenous lectins (Gabius 1997, 2001, 2006, Buzas et al. 2006, Villalobo et al. 2006). Because expression and profiling of those ligands are of prognostic relevance in several tumor types and they are supposed to be senescence-associated indicators in keratinocytes (Chovanec et al. 2004, Gabius et al. 2004, Lahm et al. 2004, Plzák et al. 2004, Szöke et al. 2005, Smetana et al. 2006), members of the adhesion/growth-regulatory family of galectins were studied by immuno- and lectin cytochemistry. What's more, galectin expression is susceptible to modulation by microenvironmental factors including growth *in vitro* or in tumors *in vivo* (Gabius & Vehmeyer 1988). These results direct attention to a methodological factor concerning the mode of cell culture. Cell growth either on coverslips or in 3D scaffolds can also influence cell features (Smalley et al. 2006). Thus, we compared cell populations kept in the classical two-dimensional (2D) culture with those maintained in three dimensional (3D) culture in a Matrigel matrix. To delineate whether the influence of stroma cells depends either on direct contact with the epithelial cells or on paracrine mechanisms we tested the cytochemical parameters mentioned above on keratinocytes physically separated from stromal cells by a microporous membrane. The given set of experiments were flanked by two approaches to infer an ontogenetic relationship between tumor cells and the stromal cell population, i.e., (i) the application of a differentiation-promoting agent, sodium butyrate, *in vitro*, and (ii) testing of stroma in tumors obtained from cells of the human FaDu line grafted to nu/nu mice *in vivo*.

Material and methods

Characterization of tumor

Attempts were made to isolate stromal cells from squamous cell carcinomas of the head and neck of three patients but only the presented cultivation was successful. This sample was the third recurrence of a squamous cell carcinoma of the head and neck in a 60-year-old male patient. The primary tumor was located in the edge region of the base of the tongue, metastases to lung and lymph nodes were present. This patient had been treated so far surgically with subsequent radiotherapy. The dissected tumor was divided into three parts. The first part was fixed with paraformaldehyde and embedded in paraffin for routine histopathological evaluation. The second part was embedded in the cryoprotective agent Tissue-Tek (Christine Grõpl, Tulm, Austria) and frozen in liquid nitrogen. This part was used for immuno- and lectin histochemistry. The third part was used for the preparation of stroma and cancer cells for experiments *in vitro*. The entire experiment was performed by strictly obeying the policy of informed consent of patients according to the Helsinki Declaration.

Detection of human papilloma virus DNA

Head and neck squamous cell carcinomas can be etiologically linked to infection with human papilloma virus (HPV) (Smith et al. 2006). To exclude a possible influence of viral infection on the studied parameters, tumors were examined for the presence of viral DNA. After removal of paraffin with xylene, sections were incubated with proteinase K-containing solution (Sigma, Prague, Czech Republic) at a final concentration of 200 μg/ml in lysis buffer (50 mM Tris-HCl, pH 8; 5 mM EDTA [ethylenediaminetetraacetic acid]; 1% Tween 20) for 2 h at 55°C. Proteinase K was then inactivated at 95°C for 10 min, and DNA was extracted using the standard phenol/chloroform mixture and stored at −20°C. A negative control was included in the process of DNA preparation.

Detection of presence and genotyping of the HPV DNA in samples were carried out using the polymerase chain reaction (PCR) with reverse-line blot hybridization enabling genotyping of 37 different HPV types in a single assay (van den Brule et al. 2002). The HPV DNA detection was performed in a PCR thermocycler PTC 200 (MJ Research, Inc, Waltham, MA, USA) with primer GP5+ and 5′-end biotin-labeled GP6+ primer, which amplify the 150 bp-long fragment of the L1 gene. PCR was performed for 40 cycles, and the resulting biotinylated PCR product was hybridized to oligonucleotide probes labeled at the 5′-terminal amino group. These probes were covalently linked to an activated, negatively charged Biodyne C membrane. After washing, the membrane was incubated for 60 min at 42°C with peroxidase-conjugated streptavidin. Chemiluminescent detection of hybridizing DNA on the membrane used the ECL detection liquid (Amersham Biosciences, Freiburg, Germany) and exposure of the membrane to LumiFilm (Roche, Indianapolis, IN, USA) for 5 min. Detection of a fragment of the human β-globin gene was used as a internal standard. It was amplified with primers PC 03 (5′ACACAACTGTGTTCACTAGC 3′) and PC 04 (5′CAACTTCATCCACGTTCACC 3′) (Saiki et al. 1985). Positive β-globin amplification proved that the sample contained a sufficient quantity of DNA and that no inhibitors of the PCR were present. Fifty microliters of the reaction mixture contained 1 × concentrated reaction buffer (Fermentas, Vilnius, Lithuania) with 4.0 mMol/l $MgCl_2$, 0.2 mMol dNTP, 0.05 pmol of each primer (PC 03 and PC 04) and 2.5 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). After an initial denaturation step for 5 min at 95°C, each of the 40 cycles comprised a 1-min period of denaturation at 95°C, primer annealing for 2 min at 55°C and chain elongation for 2 min at 72°C. In the final step, incubation for three minutes at 72°C was performed.

Cell preparation and 2D culture

Normal dermal fibroblasts (DF) and keratinocytes were prepared from skin specimens of healthy patients (with their informed consent) who underwent plastic surgery. Each skin graft was treated overnight with a 0.3% solution of trypsin at 4°C. Dermis and epidermis were separated. The tumor sample was enzymatically treated in the same way. Keratinocytes obtained from the epidermis and from the tumor samples were propagated following the modified Rheinwald-Green method (Matoušková et al. 1989). Keratinocytes from healthy donors (the first and second subcultures) were frozen in aliquots in 10% of dimethyl sulfoxide (Sigma, Praha, Czech Republic) and stored in liquid nitrogen. Fibroblast emigrating from minced dermis pieces were harvested and propagated in Dulbecco's modified Eagle's medium (DMEM) medium (Biochrom, Berlin, Germany) with 10% of fetal calf serum (Biochrom, Berlin, Germany) at 37°C and 5% CO_2. Stromal fibroblasts of the squamous cell carcinoma (SCCF) were prepared and cultured by the modified method as described (Grando et al. 1996, Lacina et al. 2007). For the experiments cells from the seventh passage cultured for 41 days, presenting a normal appearance, were used. 3T3 cells were propagated in H-MEM medium (Hanks'

salts modified Eagle's medium SevaPharma, Prague, Czech Republic) with 10% bovine serum (ZVOS, Hustopeče, Czech Republic) at 37 °C and 3.3% CO_2. Prior to co-culture with keratinocytes, proliferation of 3T3 fibroblasts was impaired by exposure to mitomycin C (Sigma, Praha, Czech Republic) at a concentration of 25 μg/ml for 3 h. These cells were seeded on glass coverslides at a density of 25,000 cells/cm^2 and cultured for 24 h. Due to their rather low proliferative activity SCCF were not exposed to mitomycin C when cultured at a density of 7000 cells/cm^2. The suspension of keratinocytes (20,000 cells/cm^2) was then added, and the cells were cultivated in a keratinocyte medium (Matoušková et al. 1989) at 37 °C and 3.3% CO_2. The SCCF phenotype was also studied after the treatment of cells with sodium butyrate (Sigma, Praha, Czech Republic) applied at concentrations of 0.4 or 0.8 mg/ml in culture medium as described elsewhere (Daehn et al. 2006) for three or six consecutive days.

The commercially available human hypopharyngeal squamous cell carcinoma line FaDu (HTB-43, American Type Culture Collection, Rockville, MD, USA) was cultured in modified Eagle's medium containing 10% fetal calf serum, antibiotics (100 units/ml of penicillin, 100 μg/ml of streptomycin; Sigma), 1.5 g/l $NaHCO_3$, 0.11 g/l sodium pyruvate, 0.292 g/l L-glutamine and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). This line was used in the animal experiment.

Cell culture in Matrigel

5×10^5 SCCF and 1×10^6 of normal keratinocytes resuspended in minimal volume (0.1 ml) and mixed with 1.5 ml of BD Matrigel™ (BD Biosciences, Erembodegen, Belgium) were placed in a culture dish (3.5 cm). This 3D system was kept under conditions as described above for seven days. After this period, the Matrigel specimen with the cells was frozen in liquid nitrogen and prepared for histochemical analysis.

Grafting of FaDu cells to nu/nu mice

Two female nu/nu CD-1 mice, aged ten weeks, were purchased from the Institute of Molecular Genetics, Academy of Science of the Czech Republic (Prague, Czech Republic). They were kept in accordance to approved guidelines and had access to food and water *ad libitum*. 1×10^6 FaDu cells were resuspended in 100 μl of phosphate-buffered saline (PBS; pH7.3) and mixed with 50 μl of BD Matrigel™ (BD Biosciences, Erembodegen, Belgium) as described. This suspension was subcutaneously injected, the animals were sacrificed after 49 days and tumor specimens were frozen as described above.

Cytogenetic analysis

SCCF from the 9th passage were subcultured for 24 h, incubated with demecolcemid (Sigma) for 4 h, detached from the substratum by applying trypsin-EDTA solution, then treated by hypotonic KCl solution and fixed by acidic ethanol. Metaphasic chromosomes were analyzed after G-/R-banding using the Ikaros version 5 (MetaSystems, Altlussheim, Germany). A total of 50 metaphases were monitored in the samples investigated.

FACS analysis of DF and SCCF

DF after a brief culture period and SCCF cultured for 41 (9th passage) days were harvested using trypsin-EDTA solution. The activity of trypsin was neutralized by adding fetal calf serum. Cells were then resuspended in fresh culture medium and analyzed for presence of the following markers: cluster of differentiation (CD)11b, CD18, CD29, CD44, CD45, CD49a, CD49d, CD63, CD90, CD106, and CD166 (all from Becton Dickinson, Prague, Czech Republic), CD11c, CD14, CD34, CD45, CD71, CD235a, CD105, and HLA-A, -B, and -C (all from Dako, Brno, Czech Republic), CXCR4 (R&D Systems, Minneapolis, MN, USA) as well as CD19e and CD49c (Chemicon, Temecula, CA, USA). IgG_1 (Dako, Brno, Czech Republic) was used as a negative control. Measurements were performed on a FACSCalibur™ instrument (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) and analyzed using Summit™ V3.3. Build 1024 software (Dako, Brno, Czech Republic).

Immuno- and lectin cytochemistry

The seven μm-thick frozen sections from human/mouse tumors and cell-containing Matrigel samples, as well as cells adherent to coverslips, were washed with PBS and briefly fixed with 5% paraformaldehyde diluted in PBS. The human galectins were purified by affinity chromatography as crucial step after recombinant production. Purity was ascertained prior to use as antigen and the resulting polyclonal antibody preparations were subjected to rigorous specificity controls including chromatographic removal of cross-reactive material, if necessary (André et al. 1999, 2004, Kayser et al. 2003a, 2003b, Kopitz et al. 2003, Dam et al. 2005, Lensch et al. 2006). Biotinylation was performed under activity-preserving conditions. Activity was ascertained by solid-phase and cell-binding assays and extent of labeling quantitated by a proteomics protocol (Gabius et al. 1984, Purkrábková et al. 2003, André et al. 2005a, 2005b, 2006, Wu et al. 2006). Staining was visualized as described previously (Froňková

et al. 1999, Plzák et al. 2001). The entire panel of monoclonal/polyclonal antibodies and the biotinylated galectins is shown in Table I. The antigen-dependent specificity was tested by replacement of the test antibody with another polyclonal or monoclonal antibody of the same isotype but against antigens not present in the studied cells/tissues. Ligand-dependent binding of galectins was tested by omission of galectin (and using the second-step reagent only) and by use of lactose to block carbohydrate-dependent binding. Nuclei of the majority of specimens were counterstained with DAPI (4′,6′-diamidino-2-phenyindole dilactate, Sigma), a DNA-specific dye. The processed specimens were finally mounted to Vectashield (Vector Laboratories, Bourlingame, CA, USA), inspected and analyzed using an Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with suitable filterblocks, a high resolution Vosskühler Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany) and a computer-assisted image analyzer (LUCIA 5.10) (Laboratory Imaging, Prague, Czech Republic). Statistical calculations using the Student's non-paired *t*-test was used to assess significance levels.

Results

The investigated stroma in this study originated from a well-differentiated keratinizing squamous cell carcinoma with keratin pearls containing keratin 10-positive cells (Figure 1). In addition, keratin 8 presence was also observed (Figure 1). Intensity of staining for galectin-7, a marker of squamous epithelium, was only weak, and keratinized tumor parts were typically reactive with galectin-3 (not shown). We performed PCR analysis to exclude the confounding influence of HPV infection. No HPV-specific DNA was found in the tumor sample (Figure 2). Next, we also checked for occurrence of karyotype abnormalities. Comparing the karyotype of SCCF with normal human dermal fibroblasts, ~80% of studied cells exhibited no differences (Figure 3). Absence of the Y chromosome was observed in 20% of studied cells (not shown). As a further comparative measure we monitored a panel of the surface markers using FACS analysis. No significant differences were observed between SCCF and normal DF including the absence of CD34 and CD105 (Figure 3). Then we proceeded to immunocytochemical monitoring.

SCCF were strongly positive for vimentin (Vim, Figure 4A) and devoid of keratin expression (K, Figure 4B). They exhibited high proliferative activity as demonstrated by detection of the proliferation marker Ki67, predominantly in nucleoli of approximately 30% of the studied cell population (Figure 4C, 4D). Nucleoli were strongly positive for nucleostemin that was detected in both the Ki67-positive and -negative nuclei (NuclS, Figure 4C). Approximately 20% of the SCCF expressed the adhesion/growth-regulatory galectin-1 in the cytoplasm, and the presence of this endogenous lectin was also detected in the extracellular matrix produced by these cells (Gal-1, Figure 4D). Using this lectin as a probe, nuclei of SCCF were positive as well as nuclei of malignant epithelial cells isolated from the tumor (Figure 4E). These malignant

Table I. Probes used for phenotypic characterization of cells.

Visualized epitope	Type of probe	Supplier/origin	Second-step reagent	Supplier
Panel of keratins (K1 = LP34) Keratin 8 Keratin 10 Keratin 19 Ki67 Vimentin (clone V9)	mMA	Dako, Brno, Czech Republic	a) SwAM-FITC b) Goat anti-mouse IgG-TRITC	a) AlSeVa, Prague, Czech Republic b) Sigma, Prague, Czech Republic
Panel of keratins Snail	rPA	Abcam, Cambridge, UK	SwAR-FITC	AlSeVa, Prague, Czech Republic
β-Catenin	rPA	Santa Cruz, Santa Cruz, CA, USA		
Nucleostemin	gPA	Neuromics, Bloomington, MN, USA	Donkey anti-goat-TRITC	Jackson Laboratories, West Grove, PA, USA
Galectin-1 Galectin-3 Galectin-7	rPA	Munich lab	SwAR-FITC	AlSeVa
Galectin-1-binding sites Galectin-3-binding sites	Biotinylated lectin	Munich lab	ExtrAvidin-TRITC	Sigma

mMA, mouse monoclonal antibody; gPA, goat polyclonal antibody; rPA, rabbit polyclonal antibody; SwAM-FITC, FITC-labeled swine anti-mouse antibody; SwAR-FITC, FITC-labeled swine anti-rabbit antibody.

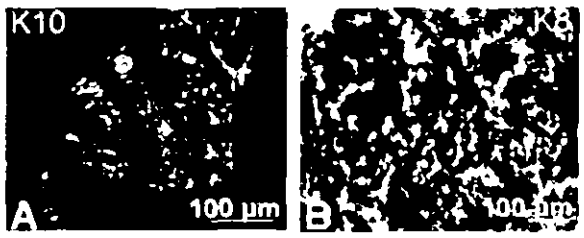


Figure 1. Immunohistochemical detection of keratin 10 (A) and keratin 8 (B) in a well differentiated keratinizing squamous cell carcinoma from which the stromal cells were prepared; scale bar: 100 μm.

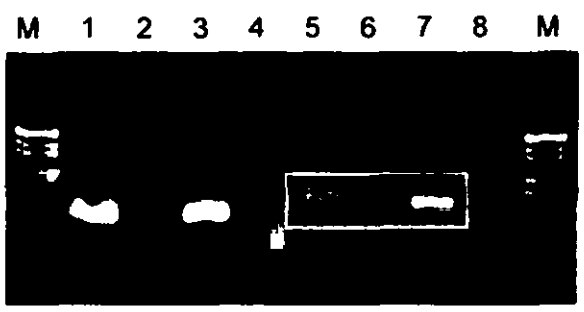


Figure 2. Search for HPV-specific DNA in the tumor by PCR. Lane M: marker; lane 1: section of β-globin gene amplified from patient material; lane 2: negative control for specific amplification of the β-globin gene; lane 3: positive control for amplification of the β-globin gene; lane 4: H_2O control; lane 5: processing of DNA sample from the tumor to detect HPV presence; lane 6: negative control of amplification of the HPV-specific DNA; lane 7: positive control of amplification of the HPV-specific DNA (type 16); lane 8: H_2O control. The rectangle denotes the region for the expected amplification product of HPV DNA, showing no signal in the sample prepared from the characterized tumor.

keratinocytes expressed keratin 8 (K8, Figure 4E), as was also observed in tissue sections. Interfollicular epidermal keratinocytes co-cultured with dermal fibroblasts or with 3T3 cells were characterized by their characteristically round morphology (Figure 4F, 4K, 4M) that contrasted with irregular shape of colonies cultured in the presence of SCCF (Figure 4L, 4N). Moreover, their phenotype was also significantly changed. While keratinocytes cultured with non-tumor stromal fibroblasts were negative for expression of keratin 8 (Figure 4F), the introduction of SCCF induced expression of this type of keratin, normally not present in the postnatal squamous epithelia (Figure 4G). Keratinocytes strongly positive for keratin 8 were observed under the influence of SCCF, mainly on the periphery of colonies (Figure 4G). These keratin 8-positive cells contained nucleoli with nucleostemin (Figure 4H). When we compared the influence of SCCF without/with treatment of mitomycin on normal keratinocytes, the keratinocytes grown together with growth-arrested SCCF were more spread than those cocultured with untreated SCCF (Figure 4H, 4I and Figure 5). Nucleostemin was detected in normal keratinocytes only if they were co-cultured with SCCF not pretreated by mitomycin C (Figure 4H). Evidently, the proliferative activity of SCCF has impact on its modulatory role. In full accordance to our previous report (Lacina et al. 2007) the stem-cell-characteristic expression of keratin 19 was observed only in keratinocytes co-cultured with SCCF (Figure 4J). Concerning the expression pattern of β-catenin, the presence of SCCF in the culture led to an intracellular shift of this protein from the cell membrane (Figure 4K) to the cytoplasm and nucleus (Figure 4L). Normal keratinocytes express keratins as a cell-type-specific form of intermediate filament, whereas the presence of vimentin is typical for fibroblasts. We visualized both cytoskeletal elements simultaneously in order to map the epithelial-mesenchymal transition. Interfollicular epidermal keratinocytes expressed keratins only (Figure 4M) when cocultured with DF and 3T3 cells, respectively. The presence of SCCF altered this expression pattern, and both types of protein, i.e. keratins and vimentin, were detected in keratinocytes, namely in cells with elongated fibroblast-like morphology (Figure 4N). Moreover, the presence of the transcription factor snail, a mediator in the control of the epithelial-mesenchymal transition, was observed in elongated epithelial cells expressing keratins (Figure 4O).

In principle, the documented influence of SCCF on interfollicular epidermal keratinocytes can be mediated by two mechanisms, i.e., by intercellular contacts or by paracrine supply of growth factors/cytokines produced by SCCF. To resolve this issue, we cultured epidermal cells, now separated from the SCCF by a microporous membrane inside the insert system. These keratinocytes also expressed keratin 8 (Figure 6A1), and cells with dual positivity for keratins and for vimentin (Figure 4P, P1) were identified in the pool of keratinocytes.

Interestingly, nucleostemin-positive nucleoli were significantly larger in keratin 8-positive than in keratin 8-negative cells (Figure $6A_{1-4}$). When considering culture methods an important aspect to be reckoned with is the mode of cell maintenance.

It is known that 2D/3D cell culture systems yield non-uniform results. Thus, we also cultivated normal interfollicular keratinocytes with SCCF in Matrigel. Whereas keratinocytes formed distinct spheroids, fibroblasts-like cells were located in their periphery (Figure 4Q). Cells in such spheres expressed keratin 8 (Figure 4R). These keratinocytes inside the spheres and cells with fibroblast-like shape expressed vimentin (Figure 4S, S_1, S_2). A rather high extent of epithelial-mesenchymal transition induced by SCCF is thus revealed as noted in the 2D system. So far, we focused on testing of SCCF as the source

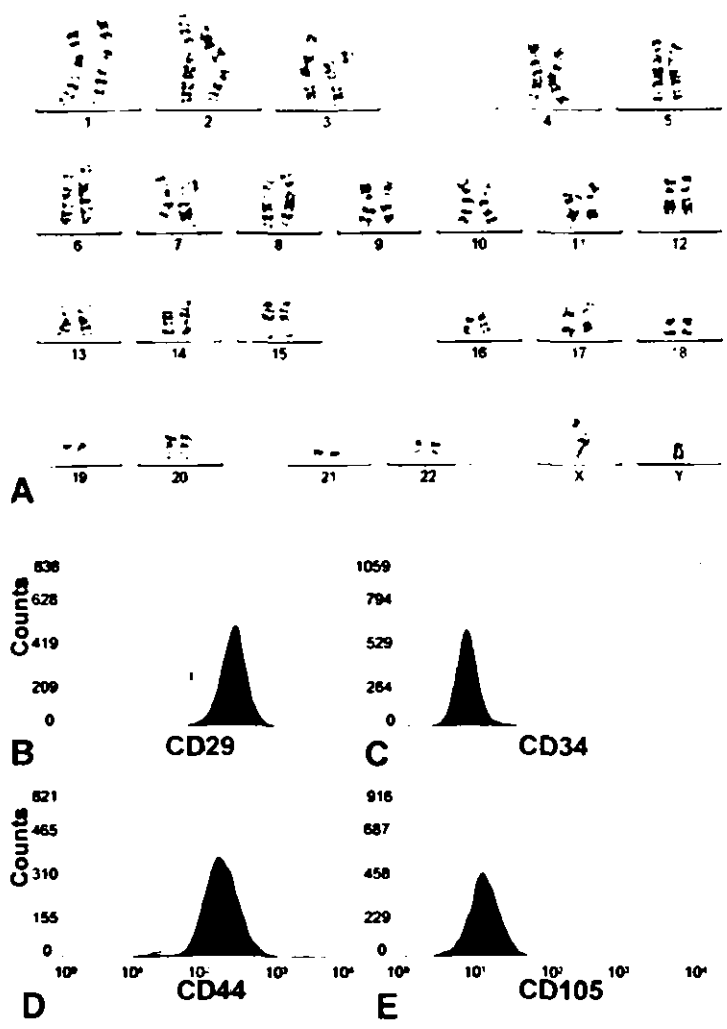


Figure 3. Karyotype of representative stromal fibroblast (A) and comparison of FACScan profiles of SCCF (black) and dermal fibroblasts (stiped). Results for CD29 (B), CD34 (C), CD44 (D) and CD105 (E) in both cell populations is presented.

for modulatory effects. To provide information on the origin of the SCCF we challenged the concept of an epithelial-mesenchymal transition by two separate experimental designs.

First, we exposed SCCF to sodium butyrate to revert the phenotype. Very few cells with keratins in addition to vimentin were present in the population of SCCF (Figure 4U), even after six days. Butyrate presence thus led to no major occurrence of reversion to epithelial cells. Should SCCF have arisen from a transition process from the pool of cancer cells, a marked extent of this process would be expected. Along this line, an *in vivo* experiment with xenotransplantation provided no evidence for such a transition. FaDu tumor cells developed large tumors in mice so that we could probe the species origin of tumor stroma. When using an antibody specific for human and porcine but not reactive with the mouse protein, no signal for vimentin expression was observed (Figure 4V). In the tested tumors stromal cells are apparently of murine nature, not a product of a transition from human tumor cells.

Discussion

We have previously initiated a study of the modulatory influence of stromal cells from a basal cell carcinoma on normal epidermal cells (Lacina et al. 2007). To extend the experimental basis of this study and to test stromal cells from squamous cell carcinoma we carried out a corresponding study. To exclude a potentially confounding factor the tumor material, which was also carefully karyotyped, was rigorously examined for the absence of HPV infection by PCR. One of the main findings of our report is the expression of keratin 8 under the influence of SCCF. This keratin is postnatally expressed in monolayer epithelia, not in squamous

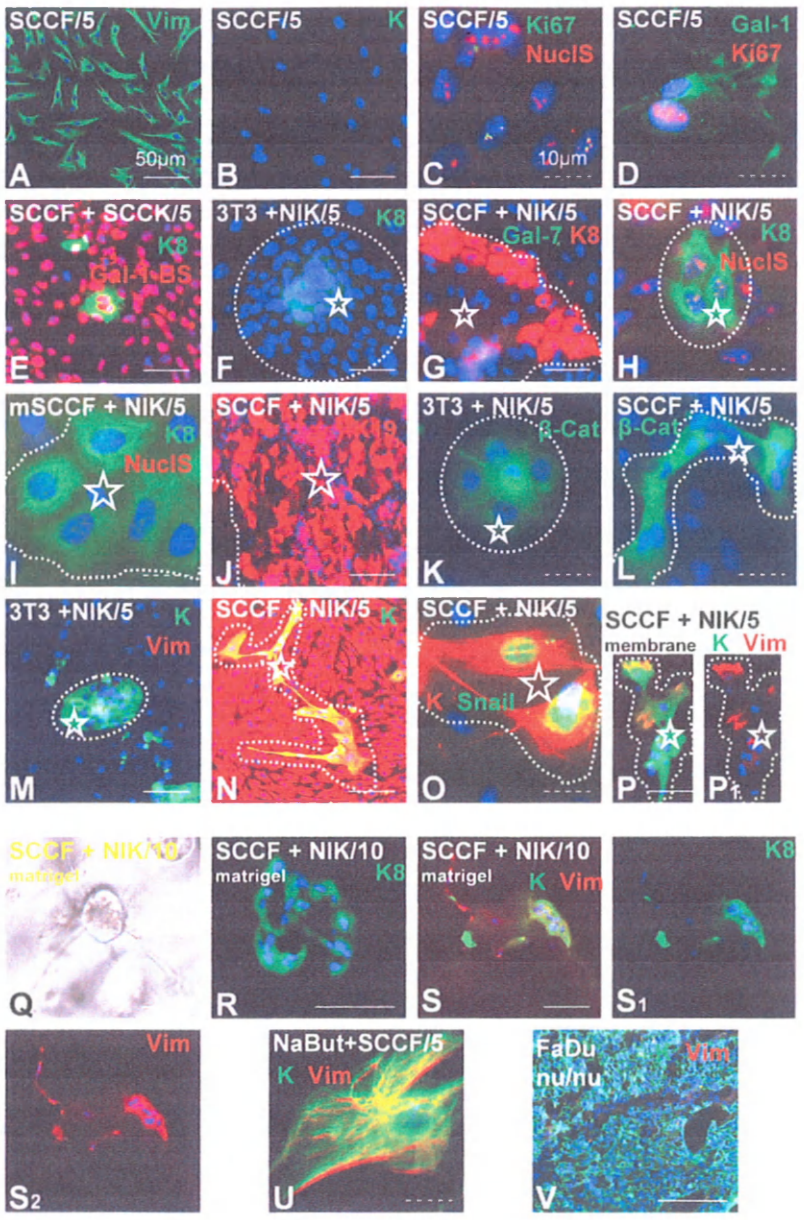


Figure 4. Immuno- and lectin cytochemical profiling of the phenotype of cultured SCCF (A-D), of cultured SCCF treated with sodium butyrate (NaBut, U), of cancer keratinocytes (SCCK) cocultured with SCCF (E), of normal interfollicular keratinocytes (NIK/5) co-cultured with 3T3 cells (F, K, M), of NIK/5 co-cultured with SCCF on coverslips (G-J, L, N, O), of NIK/5 separated from SCCF by a microporous membrane (P, P1-detection of vimentin, Vim, only), NIK co-cultured with SCCF in Matrigel (Q-S_2) and of FaDu cells in a tumor formed after xenotransplantation to nu/nu mice (V). Normal interfollicular keratinocytes were co-cultured with stromal cells or with 3T3 fibroblasts for 5 or 10 days (NIK/5, NIK/10). The applied markers are directly defined in each figure, the respective name given in the color of the detected signal. Yellow signal in panel N arises from merging red and green signals. Abbreviations: Vim, vimentin; K, panel of keratins; NuclS, nucleostemin; Gal-1, galectin-1; Gal-1-BS, binding sites for galectin-1; K8, keratin 8; Gal-7, galectin-7, K19, keratin 19; β-Cat, β-catenin. Solid Bar: 50 μm, dashed bar: 10 μm. The keratinocytes cocultured with fibroblasts on the surface of coverslips without matrigel are surrounded by the white dashed line and marked by star.

cell epithelium under physiological conditions. Moreover, keratin 8 is expressed in embryoid bodies originating from embryonic stem cells, these cells probably being precursors of epidermal stem cells (Troy & Turksen 2005). Its overexpression is associated with the malignant phenotype (Casanova et al. 2004, Raul et al. 2004). Fittingly, the increased expression correlated with poor clinical prognosis in head and neck cancer patients (Gires et al. 2004). We detected expression of this keratin in the tumor from which the stromal cells were prepared. In line with this result, nucleostemin expression is enhanced

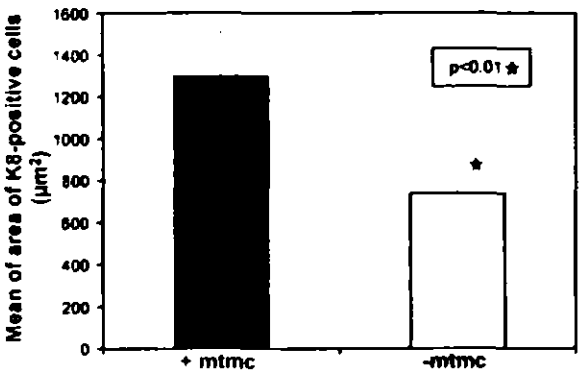


Figure 5. Comparison of the size (mean area) of normal keratinocytes (mean ± SD) cocultured with SCCF plus or minus exposure to mitomycin (mtmc), 25 µg/ml for three hours (black column: presence of mitomycin C (mtmc); white column: no mtmc). The difference is statistically significant ($p \leq 0.01$).

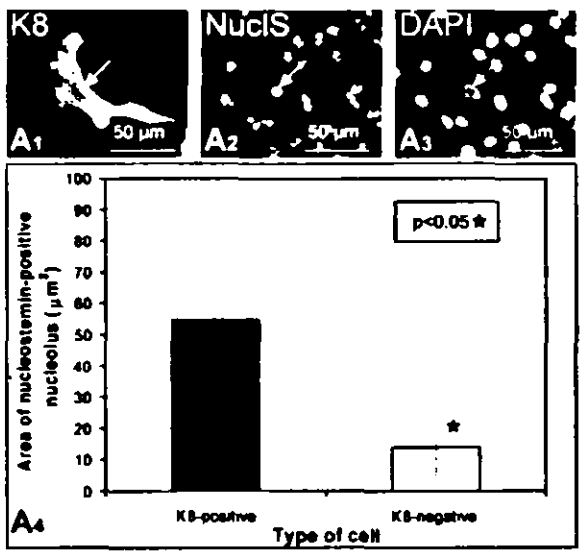


Figure 6. SCCF induced expression of keratin 8 in normal keratinocytes separated by use of a microporous membrane (A_{1-3}). These keratin 8-positive cells contained nucleostemin (NuclS) positive nucleoli significantly larger than those of the keratin 8-negative cells (mean ± SD) (A_4). The difference is statistically significant ($p \leq 0.05$).

in normal keratinocytes cultured with fibroblasts prepared from the stroma of squamous cell carcinoma. Nucleostemin expression is involved in cancer growth regulation (Liu et al. 2004, Tsai & McKay 2002, Lacina et al. 2006) and previously was reported to be up-regulated under the influence of basal-cell-carcinoma-derived stromal cells (Lacina et al. 2007). A shift of the signal for β-catenin from association with the cell membrane to the cytoplasm/nucleus can also be considered as 'protumoral' (Conacci-Sorrell et al. 2002), and, indeed, it was observed in keratinocytes cultured with SCCF. Next, monitoring of the epithelial-mesenchymal transition based on induction of vimentin and snail expression in keratin-positive keratinocytes in both the 2D and 3D culture systems revealed a clear effect of SCCF on the transition. This is consistent with the fact that it is widely accepted that vimentin is marker of mesenchymal cells and keratins are specific for epithelial cells (Petersen et al. 2003). The epithelial-mesenchymal transition is performed under the snail control (Thiery & Sleeman 2006). This process is characteristic for embryonic development and wound healing. In malignancy it represents a high-risk factor relevant for tumor spread of cancer cells (Takkunem et al. 2006, Thiery & Sleeman 2006). The absence of galectin-7, which was studied in this tumor type previously (Chovanec et al. 2005b), together with binding of galectin-1 to cell nuclei, characteristic of cells with low level of differentiation (Klima et al. 2005), added to the accumulating evidence of the strong modulation of keratinocyte properties. As a means to gauge the importance of intercellular contacts for this effect we introduced a membrane into the co-culture system providing physical separation of the two cell populations but allowing communication mediated via diffusible biochemical compounds. This arrangement did not impair the biological effect of SCCF on normal keratinocytes. Paracrine mediators thus appear sufficient to trigger changes in the monitored phenotypic characteristics. The production of growth-stimulatory/proangiogenic factors has been similarly discussed for inflammatory cells in tumors (Ichim 2005). Regarding the origin of the stromal cells, our results provide no evidence for cell fusion or an epithelial-mesenchymal transition process. In other words, local fibroblasts of the tumor have acquired special properties to affect phenotypic characteristics of keratinocytes *in vitro*.

In conclusion, stromal cells of squamous cell carcinoma are capable of altering keratinocyte properties in co-culture in a characteristic manner, namely, enhancement of keratin 8 expression and the epithelial-mesenchymal transition, among other parameters. This may even signify clinical relevance, e.g., for development of resistance mechanisms against radiotherapy (Smith & Haffty 1999, Diehn & Clarke 2006). The origin of stromal cell plasticity and the biochemical mechanisms underlying the effect on epithelial cells including tissue stem cells warrant further study.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sport of the Czech Republic, projects No. MSM0021620806 and No.

1M0021620803, by the Grant Agency of the Ministry of Public Health of the Czech Republic, project No. NR 9049-3 and by an EC Marie Curie Research Training Network grant (contract No. MRTN-CT-2005-019561). Authors are grateful to Drs J. Betka, B. Friday, R. Pytlik, T. Soukup and V. Vonka for inspiring discussions and helpful suggestions as well as to E. Vancova, I. Burdova, J. Šmahelová and V. Hajdúch for expert technical assistance.

References

André S, Kojima S, Yamazaki N, Fink C, Kaltner H, Kayser K, Gabius H-J. 1999. Galectins-1 and -3 and their ligands in tumor biology. Journal of Cancer Research and Clinical Oncology 125:461–474.

André S, Kaltner H, Furuike T, Nishimura S-I, Gabius H-J. 2004. Persubstituted cyclodextrin-based glycoclusters as inhibitors of protein-carbohydrate recognition using purified plant and mammalian lectins and wild-type and lectin-gene-transfected tumor cells as targets. Bioconjugate Chemistry 15:87–98.

André S, Kojima S, Prahl I, Lensch M, Unverzagt C, Gabius H-J. 2005a. Introduction of extended LEC-14-type branching into core-fucosylated biantennary N-glycan. Glycoengineering for enhanced cell binding and serum clearance of the neoglycoprotein. FEBS Journal 272:1986–1998.

André S, Siebert H-C, Nishiguchi M, Tazaki K, Gabius H-J. 2005b. Evidence for lectin activity of a plant recetor-like protein kinase by application of neoglycoproteins and bioinformatic algorithms. Biochim Biophys Acta 1725:222–232.

André S, Pei Z, Siebert H-C, Ramström O, Gabius H-J. 2006. Glycosyldisulfides from dynamic combinatorial libraries as O-glycoside mimetics for plant and mammalian lectins: Their reactivities in soldi-phase and cell assays and conformational analysis by molecular dynamics simulations. Bioorganic & Medical Chemistry 14:6314–6326.

Bissell MJ, LaBarge MA. 2005. Context, tissue plasticity, and cancer: Are tumor stem cells also regulated by the microenvironment? Cancer Cell 7:17–23.

Buzas EI, György B, Pásztói M, Jelinek I, Falus A, Gabius H-J. 2006. Carbohydrate recognition systems in autoimmunity. Autoimmunity 39:691–704.

Casanova ML, Bravo A, Martinez-Palacio J, Fernandez-Acenero MJ, Villanueva C, Larcher F, Conti CJ, Jorcano JL. 2004. Epidermal abnormalities and increased malignancy of skin tumors in human epidermal keratin 8-expressing transgenic mice. FASEB Journal 18:1556–1558.

Chovanec M, Smetana K Jr, Dvořánková B, Plzákova Z, André S, Gabius H-J. 2004. Decrease of nuclear reactivity to growth-regulatory galectin-1 in senescent human keratinocytes and detection of non-uniform staining profile alterations upon prolonged culture for galectins-1 and -3. Anatomy Histology Embryology 33:348–354.

Chovanec M, Smetana K Jr, Betka J, Plzák J, Brabec J, Moya-Alvarez V, André S, Kodet R, Gabius H-J. 2005a. Correlation of expression of nuclear protein pKi67 and p63 with lectin histochemical features in head and neck squamous cell cancer. International Journal of Oncology 27:409–415.

Chovanec M, Smetana K Jr, Plzák J, Betka J, Plzáková Z, Štork J, Hrdličková E, Kuwabara I, Dvořánková B, Liu F-T, Kaltner H, André S, Gabius H-J. 2005b. Detection of new diagnostic markers in pathology by focus on growth-regulatory endogenous lectins. The case study of galectin-7 in squamous epithelia. Prague Medical Report 106:209–216.

Costea DE, Tsinkalovski O, Vintermyr OK, Johannessen AC, Mackenzie IC. 2006. Cancer stem cells: New and potentially important targets for the therapy of oral squamous cell carcinoma. Oral Disease 12:443–454.

Daehn IS, Varelias A, Rayner TE. 2006. Sodium butyrate induced keratinocyte apoptosis. Apoptosis 11:1379–1390.

Dam TK, Gabius H-J, André S, Kaltner H, Lensch M, Brewer CF. 2005. Galectins bind to multivalent glycoprotein asialofetuin with enhanced affinities and a gradient of decreasing binding constants. Biochemistry 44:12564–12571.

Diehn M, Clarke MF. 2006. Cancer stem cells and radiotherapy: New insights into tumor radioresistance. Journal of National Cancer Institute 98:1755–1757.

Dvořánková B, Smetana K Jr, Chovanec M, Lacina L, Štork J, Plzáková Z, Galovičová M, Gabius H-J. 2005. Transient expression of keratin 19 is induced in originally negative interfollicular epidermal cells by adhesion of suspended cells. International Journal of Molecular Medicine 16:525–531.

Froňková V, Holíková Z, Liu F-T, Homolka J, Rijken DC, André S, Bovin NV, Smetana K Jr, Gabius H-J. 1999. Simultaneous detection of endogenous lectins and their binding capability at the single-cell level: A technical note. Folia Biologica (Praha) 45:157–162.

Gabius H-J. 1997. Animal lectins. European Journal of Biochemistry 243:543–576.

Gabius H-J. 2001. Glycohistochemistry: the why and how of detection and localization of endogenous lectins. Anatomy Histology Embryology 30:3–31.

Gabius H-J. 2006. Cell surface glycans: The why and how of their functionality as biochemical signals in lectin-mediated information transfer. Critical Reviews in Immunology 26:43–79.

Gabius H-J, Vehmeyer K. 1988. Effect of microenvironment and cell-line type on carbohydrate-binding proteins of macrophage-like cells. Biochemistry and Cell Biology 66:1169–1176.

Gabius H-J, Engelhardt R, Rehm S, Cramer F. 1984. Biochemical characterization of endogenous carbohydrate-binding proteins from spontaneous murine rhabdomyosarcoma, mammary adenocarcinoma, and ovarian teratoma. Journal of National Cancer Institute 73:1349–1357.

Gabius H-J, Siebert H-C, André S, Jiménez-Barbero J, Rüdiger H. 2004. Chemical biology of the sugar code. ChemBioChem 5:740–764.

Gires O, Münz M, Schaffrik M, Kleu C, Rauch J, Ahlemann M, Eberle D, Mack B, Wolleneberg B, Lang S, Hofmann T, Hammerschmidt W, Zeidler R. 2004. Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics-based technology. Cellular and Molecular Life Sciences 61:1198–1207.

Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A. 2002. The cadherin-catenin adhesion system in signalling and cancer. Journal of Clinical Investigation 109:987–991.

Grando SA, Schofield O, Skubitz AP, Kist DA, Zelickson BD, Zachary CB. 1996. Nodular basal cell carcinoma *in vivo* vs. *in vitro*. Archives of Dermatology 132:1185–1193.

Hayward SW, Wang Y, Cao M, Hom YK, Zhang B, Grossfeld GD, Sudilovsky D, Cunha GR. 2001. Malignant transformation in a nontumorigenic human prostatic epithelial cell line. Cancer Research 61:8135–8142.

Huber MA, Kraut N, Beug H. 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Current Opinion in Cell Biology 17:548–558.

Ichim CV. 2005. Revisiting immunosurveillance and immunostimulation: implications for cancer immunotherapy (review). Journal of Translational Medicine 3:8.

Jacobsen BM, Harrell JC, Jedlicka P, Borges VF, Vardella-Garcia M, Horwitz KB. 2006. Spontaneous fusion with, and transformation of mouse stroma by, malignant human breast cancer epithelium. Cancer Research 66:8274–8279.

Kayser K, Hoeft D, Hufnagl P, Caseliz J, Zick Y, André S, Kaltner H, Gabius H-J. 2003a. Combined analysis of tumor growth pattern and expression of endogenous lectins as a prognostic tool in primary testicular cancer and its lung metastases. Histology and Histopathology 18:771–779.

Kayser K, Nwoye JO, Kosjerina S, Goldmann T, Vollmer E, Kaltner H, André S, Gabius H-J. 2003b. Atypical adenomatous hyperplasia of lung: Its incidence and analysis of clinical, glycohistochemical and structural features including newly defined growth regulators and vascularization. Lung Cancer 42:171–182.

Keith WN. 2006. Cancer stem cells: Opportunities for novel diagnostics and drug discovery. European Journal of Cancer 42:1195–1196.

Kenny PA, Bissell MJ. 2003. Tumor reversion: Correction of malignant behavior by microenvironmental cues. International Journal of Cancer 107:688–695.

Klíma J, Smetana K Jr, Motlík J, Plzáková Z, Liu F-T, Štork J, Kaltner H, Chovanec M, Dvořánková B, André S, Gabius H-J. 2005. Comparative phenotypic characterization of keratinocytes originating from hair follicles. Journal of Molecular Histology 36:89–96.

Kopitz J, André S, von Reitzenstein C, Versluis K, Kaltner H, Pieters RJ, Wasano K, Kuwabara I, Liu F-T, Cantz M, Heck AJR, Gabius H-J. 2003. Homodimeric galectin-7 (p53-induced gene 1) is a negative regulator for human neuroblastoma cells. Oncogene 22:6277–6288.

Lacina L, Smetana K Jr, Dvořánková B, Štork J, Plzáková Z, Gabius H-J. 2006. Immunocyto- and histochemical profiling of nucleostemin expression: Marker of epidermal stem cells? Journal of Dermatological Sciences 44:73–80.

Lacina L, Smetana K Jr, Dvoranková B, Pytlik R, Kideryova L, Kucerova L, Plzakova Z, Stork J, Gabius H-J, André S. 2007. Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. British Journal of Dermatology 156:819–829.

Lahm H, André S, Hoeflich A, Kaltner H, Siebert H-C, Sordat B, von der Lieth C-W, Wolf E, Gabius H-J. 2004. Tumor galectinology: Insights into the complex network of a family of endogenous lectins. Glycoconjugate Journal 20:227–238.

Lensch M, Lohr M, Russwurm R, Vidal M, Kaltner H, André S, Gabius H-J. 2006. Unique sequence and expression profiles of rat galectins-5 and -9 as a result of species-specific gene divergence. International Journal of Biochemistry & Cell Biology 38:1741–1758.

Liu S-J, Cai Z-W, Liu Y-J, Dong M-Y, Sun L-Q, Hu G-F, Wei Y-Y, Lao W-D. 2004. Role of nucleostemin in growth regulation of gastric cancer, liver cancer and other malignancies. World Journal of Gastroenterology 10:1246–1249.

Mackenzie IC. 2005. Retention of stem cell patterns in malignant cell lines. Cell Proliferation 38:347–355.

Matoušková E, Veselý P, Königová R. 1989. Modified method of *in vitro* cultivation of human keratinocytes suitable for grafting. Folia Biologica (Praha) 35:118–123.

Michel M, Török N, Godbout M-J, Lussier M, Gaudreau P, Royal A, Germain L. 1996. Keratin 19 as a biochemical marker of skin stem cells *in vivo* and *in vitro*: Keratin 19 expressing cells are differentially located in function of anatomic sites, and their number varies with donor age and culture stage. Journal of Cell Sciences 109:1017–1028.

Motlík J, Klíma J, Dvořánková B, Smetana K Jr. 2007. Porcine epidermal stem cells as a biomedical model for wound healing and normal/malignant epithelial cell propagation. Theriogenology 67:105–111.

Owens DM, Watt FM. 2003. Contribution of stem cells and differentiated cells to epidermal tumors. Nature Reviews Cancer 3:444–451.

Petersen OW, Lind Nielsen H, Gudjonsson T, Viladsen R, Rank F, Niebuhr E, Bissell MJ, Rønnov-Jenssen L. 2003. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. American Journal of Pathology 162:391–402.

Plzák J, Smetana K Jr, Hrdličková E, Kodet R, Holíková Z, Liu F-T, Dvořánková B, Kaltner H, Betka J, Gabius H-J. 2001. Expression of galectin-3-reactive ligands in squamous cancer and normal epithelial cells as a marker of differentiation. International Journal of Oncology 19:59–64.

Plzák J, Betka J, Smetana K Jr, Chovanec M, Kaltner H, André S, Kodet R, Gabius H-J. 2004. Galectin-3: An emerging prognostic indicator in advanced head and neck carcinoma. European Journal of Cancer 40:2324–2330.

Purkrábková T, Smetana K Jr, Dvořánková B, Holiková Z, Böck C, Lensch M, André S, Pytlik R, Liu F-T, Klíma J, Smetana K, Motlík J, Gabius H-J. 2003. New aspects of galectin functionality in nuclei of cultured bone marrow stromal and epidermal cells: Biotinylated galectins as tool to detect specific binding sites. Biology of the Cell 95:535–545.

Raul U, Sawant S, Dange P, Kalraiya R, Ingle A, Vaidya M. 2004. Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: Induction of transformed phenotype. International Journal of Cancer 111:662–668.

Reya T, Morrison SJ, Clake MF, Weissman IL. 2001. Stem cells, cancer and cancer stem cells. Nature 414:105–111.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354.

Sell S. 2004. Stem cell origin of cancer and differentiation therapy. Critical Reviews in Oncology Hematology 51: 1–28.

Smalley KS, Lioni M, Herlyn M. 2006. Life isn't flat: Taking cancer biology to the next dimension. In Vitro Cellular & Developmental Biology-Animal 42:242–247.

Smetana K Jr, Dvořánková B, Chovanec M, Bouček J, Klíma J, Motlík J, Lensch M, Kaltner H, André S, Gabius H-J. 2006. Nuclear presence of adhesion-/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin. Histochemistry and Cell Biology 125:171–182.

Smith BD, Haffty BG. 1999. Molecular markers as prognostic factors for local recurrence and radioresistance in head and neck squamous cell carcinoma. Radiation Oncology Investigation 7:125–144.

Smith EM, Ritchie JM, Pawlita M, Rubenstein LM, Haugen TH, Turek LP, Hamšiková E. 2006. Human papilloma virus seropositivity and risks of head and neck cancer. International Journal of Cancer 120:825–832.

Szöke T, Kayser K, Baumhäkel J-D, Trojan I, Furak J, Tiszlavicz L, Horvath A, Szluka K, Gabius H-J, André S. 2005. Prognostic significance of adhesion/growth-regulatory lectins in lung cancer. Oncology 69:167–174.

Takkunem M, Grenman R, Hukkanen M, Korhonen M, Garcia de Herreros A, Virtanen I. 2006. Snail-dependent and -independent epithelial-mesenchymal transition in oral squamous carcinoma cells. Journal of Histochemistry & Cytochemistry 54:1263–1275.

Thiery J-P, Sleeman JP. 2006. Complex networks orchestrate epithelial-mesenchymal transitions. Nature Reviews Molecular Cell Biology 7:131–142.

Troy T-C, Turksen K. 2005. Commitment of embryonic stem cells to an epidermal cell fate and differentiation. Developmental Dynamics 232:293–300.

Tsai RYL, McKay RDG. 2002. A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Development 16:2991–3003.

Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E. 2004. Defining the epithelial stem cell niche in skin. Science 303:359–363.

van den Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. 2002. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. Journal of Clinical Microbiology 40:779–787.

Villalobo A, Nogales-González A, Gabius H-J. 2006. A guide to signaling pathways connecting protein-glycan interaction with the emerging versatile effector functionality of mammalian lectins. Trends in Glycoscience and Glycotechnology 18:1–37.

Watt FM, Hoggan BL. 2000. Out of Eden: Stem cells and their niches. Science 287:1427–1430.

Weber F, Shen L, Fukino K, Patocs A, Mutter GL, Caldes T, Eng C. 2006. Total-genome analysis of BRCA1/2-related invasive carcinomas of the breast identifies tumor stroma as potential landscaper for neoplastic initiation. American Journal of Human Genetics 78:961–972.

Wu AM, Singh T, Wu JH, Lensch M, André S, Gabius H-J. 2006. Interaction profile of galectin-5 with free saccharides and mammalian glycoproteins: Probing its fine specificity and the effect of naturally clustered ligand presentation. Glycobiology 16:524–537.

ONCOLOGY REPORTS 00: 0-00, 0000

Human hair follicle and interfollicular keratinocyte reactivity to mouse HPV16-transformed cells: An *in vitro* study

KAREL SMETANA Jr[1,2], BARBORA DVORÁNKOVÁ[1,2], LUKÁS LACINA[1,2,3], ZDENEK CADA[1,2,4] and VLADIMÍR VONKA[5]

[1]Charles University in Prague, First Faculty of Medicine, Institute of Anatomy; [2]Charles University in Prague, Second Faculty of Medicine, Center of Cell Therapy and Tissue Repair; [3]Charles University in Prague, First Faculty of Medicine, Department of Dermatovenereology; [4]Charles University in Prague, First Faculty of Medicine, Department of Otorhinolaryngology and Head and Neck Surgery; [5]Institute of Hematology and Blood Transfusion, Prague, Czech Republic

Abstract. The role of stem cells in cancer formation and spreading has been established. As with normal tissue, the cancer stem cells need a special microenvironment to support their growth. This microenvironment may be represented by the tumor stroma. One of the possible ways of tumor stromal formation is the epithelial-mesenchymal transition of tumor epithelium. Following this mechanism, stromal cells must share the basic genetic alterations with the tumor cells. In an attempt to create a system capable of testing some aspects of the mesenchymal cell-keratinocyte interactions, we studied the effects of the fibroblastoid mouse TC-1 cells that were prepared by the introduction of human papillomavirus type 16 (HPV16) genes *E6* and *E7* to lung epithelial cells on the phenotype of normal human interfollicular and hair follicle keratinocytes. From this point of view, they may resemble stromal cells formed by the epithelial-mesenchymal transition of cells from HPV-induced squamous cell carcinoma. In contrast to 3T3 murine embryonic fibroblasts which were used as control cells, TC-1 cells influenced not only the size of the keratinocytes and the shape of their colonies, but also induced the expression of keratins 8 and 19 and vimentin. In conclusion, TC-1 cells exhibited a marked biological activity by influencing the behavior of the normal human follicular and intefollicular keratinocytes. This observation is compatible with the hypothesis that stromal cells play an important role in tumor progression and spreading.

Correspondence to: Professor Karel Smetana Jr. Charles University, First Faculty of Medicine, Institute of Anatomy, U nemocnice 3, 128 00 Prague 2, Czech Republic
E-mail: karel.smetana@lf1.cuni.cz

Key words: keratinocyte, cancer stem cell, tumor stroma, keratin 8, keratin 19, vimentin

Introduction

The volume of data indicating a role of genetically altered adult tissue stem cells in cancer formation (including basal and squamous cell carcinoma) is rapidly increasing (1-8). According to our previous studies, very poorly differentiated malignant keratinocytes are present, with some phenotypical markers of epidermal stem cells on the periphery of the squamous cell carcinoma node/nodule where the cells with features of differentiated elements are located in the centre of the tumor (9,10). The majority of epithelial cancer cell lines, which are those that can form tumors after grafting to immunocomprimed mice, exhibit markers of stem cells that gradually disappear during the prolonged cultivation (11,12). Normal embryonic stem cells introduced to the adult organism are highly tumorigenic as observed for more than 30 years (13). Since these malignant cells are able to form normal tissue in mice after their introduction to mouse blastocysts, it can be concluded that these cells are genetically normal and their malignant conversion is influenced by the specific microenvironment of adult tissue that is non-physiological for embryonic cells. It is widely accepted that the maintenance of stemness in adult tissue stem cells is controlled by the specific microenvironment, known as the niche (14,15). Although the stem cell niche in some adult stem cells, such as the epidermal ones has been quite well characterized at the molecular level (16), niche modeling in tissue culture conditions and a prolonged expansion of stem cells has not been successful thus far.

When we accept the hypothesis on the existence of cancer stem cells (as mentioned above), the special micro-environment or niche for propagation of the cancer stem will also be necessary for their function and it can be provided by the cells of cancer stroma (17). The impact of stromal cells on the tumor elements was observed predominantly in breast (18), prostate gland (19,20) and colon cancer (21). The fibro-blasts isolated from basal or squamous cell carcinoma are able to influence significantly the phenotype of cocultured normal interfollicular keratinocytes to be similar to the phenotype of malignant epithelial tumor cells from which the

stromal cells were prepared (22,23). Although the molecular mechanism of the stromal cell action to cancer cells is not clear, the role of specific growth factors/cytokines can be expected because the direct contact between stromal and epithelial cells is not necessary (22,23).

The nature of tumor-associated fibroblasts is not clear as yet, but in principle they can arise from three different mechanisms or their combination: i) the cytokine/growth factors produced by the malignant cells can influence the local fibroblast population that will consequently support the growth of cancer cells (21); ii) the cancer cells can fuse with the local fibroblasts and form polyploid stromal elements (24) and iii) the direct formation of stromal fibroblasts by the epithelial-mesenchymal transition of malignant epithelium to fibroblastoid stromal cells (25).

Epidermal stem cells are located in the bulge region of the outer root sheath of a hair follicle and also in the basal layer of the interfollicular epidermis (26). In this study we cultured the normal human hair follicle and interfollicular keratinocytes with TC-1 cells. TC-1 cells were isolated after the co-transfection of mouse (C57/B6) lung epithelial cells with *E6* and *E7* genes of human papillomavirus type 16 (HPV16) and by an activated *H-ras* oncogene to maintain their malignant properties (27). These cells were employed as they acquire a fibroblast-like morphology that includes the disappearance of keratins and are highly oncogenic for syngeneic animals. When the stromal cells originate in cancer epithelium, they will carry the same genetic alterations as the epithelial tumor cells. TC-1 cells that were originally epithelial exhibit the properties of fibroblast and they have the same basic genetic alterations as the putative original cancer epithelium where the *E6* and *E7* genes are present and expressed (28). Therefore, we employed these cells as a model of stromal cells formed from tumor epithelium because they are the fibroblasts containing gene sequences typical for HPV16-transformed squamous epithelial cells from which they originated. The phenotype of the two cell types, i.e. normal hair follicle (NHF) and normal interfollicular epidermal (NIF) keratinocytes, cultured under the influence of TC-1 cells was monitored and compared with the phenotype of the same cells cultured on a mouse 3T3 cell standard feeder (29). These cells are also of mouse origin and are known to support the growth and physiological differentiation pattern in the normal human keratinocytes under *in vitro* conditions (22,23).

Materials and methods

Preparation of normal hair follicle and interfollicular keratinocytes. Skin samples were obtained from the Department of Aesthetic Surgery of the Third Faculty of Medicine of the Charles University in Prague according to the criteria of the Helsinki Declaration. Informed consent of patients was obtained and the study was approved by the local ethics committee. Skin samples from the breast of 2 female donors were employed. NHF and NIF keratinocytes were prepared and subcultured as described previously (30).

Cell lines. TC-1 cells kindly provided by Dr T.C. Wu (Johns Hopkins University, Baltimore) were prepared by the transformation of C57BL/6 primary mouse lung epithelial cells by HPV16 *E6/E7* oncogenes and the activated *H-ras* oncogene (27). They were maintained as previously described (31). In the present experiments cells from the 3rd passage, derived from a large frozen stock, were used. 3T3 as mouse embryonic fibroblasts (32) were propagated in H-MEM (Hanks' salts modified Eagle's medium, SevaPharma, Prague, Czech Republic) with 10% bovine serum (ZVOS, Hustopece, Czech Republic) at 37°C and 3.3% CO_2.

Coculture of keratinocytes with TC-1 and 3T3 cells. The two types of keratinocytes were cocultured with TC-1 for 6 or 11 days, respectively. The proliferation activity of the feeder cells was stopped using mitomycin C (Sigma-Aldrich, Prague, Czech Republic) at a concentration of 25 $\mu g/ml^{-1}$ for 3 h prior to cocultivation. Feeder cells were seeded on cover glasses at a density of 25,000 cells/cm^2 and cultured for 24 h. Then the suspension of freshly prepared NHF and NIF keratinocytes (20,000 cells/cm^2) was added. They were allowed to adhere for 15 min on the surface of preseeded cover glasses and all non-adherent cells were washed out. The cells were cultivated in the keratinocyte medium at 37°C and 3.3% CO_2 (22). Keratinocytes cocultured with 3T3 fibroblasts under the same conditions were used as a control. The growth and size of the quickly adhered keratinocytes were compared with the exposed cells.

To distinguish between the effect of direct contact of the keratinocytes with experimental TC-1 fibroblasts and the role of the medium conditioned by their products, we evaluated the phenotype of keratinocytes separated from the fibroblasts during their coculture by a microporous membrane (Transwell Inserts, Corning, Acton, USA) as described (22).

Immunocytochemistry. The procedure of multiple labeling at a single cell level was employed (33). Cells were briefly fixed with paraformaldehyde, washed in PBS and permeabilized by Triton X-100 (Sigma-Aldrich). All antibodies were diluted as recommended by the supplier. A panel of keratins was visualized using the mouse monoclonal antibody LP34, by anti-high molecular weight keratins (both from Dako, Brno, Czech Republic) and by rabbit polyclonal antibody (Abcam, Cambridge, UK). It should be recalled that keratin type 8 is physiologically not present in squamous cell epithelia, but its expression in the cells of squamous cell carcinoma is a marker of poor clinical prognosis for the respective patient (34,35). Keratin 19 was also detected by the mouse monoclonal antibody (Dako) as it is expressed in epidermal stem cells (36). Vimentin which is normally not present in epithelial cells, can be used in colocalization with keratins as a marker of epithelial-mesenchymal transition (25). This process is important for tumor spreading in organisms (37). Such colocalization was studied in cultured cells using the mouse monoclonal antibody (Dako). Nucleostemin, a protein important for the control of stemness and proliferation, was detected by the goat polyclonal antibody (Neuromics, Bloomington, MN, USA). Although nucleostemin is not an exclusive marker of epidermal stem cells, it is characteristic of a distinct population of keratinocytes cultivated *in vitro* (22,23,38). The well-known marker of proliferating cells, Ki67, was detected by the mouse monoclonal antibody (Dako).

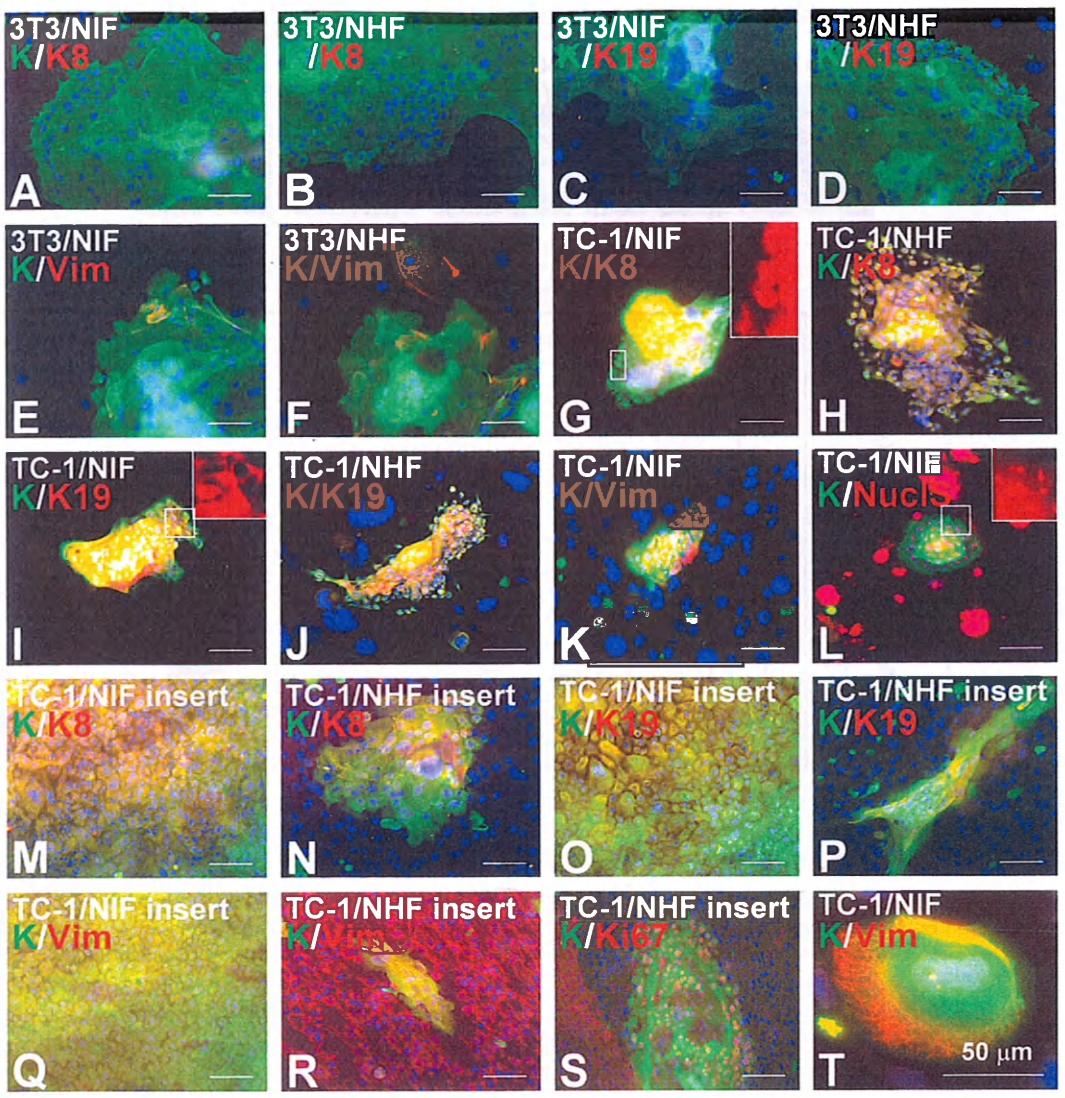
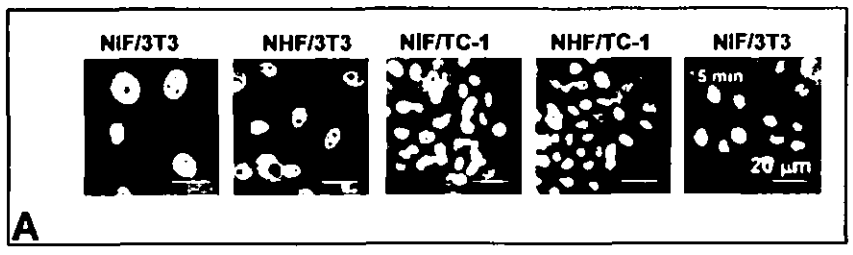


Figure 1. Detection of the panel of keratins (green signal: A-T), of keratin 8 (red signal: A, B, G, H, M and N), of keratin 19 (red signal: C, D, I, J, O and P), of vimentin (red signal: E, F, K, Q, R and T), of nucleostemin (red signal: L) and of Ki67 (red signal: S) in normal interfollicular keratinocytes (NIF: A, C, E, G, I, K, L, M, O, Q and T) and in normal hair follicle keratinocytes (NHF: B, D, F, H, J, N, P, R and S) cultured in the presence of 3T3 (A-F) and TC-1 cells (G-T), respectively. The inset shows details of K8 (G), K19 (I) and nucleostemin (L) expression in the red canal only. The yellow color indicates the co-localization of signals of similar intensity. The nuclei were stained with DAPI and the scale is 50 μm.

The specificity of immunohistochemical reaction was tested by omission of the first step antibodies or by their replacement with antibodies against thyreoglobulin which do not normally occur in the studied cells. In the case of monoclonals, an antibody of the same isotype was used as a control. FITC-labeled swine anti-mouse immunoglobulins (AlSeVa, Prague, Czech Republic), FITC-labeled swine anti-rabbit immunoglobulins (AlSeVa), TRITC-labeled goat anti-mouse immunoglobulins (Sigma-Aldrich) and TRITC-labeled donkey anti-goat immunoglobulins (Jackson Laboratories, West Grove, PA, USA), respectively, were used as the second step antibody. Nuclear DNA was counterstained with DAPI (4',6'-diamidino-2-phenylindole dilactate, Sigma-Aldrich). Specimens were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined by fluorescence microscopy using a Nikon Eclipse 90i microscope (Nikon, Prague, Czech Republic) equipped with filterblocks specific for FITC, TRITC and DAPI, respectively, a high resolution CCD camera Cool-1300Q (Vosskühler, Osnabrick, Germany) and a Lucia 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic). This equipment was also used for measuring the mean area of keratinocyte nuclei. The results were evaluated using Student's unpaired t-test.

Results

The influence of TC-1 cells on NHF and NIF keratinocyte growth and cell size. The two cell types, i.e. NHF and NIF keratinocytes formed typical flat colonies when they had been cocultured with 3T3 feeder cells (Fig. 1A-F). In contrast, NHF and NIF keratinocytes cultured on TC-1 feeder cells formed very small and dome-shaped colonies with papilloma-like morphology (Fig. 1G-L). As expected, the



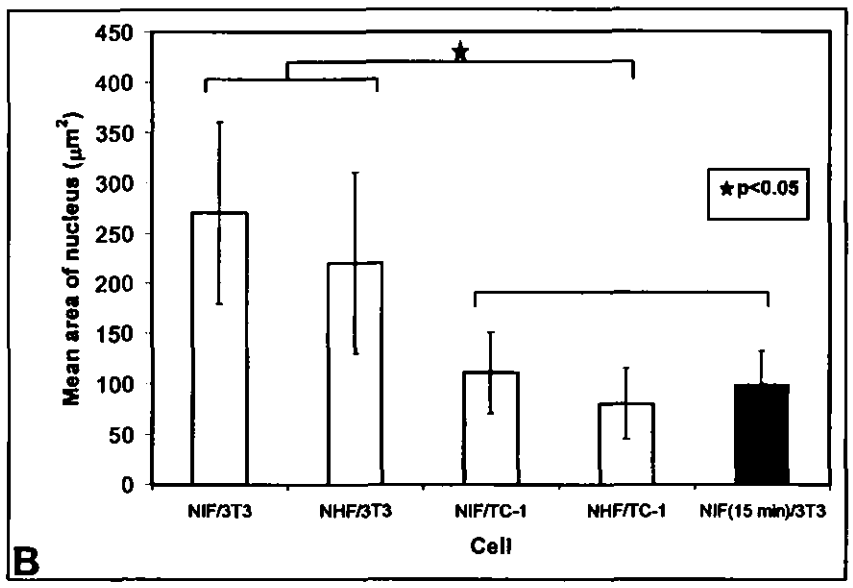


Figure 2. The comparison of size of DAPI-positive nuclei of normal interfollicular (NIF) and normal hair follicle keratinocytes (NHF) cultured in the presence of 3T3 fibroblasts and TC-1 cells. Interfollicular keratinocytes that adhered only for 15 min are indicated as '15 min'. Subsequently, non-attached cells were removed and only those which adhered were cultured (A). The graph (B) compares the mean area of keratinocyte nuclei cultured as described above. The scale is 20 μm and the statistical significance was estimated using unpaired Student's t-test at a significance level of $p<0.05$.

colonies became enlarged during the cultivation period from days 6 to 11 (data not shown). While NIF keratinocytes formed large colonies when they were separated from TC-1 cells by a microporous membrane, only small colonies were observed if NHF keratinocytes were cultured under the same conditions (Fig. 1M-S). A possible role of the mitomycin C pretreatment of TC-1 in direct coculture experiments on keratinocyte growth was tested in an experiment in which the two cell populations were separated by a microporous membrane. No significant differences were observed, indicating a negligible influence of this procedure on the behavior of keratinocytes. Notably, NHF and NIF keratinocytes cultured in the presence of TC-1 were very small (Figs. 1 and 2). Since the cell borders were not distinguishable in all cells, we measured the areas of DAPI-positive nuclei. This reactivity reflects the size of the cells (Fig. 2). When the keratinocytes cocultured with TC-1 cells and the rapidly adhering NIF keratinocytes were compared, the nuclear areas were quite similar (Fig. 2).

Effects of TC-1 on NHF and NIF keratinocyte phenotype. In comparison with cultures in which 3T3 feeder cells were used, we observed an increased number of dead cells with an unspecific positive signal for the studied markers and with no signal for the DAPI staining of DNA when the TC-1 feeder was employed. The phenotype of NHF and NIF keratinocytes cultured in the presence of TC-1 cells was greatly different from those maintained in the presence of 3T3 cells (Table I and Fig. 1A-F). In principle, no qualitative differences between the sensitivity of NHF and NIF to TC-1 were found concerning the studied phenotype (Fig. 1G-J). Nearly all keratinocytes in the presence of TC-1 exhibited a signal for the keratins 8 and 19 (Fig. 1G-J) and for vimentin (Fig. 1K). They contained

Table I. Phenotype of keratinocytes cultured under the influence of 3T3 and TC-1 as a direct coculture.

	3T3		TC-1	
Marker	NHF	NIF	NHF	NIF
K	++	++	++	++
K8	-	-	++	++
K19	-	-	++	++
VIM	-	-	++	++
NuclS	+	-	++	++
Ki67	+	+	+	+

-, no positive cells; +, 25-50% of positive cells and ++, >50% of positive cells.

nucleostemin-positive nucleoli (Fig. 1L) that were also present in the nuclei of NHF cultured under the influence of 3T3, but not in the nuclei of NIF cocultured with 3T3 fibroblasts. A marked portion of keratinocytes was actively proliferating as estimated by the expression of Ki67 in the two types of keratinocytes cultured with 3T3 and TC-1 (data not shown).

The separation of cell populations (i.e. TC-1 fibroblasts and keratinocytes) by a microporous membrane had no effect on the high expression of the two studied keratin types (8 and 19), vimentin and Ki67 (Fig. 1M-S).

A small proportion of cells (<5%) in the coculture of TC-1 and keratinocytes was large (>100 μm in diameter) with the large nuclei exhibiting keratins and vimentin (Fig. 1T).

Discussion

This study demonstrated the influence of TC-1 cells (HPV16-transformed mouse lung epithelial cells acquiring the fibroblast properties) on two types of keratinocytes (NHF and NIF). At variance with the control 3T3 cells, TC-1 cells induced a formation of highly abnormal papilloma-like colonies. The size of NHF and NIF cells was significantly reduced under the TC-1 cell influence. It remained similar to the size of quickly adhering keratinocytes. This observation appears to be important, as stem cells, including epidermal ones are known to be very small (39-42).

Concerning the phenotype of normal human hair follicle and interfollicular keratinocytes, TC-1 cells but not the control 3T3 cells induced a high level expression of keratins 8 and 19, vimentin and nucleostemin. These phenotype changes indicated the important effect of TC-1 on the differentiation of normal keratinocytes, because keratin 19 has not been observed in postnatal interfollicular epidermis (36,43). Its presence in keratinocytes of the bulge region of the hair follicle appears to be restricted to the stem epidermal stem cell (36). Under pathological conditions this has been observed in some basal cell carcinomas (40). Keratin 8 which is induced by TC-1 is not expressed by the cells of squamous epithelia in the postnatal period under physiological conditions but its expression in the squamous cell carcinoma has been recognized as a marker of poor clinical prognosis of patients (44). The two keratins are not expressed in human keratinocytes during the prolonged cultivation *in vitro* except for the keratinocytes cocultured with stromal cells from basal or squamous cell carcinomas exhibiting the presence of keratins 8 or 19 (22,23). The coexpression of keratins and vimentin in keratinocytes cocultured with TC-1 cells can be interpreted as evidence of epithelial-mesenchymal transition (25,45) of the normal keratinocytes. However, additional evidence substantiating this conclusion are needed. This phenomenon can be important for the spreading of the tumor in the organism (25). The results described above were observed even if the two cell populations had been separated by a microporous membrane, thereby indicating the involvement of some soluble bioactive factors produced by TC-1 cells.

These results are in agreement with the concepts which assume that the mesenchymal cells play a leading role in the control of the development of epidermis including appendages such as hair or teeth (46,47). Even postnatally, fibroblasts significantly influence the expression of specific keratins in epidermal cells (48). The strong influence of TC-1 cells on normal keratinocytes, as demonstrated in the present experiments, is compatible with the concept that tumor stroma play a fundamental role in the biology of cancer arising from squamous epithelia. The presented results indicate that fibroblastoid cells formed by mesenchymal transition from cancer epithelium can strongly influence the properties of epithelial cells by paracrine fashion. Since the present data were obtained using human keratinocytes and mouse-transformed fibroblastoid cells, the putative factors involved in the events reported are not species-specific.

In conclusion, the model described in this study can help us to understand the biology of squamous cell carcinomas induced by HPV infection, such as some head and neck cancers and all or nearly all carcinomas of the uterine cervix (28,49). The fibroblastoid but originally epithelial cells expressing the E6/E7 proteins were highly active as is demonstrated by their influence on keratinocyte differentiation and epithelial-mesenchymal transition, a phenomenon supporting tumor growth and spreading in the organism.

Acknowledgements

This study was supported by the Ministry of Education Youth and Sport of the Czech Republic, project No. MSM0021620806 and 1M0021620803 as well as by the Internal Grant Agency of the Ministry of Health, project No. NR 9049-3 and NR9075-3. The authors would like to thank Eva Vancová, Iva Burdová and Vít Hajdúch for their technical assistance.

References

1. Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. Nature 414: 105-111, 2001.
2. Sell S: Stem cell origin of cancer and differentiation therapy. Crit Rev Oncol Hematol 51: 1-28, 2004.
3. Morris RJ: A perspective on keratinocyte stem cells as targets for skin carcinogenesis. Differentiation 72: 381-386, 2004.
4. Burkert J, Wright NA and Alison MR: Stem cells and cancer: an intimate relationship. J Pathol 209: 287-297, 2006.
5. Costea DE, Tsinkalovsky O, Vintermyr OK, Johannessen AC and Mackenzie IC: Cancer stem cells - new and potentially important targets for the therapy of oral squamous cell carcinoma. Oral Dis 12: 443-454, 2006.
6. Finlan LE and Hupp TR: Epidermal stem cells and cancer stem cells: insights into cancer and potential therapeutic strategies. Eur J Cancer 42: 1283-1292, 2006.
7. Janes SM and Watt FM: New roles for integrins in squamous cell carcinoma. Nature Rev Cancer 6: 175-183, 2006.
8. Motlík J, Klíma J, Dvoránková B and Smetana K Jr: Porcine epidermal stem cells as a biomedical model for wound healing and normal/malignant epithelial cell propagation. Theriogenology 67: 105-111, 2007.
9. Plzák J, Betka J, Smetana K Jr, Chovanec M, Kaltner H, André S, Kodet R and Gabius H-J: Galectin-3: an emerging prognostic indicator in advanced head and neck carcinoma. Eur J Cancer 40: 2324-2330, 2004.
10. Chovanec M, Smetana K Jr, Betka J, Plzák J, Brabec J, Moya-Álvarez V, André S, Kodet R and Gabius H-J: Correlation of expression of nuclear proteins pKi67 and p63 with lectin histochemical features in head and neck squamous cell cancer. Int J Oncol 27: 409-415, 2005.
11. Mackenzie IC: Stem cell properties and epithelial malignancies. Eur J Cancer 42: 1204-1212, 2006.
12. Smetana K Jr, Dvoránková B Chovanec M, Boucek J, Klíma J, Motlík J, Lensch M, Kaltner H, André S and Gabius H-J: Nuclear presence of adhesion-/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin. Histochem Cell Biol 125: 171-182, 2006.

13. Mintz B and Illmensee K: Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc Natl Acad Sci USA 72: 3585-3589, 1975.
14. Watt FM and Hogan BL: Out of Eden: stem cells and their niches. Science 287: 1427-1430, 2000.
15. Li Z and Li L: Understanding hematopoietic stem-cell microenvironments. Trends Biochem Sci 31: 589-595, 2006.
16. Fuchs E, Tumbar T and Guasch G: Socializing with the neighbors: review stem cells and their niche. Cell 116: 769-778, 2004.
17. Li L and Neaves WB: Normal stem cells and cancer stem cells: the niche matters. Cancer Res 66: 4554-4557, 2006.
18. Bissell MJ and LaBarge MA: Context, tissue plasticity, and cancer: Are tumor stem cells also regulated by the microenvironment? Cancer Cell 7: 17-23, 2005.
19. Cunha GR, Hayward SW, Wang YZ and Rickey WA: Role of the stroma microenvironment in carcinogenesis of the prostate. Int J Cancer 107: 1-10, 2003.
20. Condon MS: The role of the stromal microenvironment in prostate cancer. Semin Cancer Biol 15: 132-137, 2005.
21. Nakagawa H, Liyanarchchi S, Davuluri RD, Auer H, Martin EW Jr, de la Chapelle A and Frankel WL: Role of cancer-associated stroma fibroblasts in metastatic colon cancer to the liver and their expression profiles. Oncogene 23: 7366-7377, 2004.
22. Lacina L, Smetana K Jr, Dvoránková B, Pytlík R, Kideryová L, Kucerová L, Plzáková Z, Stork J, Gabius H-J and André S: Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. Br J Dermatol 156: 819-829, 2007.
23. Lacina L, Dvoránkova B, Smetana K Jr, Chovanec M, Plzák J, Tachezy R, Kideryová L, Kucerová L, Cada Z, Boucek J, Kodet R, André S and Gabius H-J: Marker profiling of normal keratinocytes identifies the stroma from squamous cell carcinoma of the oral cavity as a modulatory microenvironment in co-culture. Int Radiation Biol 83: 837-848, 2007.
24. Jacobsen BM, Chuck Harrell JC, Jedlicka P, Borgis VF, Varella-Garcia M and Horwitz KB: Spontaneous fusion with, and transformation of mouse stroma by, malignant human breast cancer epithelium. Cancer Res 66: 8274-8279, 2006.
25. Petersen OW, Nielsen HL, Gudjonsson T, Villadsen R, Rank F, Niebuhr E, Bissell MJ and Rønnov-Jessen L: Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. Am J Pathol 162: 391-402, 2003.
26. Watt FM, Celso CL and Silva-Vargas V: Epidermal stem cells: an update. Current Opinion Genet Develop 16: 518-524, 2006.
27. Lin K-Y, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM and Wu TC: Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 56: 21-26, 1996.
28. Doorbar J: Molecular biology of human papilloma virus infection and cervical cancer. Clin Sci 110: 525-541, 2006.
29. Rheinwald JG and Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6: 331-343, 1975.
30. Purkrábková T, Smetana K Jr, Dvoránková B, Holíková Z, Böck C, Lensch M, André S, Pytlík R, Liu F-T, Klíma J, Smetana K, Motlík J and Gabius H-J: New aspects of galectin functionality in nuclei of cultured bone marrow stromal and epidermal cells: biotinylated galectins as tool to detect specific binding sites. Biol Cell 95: 535-545, 2003.
31. Smahel M, Síma P, Ludvíková V, Marinov I, Pokorná D and Vonka V: Immunisation with modified HPV16 E7 genes against mouse oncogenic TC-1 cell sublines with downregulated expression of MHC class I molecules. Vaccine 21: 1125-1136, 2003.
32. Todaro GJ and Green H: Quantitative studies of the growth mouse embryo cells in culture and their development into established lines. J Cell Biol 17: 299-313, 1963.
33. Fronková V, Holíková Z, Liu F-T, Homolka J, Rijken DC, André S, Bovin NV, Smetana K Jr and Gabius H-J: Simultaneous detection of endogenous lectins and their binding capability at the single cell-level-a technical note. Folia Biol 45: 157-162, 1999.
34. Carrilho C, Alberto M, Buane L and David L: Keratins 8, 10, 13, and 17 are useful markers in the diagnosis of human cervix carcinomas. Hum Pathol 35: 546-551, 2004.
35. Fillies T, Werkmeister R, Packeisen J, Brandt B, Morin P, Weingart D, Joos U and Berger H: Cytokeratin 8/18 expression indicates a poor prognosis in squamous cell carcinomas of the oral cavity. BMC Cancer 6: 10-18, 2006.
36. Michel M, Török N, Godbout M-J, Lussier M, Gaudreau P, Royal A and Germain L: Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially located in function of anatomic sites, and their number varies with donor age and culture stage. J Cell Sci 109: 1017-1028, 1996.
37. Thiery JP and Sleeman JP: Complex networks orchestrace epithelial-mesenchymal transitions. Nature Rev Mol Cell Biol 7: 131-142, 2006.
38. Lacina L, Smetana K Jr, Dvoránková B, Stork J, Plzáková Z and Gabius H-J: Immunocyto- and histochemical profiling of nucleostemin expression: marker of epidermal stem cells? J Dermatol Sci 44: 73-80, 2006.
39. Barrandon J and Green H: Cell size as a determinant of the clone-forming ability of human keratinocytes. Proc Natl Acad Sci USA 82: 5390-5394, 1985.
40. Dvoránková B, Smetana K Jr, Chovanec M, Lacina L, Stork J, Plzáková Z, Galovicová M and Gabius H-J: Transient expression of keratin K19 is induced in originally negative interfollicular epidermal cells by adhesion of suspended cells. Int J Mol Med 16: 525-531, 2005.
41. Kucia M, Reca R, Campbell FR, Zuba-Surma R, Majka M, Ratajczak J and Ratajczak MZ: A population of very small embryonic-like (VSEL) CXCR4+ SSEA-1+ OCT-4+ stem cells identified in adult bone marrow. Leukemia 20: 857-869, 2006.
42. Klíma J, Motlík J, Gabius H-J and Smetana K Jr: Phenotypic characterization of porcine interfollicular keratinocytes separated by elutriation: a technical note. Folia Biol 53: 33-36, 2007.
43. Commo S, Gaillard O and Bernard BA: The human hair follicle contains two distinct K19 positive compartments in outer root sheath: a unifying hypothesis for stem cell reservoir? Differentiation 66: 157-164, 2000.
44. Gires O, Münz M, Schaffrik M, Kleu C, Rauch J, Ahlemann M, Eberle D, Mack B, Wolleneberg B, Lang S, Hofmann T, Hammerschmidt W and Zeidler R: Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics-based technology. Cell Mol Life Sci 61: 1198-1207, 2004.
45. Kong W, Li S, Liu C, Bari AS, Longaker M and Lorenz HP: Epithelial-mesenchymal transition occurs after epidermal development in mouse skin. Exp Cell Res 312: 3959-3968, 2006.
46. Peterková R, Peterka M, Viriot L and Lesot R: Dentition development and budding morphogenesis. J Craniofac Genet Dev Biol 20: 158-172, 2000.
47. Millaire SE: Molecular mechanisms regulating hair follicle development. J Invest Dermatol 118: 216-225, 2002.
48. Yamaguchi Y, Itami S, Tarutani M, Hosokawa K, Miura H and Yoshikawa K: Regulation of keratin 9 in nonpalmoplantar keratinocytes by palmoplantar fibroblasts through epithelial-mesenchymal interactions. J Invest Dermatol 112: 483-488, 1999.
49. Kreimer AR, Clifford GM, Boyle P and Franceschi S: Human papilloma virus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev 14: 467-475, 2005.

PŘEHLEDOVÝ ČLÁNEK

Galektiny v dlaždicových karcinomech hlavy a krku

Čada Z.[1,2], Plzák J.[1,2,3], Chovanec M.[1,2,3], Dvořánková B.[1,3], Lacina L.[1,3,4], Szabó P.[1], Smetana K., Jr.[1,3], Betka J.[2]

[1]Karlova Univerzita, 1. lékařská fakulta, Anatomický ústav, Praha
[2]Karlova Univerzita, 1. lékařská fakulta, Klinika otorinolaryngologie a chirurgie hlavy a krku FN Motol, Praha
[3]Karlova Univerzita, 2. lékařská fakulta, Centrum buněčné terapie a tkáňových náhrad, Praha
[4]Karlova Univerzita, 1. lékařská fakulta, Dermatovenerologická klinika VFN, Praha

SOUHRN

Nádory hlavy a krku tvoří kolem 5 % všech nádorů. Osmdesát až devadesát procent těchto nádorů je představováno dlaždicovými karcinomy. I přes rozvoj chirurgických poznatků a metod včetně onkologických léčebných režimů, je terapie těchto tumorů svízelná a pětileté přežití u pokročilých nádorů je stále velmi nízké. Řešením situace je hledání nových znaků (markerů), které by lépe charakterizovaly tyto nádory a napomohly tak při upřesnění léčebné strategie. Jedním z těchto znaků by mohly být endogenní lektiny zvané galektiny a jejich ligandy. V patologii dlaždicových karcinomů hlavy a krku se nejvíce uplatňují galektin-1, -3 a -7.

Klíčová slova: dlaždicové karcinomy hlavy a krku, lektiny, galektiny, prognostické znaky.

SUMMARY

Čada Z., Plzák J., Chovanec M. et al.: Galectins in Squamous Cell Carcinomas of the Head and Neck Cancers

Cancers of head and neck represents about 5% of all tumors. 80 to 90% of these tumors are constituted of squamous cell carcinomas. Despite a rapid progress in diagnostics and therapy the overall 5-year survival of this type of cancer is among the lowest of the major cancer types. This unfavourable situation needs the extensive research to found new markers to better characterize biological behavior of tumors as a rational background for more sophisticated therapeutic modalities. Among the most promising markers are endogenous lectins called galectins and their ligands. Especially galectin-1, -3 and -7 play a key role in pathology of squamous cell carcinomas.

Key words: squamous cell carcinomas, lectins, galectins, prognostic markers. *Ča.*

Čas. Lék. čes., 2008, 147, pp. 559–563.

DLAŽDICOVÉ KARCINOMY HLAVY A KRKU

Dlaždicové karcinomy hlavy a krku představují kolem 5 % všech tumorů. Naprostou většinu z nich (90 %) tvoří dlaždicové karcinomy vycházející ze sliznic horních cest dýchacích a polykacích. Z klinického hlediska se dělí především dle lokalizace na karcinomy dutiny ústní, orofaryngu, epifaryngu, hypofaryngu, dutiny nosní, hrtanu a slinných žláz. Jedním z nejvíce rizikových faktorů pro vznik těchto nádorů je kouření. Více než 80 % nádorů hlavy a krku je spojeno s expozicí tabákovému kouři (1). Mezi další rizikové faktory dále patří především alkohol, lidský papiloma virus (HPV, sérotypy 2, 6, 11, 16 a další), virus Epsteina a Barrové (EBV), dietní faktory (nedostatek ß-karotenů, vitaminu A), faryngolaryngeální reflux, genetická predispozice (genetický polymorfismus genů enzymů, jež se podílejí na neutralizaci kancerogenů, např. CYP1A1, GSTM1 a další) (1), vlivy zevního prostředí (azbest, chrom, dřevný prach, prach v kožedělném průmyslu). Dlaždicové karcinomy hlavy a krku se nejčastěji vyskytující v orofaryngu a laryngu a jsou charakterizovány lokálním agresivním chováním a časným metastazováním do regionálních uzlin. Systémové metastázy jsou především v plicích a játrech. Terapie je chirurgická, onkologická nebo kombinace obou modalit. Cílem terapie je zajistit radikální odstranění nádoru a dosažení uspokojivé kvality života (2).

prof. MUDr. Karel Smetana, DrSc.
Anatomický ústav 1. LF UK
U Nemocnice 3, 128 00 Praha 2
fax: +420 224 965 770, e-mail: karel.smetana@lf1.cuni.cz
prof. MUDr Jan Betka, DrSc.
Klinika otorinolaryngologie a chirurgie hlavy a krku 1. LF UK a FNM
V Úvalu 84, 150 06 Praha 5
fax +420 224 434 319, e-mail jan.betka@lfmotol.cuni.cz

Navzdory diagnostickým i terapeutickým pokrokům zůstává stále prognóza pacientů s karcinomy hlavy a krku vážná. Při léčbě je nutné zachování dostatečné radikality a zároveň ochrana pacientů před zbytečně agresivními postupy, které zhoršují funkční výsledky (3).

Proto je nutné hledat nějaké prognostické znaky-markery, které by lépe charakterizovaly tyto nádory. Molekulami, které by se mohly stát nadějnými prognostickými znaky, jsou členové rodiny endogenních živočišných lektinů-galektiny a jejich ligandy (4).

LEKTINY

Lektiny jsou proteiny, které nemají charakter enzymů či protilátek a jsou schopné specificky rozpoznat sacharidové struktury (5). S lektiny se setkáváme u všech živých organismů od virů po živočichy. Nejdůležitější strukturní součástí molekuly každého lektinu je **doména rozpoznávající sacharidy** (Carbohydrate Recognition Domain – CRD). Naším zájmem byly a jsou **živočišné (endogenní) lektiny**, které dělíme na základě strukturního uspořádání na pět tříd (tab. 1) (4, 6), a to především galektiny.

GALEKTINY

Galektiny patří mezi endogenní lektiny dříve nazývané S-lektiny, které jsou charakterizovány specifickou CRD a afinitou k β-galaktosidům. Nacházejí se především v extracelulární matrix, v buněčném jádru, cytoplazmě a buněčné membráně. Doposud bylo popsáno minimálně 14 zástupců rodiny galektinů. Dle struktury se dají rozdělit do 3 skupin (tab. 2).

Galektiny se uplatňují v široké škále biologických dějů, kde se podílejí na regulaci proliferace, diferenciace, apoptózy a modulaci mezibuněčné interakce a interakce s extracelulární matrix, a to jak v normě, tak i za patologických stavů. V kancerogenezi se uplatňují především galektin-1, -3, -7 (7, 8).

Galektin-1

Galektin-1 (molekulární hmotnosti 14,5 kDa) se vyskytuje v mnoha tkáních (kostní, svalová, srdeční, placenta, lymfatická). Jeho funkce lze shrnout do následujících bodů: 1. buněčná adheze a mezibuněčné interakce; 2. imunomodulace, zánětlivé procesy; 3. regulace buněčného růstu; 4. apoptóza; 5. sestřih pre-mRNA.

Galektin-1 vykazuje jak pozitivní, tak negativní efekt na buněčnou adhezi. Příkladem takového antagonistického chování je zesílení adheze u buněk melanomové linie, buněk čichového epitelu či rabdomyosarkomu ve srovnání s normálními myoblasty, kde adhezi inhibuje (9). Galektin-1 je popisován jako významně proapoptotický lektin, který má zřejmě důležitou úlohu při selekci a vyzrávání T-lymfocytů. Je zvýšeně exprimován v imunologicky privilegovaných orgánech, jako je placenta a oko. Pravděpodobně se uplatňuje jako protektivní faktor autoimunitních chorob právě pro

Tab. 1. Klasifikace živočišných lektinů (připraveno z dat získaných zejména z Gabius, 1997 a Smetana a André, 2008)

Rodina	Charakteristika	Sacharidové ligandy
C-lektiny	konzervativní CRD, pro vazbu se sacharidem potřebují divalentní kationty jako kofaktor	různé (manóza, galaktóza, fukóza, heparinový tetrasacharid)
I-lektiny	vykazují strukturální homologii s imunoglobuliny	různé (hyaluronová kyselina, α2,3/α2,6-sialyllaktóza, manóza-6N-acetylglukóza), β-galaktosidy
galektiny (S-lektiny)	konzervativní CRD, postrádají transmembránové hydrofobní úseky,pro vazbu nepotřebují divalentní kationty	
pentraxiny	pentamerové uspořádání	4,6 cyklický acetal, β-galaktózy, galaktóza, sulfonylované a fosforylované monosacharidy
P-lektiny	konzervativní CRD	manóza-6-fosfát

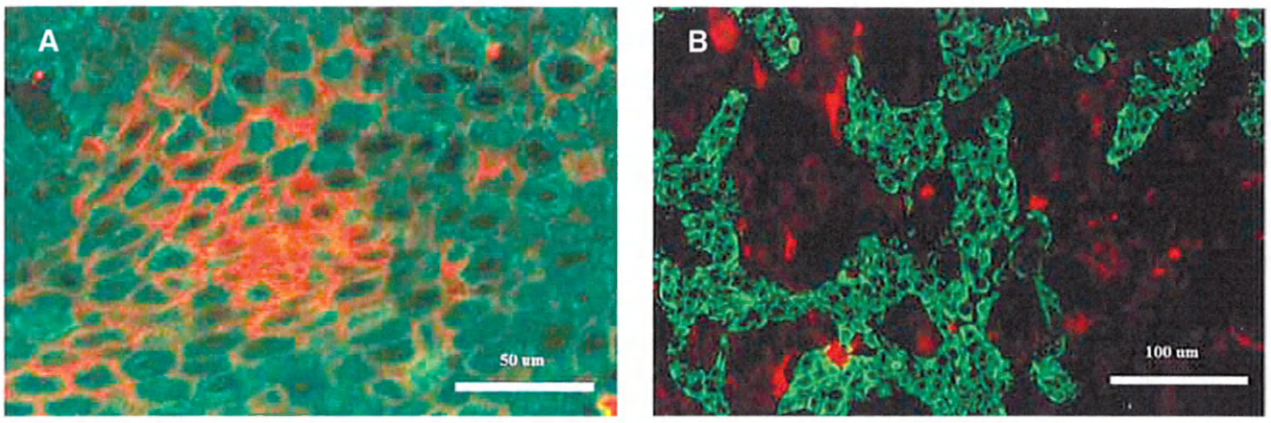
CRD – karbohydráty rozpoznávající doména (carbohydrate recognition domain)

Tab. 2. Klasifikace galektinů dle struktury (dle různých autorů)

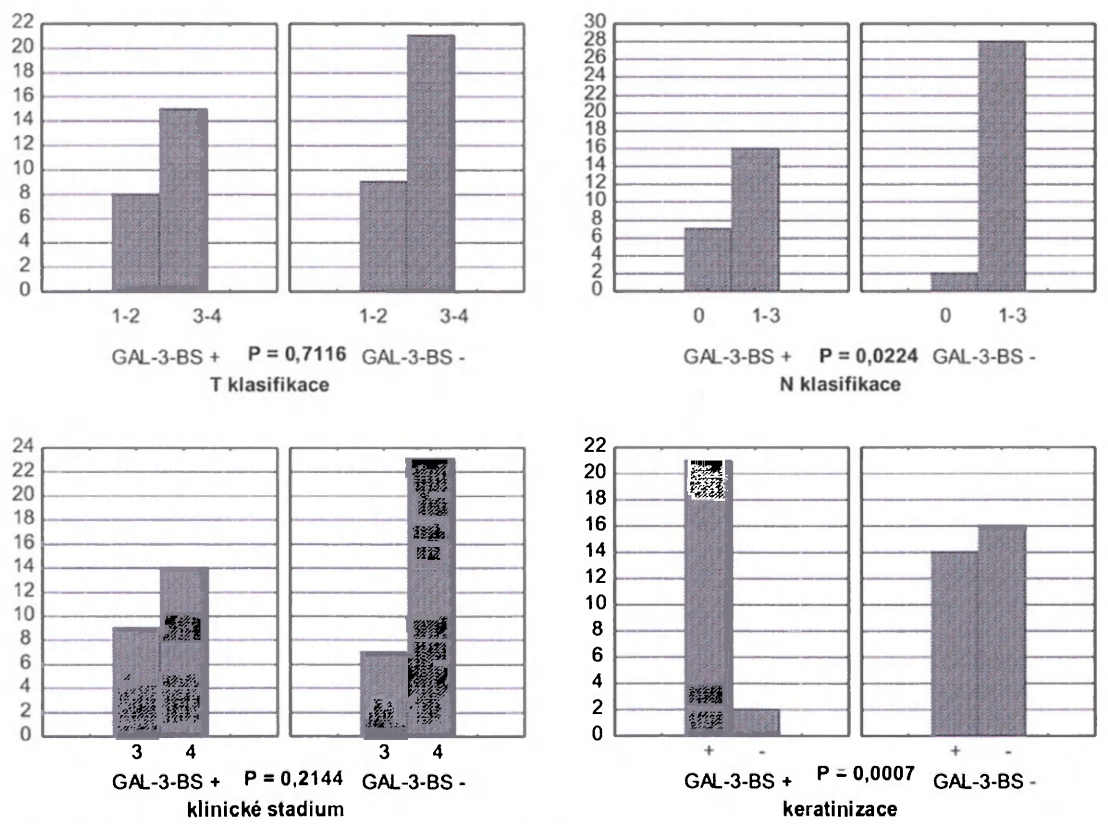
Typ galektinu	Schéma struktury	Zástupci
Prototyp – nekovalentní homodimery, obě části mají stejnou CRD se specificitou *pro stejný oligosacharid*	OO	galektin-1, -2, -5, -7, -10, -11, -13, -14
Chimera typ – obsahuje CRD na C konci aminokyselinového řetězce, N konec oligosacharid neváže	O–	galektin-3
Tandem-repeat typ – obsahuje dvě kovalentně vázané CRD s různou specifitou	O–O	galektin-4 -6, -8, -9, -12

O – karbohydráty rozpoznávající doména (CRD)

jeho indukční vlastnosti apoptózy u aktivovaných autoagresivních klonů T-lymfocytů (10). Protichůdné je působení galektinu-1 na buněčnou proliferaci. Jeho exprese stimuluje proliferaci endotelových buněk (11). Rovněž přidání nízkých dávek exogenního lektinu jejich proliferaci stimuluje. Naopak vysoká ji inhibuje (12). Galektin-1 je asociován s ribonukleproteiny buněčného jádra (RNP), které jsou součástí sestřihových komplexů a podílejí se na vzniku definitivní podoby mRNA (13). Karcinomy hlavy a krku vykazují heterogenní expresi galektinu-1 (14). Exprese galektinu-1 je v literatuře popisována u karcinomů s výrazně maligním fenotypem (vysoce atypická exprese diferenciačních znaků, například přítomnost keratinu-8) a zvýšenou tendencí k metastazování především v karcinomech jazyka. V karcinomech laryngu a hypofaryngu se galektin-1 vyskytuje heterogenně, kdy je jeho výskyt ovlivněn hypoxií v nádoru. Tyto nálezy u nádorů je možno dát do souvislosti s výskytem galektinu-1 u kmenových buněk dlaždicových epitelů, neboť se zdá, že se kmenové buňky podílejí na vzniku nádorů vycházejících z dlaždicových epitelů (15). Vysoký výskyt



Obr. 1. Dobře diferencovaný dlaždicový karcinom tonzily
Silná vazba galektinu-3 na povrchu buněk v centrální oblasti nádoru (A); regionální lymfatická metastáza dlaždicového karcinomu tonzily. Metastatické buňky obsahují cytokeratiny, ale nevážou galcktin-3 (B). Dvojité fluorescenční značení: LP-34+ cytokeratiny zeleně vazba galektinu-3 červeně.



Obr. 2. Gal-3-BS-: Absence vazebných míst pro galektin-3 na nádorových buňkách (z angl. překladu galectin-3 binding sites);
Gal-3-BS+: Přítomnost vazebných míst pro galektin-3 na nádorových buňkách

galektinu-1 ve stromatu dlaždicových karcinomů hlavy a krku je charakteristický (16) a může se podílet na indukci apoptózy lymfocytů infiltrujících oblast nádoru (viz výše).

Galektin-3

Galektin-3 se podobně jako galektin-1 vyskytuje v buňkách (jádro/cytoplazma) a v mezibuněčné hmotě. Podílí se rovněž se na adhezi buněk i interceluláních interakcích, regulaci dělení a apoptózy a sestřihu pre-mRNA. V dlaždicových epitelech je typická jeho přítomnost v suprabazálních vrstvách. Kromě toho je přítomen v makrofázích a Langerhansových buňkách (17). Je exprimován v karcinomech prostaty a štítné žlázy. Exprese galektinu-3 má proproliferační a antiapoptotický účinek (7, 18). Karcinomy hlavy a krku vykazují rozdílný výskyt galektinu-3 v závislosti na oblasti, z níž tumor pochází. Rovněž subcelulární lokalizace galektinu (jádro/cytoplazma/membrána) může přinést cenné informace o biologickém chování nádoru a prognóze. Příkladem jsou práce, které poukazují na větší počet recidiv u karcinomů jazyka se současným zvýšením exprese galektinu-3 v cytoplazmě a snížením v jádru (19, 20). Podobný význam má i průkaz vazebných míst pro galektin-3, která se nacházejí zejména v mezibuněčných kontaktech buněk dobře diferencovaných dlaždicových karcinomů (obr. 1A), naopak snížená vazba galektinu-3 na buněčnou membránu je typická pro méně diferencované karcinomy a metastázy do uzlin (obr. 1B). S těmito výsledky je v souladu pozorování, které ukazuje zvýšenou vazbu galektinu-3 v korelaci s keratinizací tumorů a metastazováním do lymfatických (obr. 2). Tyto nálezy se odrazily v lepším přežívání pacientů s vysokou expresí vazebných míst pro galektin-3 (21).

Galektin-7

Galektin-7 představuje endogenní lektin prototypního typu exprimovaný ve všech vrstvách dlaždicového epitelu. Za fyziologických podmínek se uplatňuje v procesech regulace proliferace, apoptózy a stratifikace dlaždicových epitelů. Předpokládá se, že hraje důležitou roli v embryonálním vývoji vrstevnatých epitelů (22). Tyto výsledky naznačují, že galektin-7 by mohl být dobrým markerem normální stratifikace dlaždicových epitelů. Velice zřídka je detekován v bazocelulárních karcinomech (23). Zvýšená exprese mRNA byla zaznamenána u linie keratinocytů po expozici UVB záření a po aplikaci prodiferenciačních činidel (24). Je proto popisován jako p53 inducibilní gen 1 a jeho podíl na spuštění apoptózy, zejména u buněk s poškozenou DNA je zřejmý. Exprese tohoto lektinu v dlaždicových karcinomech je popisována s rozdílnými výsledky a prognostickými výhledy pro pacienta (25, 26).

ZÁVĚR

Hledání nových prognostických znaků karcinomů hlavy a krku by mohlo přispět k lepší charakterizaci těchto zhoubných tumorů a k přípravě terapie „na míru" pro konkrétního pacient. Galektiny představují zajímavé proteiny, které by mohly rozšířit spektrum znaků pro jejich detailní biologickou charakterizaci (24, 27). Zejména reaktivita diferencovaných nádorových buněk pro značený exogenní galektin-3 se zdá být z hlediska stanovení další perspektivy pacienta velmi slibná (21). Pro širší zavedení průkazu galektinů a jejich ligandů do klinické praxe je však nezbytný jejich další podrobný výzkum.

Zkratky

CRD – karbohydráty rozpoznávající doména (carbohydrate recognition domain)
EBV – virus Epsteina a Barrové
HPV – lidský papiloma virus
RNP – ribonukleproteiny buněčného jádra

LITERATURA

1. **Myers, E. N., Suen, J. Y., Myers, J. N., Hanna, E. Y. N.:** Cancer of the head and neck. Philadelphia, Pennsylvania, Saunders, 2003, s. 5–28.
2. **Boring, C. C., Squires, T.S., Ton, T.:** Cancer statistics, 1992. CA Cancer J. Clin., 1992, 42, s. 19–38.
3. **Ogawa, T., Tsurusako, Y., Kimura, N. et al.:** Comparison of tumor markers in patients with squamous cell carcinoma of the head and neck. Acta Otolaryngol (Stockh), 1999, 540 (Suppl.), s. 72–76.
4. **Smetana, K., Jr., André, S.:** Mammalian lectin as tool in glycochemistry and histochemistry with relevance for diagniostic procedure. In: McMahn, R. J. (Ed.) Avidin-Biotin Interactions, Methods and Applications. Humana Press, Totowa, NJ, USA, 2008, s. 171–185.
5. **Kocourek, J., Hořejší, V.:** Defining a lectin. Nature, 1981, 290, s. 188.
6. **Gabius, H.-J.:** Animal lectins. Eur J Biochem., 1997, 243, s. 543–576.
7. **Plzák, J., Smetana, K. Jr., Hrdličková, E. et al.:** Expression of galectin-3-reactive glycoligands in squamous cell cancer and normal epithelial cells as a marker of differentiation. Int. J. Oncol., 2001, 19, s. 59–64.
8. **Lohr M., Lensch M., Andre S. et al.:** Murine Homodimeric Adhesion/Growth-Regulatory Galectins-1, -2 and -7: Comparative Profiling of Gene/Promoter Sequences by Database Mining, of Expression by RT-PCR/Immunohistochemistry and of Contact Sites for Carbohydrate Ligands by Computational Chemistry. Folia Biologica, 2007, 53, s. 109–128.
9. **Cooper, D. N. W., Massa, S. M., Barondes, S. H.:** Endogenous muscle lectin inhibit myoblast adhesion to laminin. J. Cell Biol., 1991, 115, s. 1437–1448.
10. **Perillo, N. L., Uittenbogaart, C. H., Nguyen, J. T. et al.:** Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes. J. Exp. Med., 1997, 185, s. 1851–1858.
11. **Sanford, G. L., Harris-Hooker, S.:** Stimulation of vascular cell proliferation by ß-galactoside-binding lectins. FASEB J., 1990, 4, s. 2912–2918.
12. **Adams, L., Scott, G.K., Weinberg, C.:** Biphasic modulation of cell growth by recombinant human galectin-1. Biochem. Biophys. Acta, 1996, 1312, s. 137–144.
13. **Vyakarman, A., Daggher, S. F., Wang, J. L. et al.:** Evidence for a role for galectin-1 in pre-mRNA splicing. Mol. Cell Biol., 1997, 17, s. 4730–4737.
14. **Plzák, J., Smetana, K. Jr., Chovanec, M., Betka, J.:** Glycobiology of head and neck squamous epithelia and carcinomas. ORL, 2005, 67, s. 61–69.
15. **Motlík, J., Klíma, J., Dvořánková, B., Smetana, K. Jr.:** Porcine epidermal stem cells as a biomedical model for wound

healing and normal/malignant epithelial cell propagation. Theriogenology, 2007, 67, s. 105–111.
16. **Čada, Z., Bouček, J., Dvořánková, B. et al.:** Nucleostemin expression in squamous cell carcinoma of the head and neck. *Anticancer research*, 2007, 27, s. 3279–3284.
17. **Smetana, K. Jr., Holíková, Z., Klubal, R. et al.:** Coexpression of binding sites for A(B) histo-blood group trisaccharides with galectin-3 and Lag antigen in human Langerhans cells. J. Leukocyte Biol., 1999, 66, s. 644–649.
18. **Polyak, K., Xia, Y., Zweier, J. L. et al.:** A model for p53-induced apoptosis. Nature, 1997, 389, s. 300–305.
19. **Honjo, Y., Inohara, H., Akahani, S. et al.:** Expression of cytoplasmic galectin-3 as a prognostic marker in tongue carcinoma. Clin. Cancer Res., 2000, 6, s. 4635–4640.
20. **Piantelli, M., Iacobelli, S., Almadori, G. et al.:** Lack of expression of galectin-3 is associated with a poor outcome in node-negative patients with laryngeal squamous-cell carcinoma. *J. Clin. Oncol.*, 2002, 20, s. 3850–3856,
21. **Plzák, J., Betka, J., Smetana, K. Jr. et al.:** Galectin-3 – an emerging prognostic indicator in advanced head and neck carcinoma, European. Journal of Cancer, 2004, 40, s. 2324–2330.
22. **Magnaldo, T., Fowlis, D., Darmon, M.:** Galectin-7, a marker of all types of stratified epithelia. 1998 63, s. 159–168.
23. **Chovanec M., Smetana K. Jr., Plzák J. et al.:** Detection of new diagnostic markers in pathology by focus on growth-regulatory endogenous lectins. The case study of galectin-7 in squamous epithelia. Prague Med. Rep., 2005, 106, s. 209–216.
24. **Bernerd, F., Sarasin, A., Magnoldo, T.:** Galectin-7 overexpression is associated with the apoptotic process in UVB-induced sunburn keratinocytes. Cell Biology, 1999, 96, s. 11329–11334.
25. **Saussez, S., Cucu, D. R., Decaestecker, C. et al.:** Galectin 7 (p53-induced gene 1): a new prognostic predictor of recurrence and survival in stage IV hypopharyngeal cancer. Ann. Surg. Oncol., 2006, 13, s. 999–1009.
26. **Čada, Z., Chovanec, M., Smetana, K., Jr. et al.:** Growth/malignancy-regulatory galectin-7: will the lectines activity *in vitro* establish clinical correlations in head and neck squamous cell and basal cell carcinomas? Histol. Histopathol., 2008 (in press).
27. **Liu, M., Weynand, B., Delos, M.:** Prognostic factors in squamous cell carcinomas of the head and neck. Acta Oto-rhino-laryngol Belg., 1999, 53, s. 155–160.

Část výsledků shrnutých v tomto přehledném článku byla získána s podporou grantu IGA MZČR NR 9049-3, GA ČR 304/08/P175.

KNIHY

Velemínský, M.:
NAŠE DÍTĚ ŠPATNĚ SPÍ – NESPRÁVNÉ NASTAVENÍ VNITŘNÍCH HODIN

Praha, Triton 2007, 142 s., 1. vydání, vázané, barevné, cena neuvedena.
ISBN 978-80-7254-936-8.

Stížností na poruchu spánku slyší dětský lékař dosti často. I když se týká především starších kojenců a batolat, ani děti v pozdějším věku nejsou této poruchy ušetřeny. Podle literárních údajů se týká asi 50 % kojenců (!). Porucha spánku dítěte se však netýká jen jeho (tomu nespavost často vůbec nevadí), ale unavuje především rodiče. Zvláště matky, které musí vstávat k nespícímu dítěti i několikrát za noc, bývají vyčerpané. A tak dlouhodobá nespavost dítěte ovlivňuje negativně i pohodu rodičů, a může tak narušit klidnou atmosféru domova.

Lékaře–nepediatry možná překvapí, že poruchu nočního spánku dítěte je možné zařadit do skupiny civilizačních chorob.

Referovaná kniha přináší úvahy lékařů, psychologů, osobní zkušenosti i informace literárních zdrojů. Obsah je rozdělen do 17 samostatných kapitol různého rozsahu (8–31 s.) Zahrnují problematiku ve značné šíři. Kapitoly 2–10 jsou věnovány fyziologickým informacím o spánku (*Základy teorie spánku*, Vývoj spánkových vzorců u dětí, Význam přístupu rodičů k dítěti, Základní terminologie spojená se spánkem kojenců, Doba spánku, Usínání, Ranní vstávání, Denní spánek a Místo a způsob spánku). Kapitoly 11–16 se věnují různým patologickým stavům, které mohou spánek provázet (Náměsíčnost, Noční děs, Noční strachy a noční můry, Tlučení hlavou a houpání, Syndrom spánkové apnoe a Bolest). Kapitoly 17 a 18 (Děti s duševní poruchou a Náhlá úmrtí kojenců) odbornou část uzavírají.

Text knihy je psán velmi dobrou češtinou a lehce se čte. Je v něm minimum odborných výrazů – a ty jsou ještě vysvětleny ve Slovníčku na konci knihy. Zvláštností je přenesení (obvykle sdělovaného) textu do formy „otázek rodičů" a „odpovědí lékaře". Tato forma uspořádání je náročná pro autora, ale za to velmi přibližuje text rodičům–laikům.

Komu knihu doporučit? Samozřejmě by se s ní měli seznámit pediatři, i když z ní mohou čerpat cenné informace i rodiče, „jejichž dítě špatně spí".

Jan Petrášek
U Nemocnice 1, 128 08 Praha 2

Histol Histopathol (2009) 24: 000-000

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Galectin-7 with the lectin's activity establish clinical correlations in head and neck squamous cell and basal cell carcinomas?

Z. Čada[1,2], M. Chovanec[1,2,3], K. Smetana Jr[1,3], J. Betka[2], L. Lacina[1,4], J. Plzák[1,2,3], R. Kodet[5], J. Štork[4], M. Lensch[6], H. Kaltner[6], S. André[6] and H-J. Gabius[6]

[1]Charles University in Prague, First Faculty of Medicine, Institute of Anatomy, [2]Charles University in Prague, First Faculty of Medicine and Faculty Hospital Motol, Department of Otorhinolaryngology and Head and Neck Surgery, [3]Center for Cell Therapy and Tissue Repair, Faculty Hospital Motol, [4]Charles University in Prague, First Faculty of Medicine and General Teaching Hospital, Department of Dermatovenerology, [5]Charles University in Prague, Second Faculty of Medicine and Faculty Hospital Motol, Institute of Pathology and Molecular Medicine, Prague, Czech Republic, [6]Ludwig-Maxmillians-University in Munich, Faculty of Veterinary Medicine, Institute for Physiological Chemistry, Munich, Germany

Summary. The human lectin galectin-7 (Gal-7; p53-induced gene-1) has anti- and pro-malignant features in different *in vitro* models. We tried to clarify relation of its expression to cellular and clinical parameters in head and neck squamous and basal cell carcinomas. Using a non-cross-reactive antibody, immunohistochemical staining in squamous cell epithelia (epidermis, epithelium of oropharynx and larynx) (n = 57), squamous cell carcinomas (n = 47) and lymph node metastases (n = 25), as well as basal cell carcinomas (n = 10) were studied. This monitoring was flanked by processing to assess the level of differentiation (cytokeratins 10 and 14), proliferation (Ki67) and basal lamina formation (collagen IV). The results were correlated with clinical and pathological findings (grading, TNM-staging, extracapsular spread, angio- and lymphangioinvasion, perineural invasion, recurrence and survival). Gal-7 resides in all layers of epithelia with cytoplasmic and nuclear localization in normal specimens. Basal cell carcinomas were devoid of the Gal-7 respective signal. Squamous cell carcinomas were positive, presenting different staining profiles. Intense staining was predominantly found in squamous cell cancers with high degrees of differentiation and keratinization. Fittingly, poor level of differentiation (P = 0.0009), absence of keratinization (P = 0.0105) and significant discontinuity or absence of collagen IV expression in the peritumoral basal lamina (P = 0.0024) was found in Gal-7-negative tumors. Gal-7 presence was not related to gender, primary tumor site, T-stage, N-stage, clinical stage, extracapsular spread, angio- and lymphangioinvasion, perineural spread or treatment outcome at a statistically significant level. Immunohistochemical analysis revealed a positive correlation for differentiation and keratinization to Gal-7 presence in squamous cell carcinomas. Absence of Gal-7 expression was detected in basal cell carcinomas. These clinical data delineate Gal-7 influence on differentiation in vivo, without evidence for a role in dissemination reported for lymphoma.

Key words: Carcinoma, Collagen IV, Galectin, Keratinization, Lectin

Offprint requests to: Martin Chovanec, M.D., Ph.D., Charles University, First Faculty of Medicine, Department of Otorhinolaryngology and Head and Neck Surgery, V Úvalu 84, 150 06 Prague 5, Czech republic. e-mail: martin.chovanec@lf1.cuni.cz

Introduction

The malignant process is known to be associated with aberrant glycosylation. Because the emerging concept of the sugar code ascribes a role as biochemical signals to glycan epitopes of glycoconjugates from normal and tumor cells, these changes may not serve just as phenotypic markers. They also convey new properties to the cells which can be decoded by tissue lectins (Gabius, 1997a, 2006). In fact, these glycan-binding proteins are capable of "reading" even rather subtle modifications in glycan structures, such as the presence of core fucosylation or alterations in epitope density, and translate them into responses, affecting e. g. cell adhesion, growth or migration (Villalobo et al., 2006; Wu et al., 2006; André et al., 2007a). Homing especially

in on spatially accessible branch-end ß-galactosides, the members of the galectin family belong to these endogenous effectors (Kasai and Hirabayashi, 1996; Gabius, 1997b; Cooper, 2002). The recent finding that a tumor suppressor modulates in an orchestrated manner the expression of a galectin and glycan tailoring of its ligands for acquisition of susceptibility to anoikis underscores the effectiveness of such interactions in tumor biology (André et al., 2007b). In addition to sensing changes in glycan profiles, these endogenous lectins are known to exert activities also in the cytoplasm and nucleus by virtue of peptide binding, for example, regulating transcriptional activity, transformation or apoptosis (Rotblat et al., 2004; Wang et al., 2004; Smetana et al., 2005). This background explains our interest in galectins and their presence in tumors.

The homodimeric galectin-7 (Gal-7) was initially detected in studies aimed at identifying markers associated with the normal keratinocyte phenotype, its expression was sensitive to SV40 transformation and linked to p53-related induction of apoptosis in epidermis and human DLD-1 colon carcinoma cells (Madsen et al., 1995; Magnaldo et al., 1995; Polyak et al., 1997; Bernerd et al., 1999; Saussez and Kiss, 2006). *In vitro*, the lectin inhibits growth of neuroblastoma cells and induces apoptosis in activated T cells, but, in stark contrast, is associated with an aggressive phenotype in murine 164T2 lymphoma, characterized by increased matrix metalloproteinase-9 expression (Kopitz et al., 2003; Moisan et al., 2003; Sturm et al., 2004; Demers et al., 2007). Proteomic profiling raised evidence for a relation to differentiation in bladder squamous cell carcinomas and, conversely, tumorigenesis in buccal squamous cell cancer (Østergaard et al., 1997; Chen et al., 2004). Differential display of mRNA populations to chemical carcinogenesis was described in rat mammary gland but not colon (Lu et al., 1997). Thus, Gal-7 activities and relations to disease progression in models appear to indicate contextual functionality, making predictions for clinical correlations on the basis of in vitro data difficult. In view of the same aim, the immunohistochemical analysis of the anti-apoptotic Gal-3 in breast cancer has recently revealed that in vitro activities cannot simply be extrapolated to the clinical situation (Moisa et al., 2007). The question is thus open to define associations between lectin expression and clinical parameters in tumor specimens. Toward this end, we analyzed head and neck squamous cell and basal cell carcinomas, using an antibody preparation non-cross-reactive to other members of the galectin family.

Materials and methods

Tissue processing

Samples of human tissue were obtained with the explicit informed consent of patients according to the Helsinki Declaration during surgical procedures for head and neck squamous cell carcinomas (Department of Otorhinolaryngology and Head and Neck Surgery, First Faculty of Medicine and Faculty Hospital Motol, Charles University in Prague) and basal cell carcinomas (Department of Dermatovenerology, First Faculty of Medicine and General Teaching Hospital, Charles University in Prague). Each sample was divided to two parts. First part was routinely embedded to paraffin and used for histopathologic inspection, second was prepared for preparation of frozen sections. These samples (Table 1) were frozen in liquid nitrogen using Tissue-Tek (Sakura-Finetek Europe B.V., Zoeterwoude, The Netherlands). 7-µm-thick frozen sections were prepared by a Cryocut-E microtome (Reichert-Jung, Vienna, Austria). Tissue-Tek was removed by rinsing in phosphate-buffered saline (pH 7.2; PBS) immediately before starting immunohistochemical processing. Sections were routinely fixed with 2% (w/v) paraformaldehyde in PBS. Carbohydrate-free bovine serum albumin (BSA; Sigma, Prague, Czech Republic) was used to block non-specific protein-protein interactions.

Sections from paraffin embedded tissue were routinely analyzed after the staining by hematoxylin and eosin. Parameters such as differentiation grading, extracapsular spread, angio- and lymphangioinvasion, perineural invasion were evaluated as described (Bryne et al., 1989; Ravasz et al., 1993).

Immunohistochemical processing

After recombinant production Gal-7 was purified using affinity chromatography as a crucial step, and purity was ascertained by one- and two-dimensional gel electrophoresis, gel filtration and mass spectrometry (Kopitz et al., 2003; André et al., 2004). The quality-controlled protein was used as antigen in rabbits, and the resulting polyclonal antibodies were thoroughly checked for any cross-reactivity against other members of the galectin family by Western blotting and ELISA, especially the proto-type proteins, then removing any traces by chromatographic affinity depletion (Kayser et al., 2003; Lohr et al., 2007). Double labeling using commercial antibodies was performed to characterize

Table 1. Number of tissue samples.

Tissue	Number of donors
Human epidermis	10
Basal cell carcinomas	10
Squamous cell epithelia (oral cavity, oropharynx, larynx, hypopharynx)	47
Primary squamous cell cancer (oral cavity, oropharynx, larynx, hypopharynx)	47
Lymph node metastases of squamous cell carcinomas (oral cavity, oropharynx, larynx, hypopharynx)	25

cell characteristics. Cytokeratins 10 and 14 were detected by mouse monoclonal antibodies (Dako, Brno, Czech Republic; SIGMA, Prague, Czech Republic), as were the nuclear Ki67 antigen of proliferating cells (Dako, Prague, Czech Republic) and collagen IV (Sigma, Prague, Czech Republic). Commercially available ExtrAvidin-tetramethylrhodamine isothiocyanate (TRITC) (Sigma, Prague, Czech Republic) and fluorescin isothiocyanate (FITC)-labeled swine-anti mouse and swine-anti rabit immunoglobulins (SwAM-FITC, SwAR-FITC, ALSEVA, Prague, Czech Republic; GoAM-TRITC: Sigma, Prague, Czech Republic) were used as second-step reagents. To exclude a false-positive reaction by non-specific binding of immunoglobulins via Fc receptors, an antibody specific for CD1a (Immunotech, Prague, Czech Republic) not present on epithelial cells was tested in parallel. This reagent replaced the first-step markers during routine processing in a control section. Finally, the specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA, USA.) and then visually inspected and analyzed using an Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with respective filter blocks, a high-resolution CCD camera (Cool-1300Q; Vossküller, Osnabrück, Germany) and a computer-assisted image analyser (LUCIA 5.10) (Laboratory Imaging, Prague, Czech Republic). All sections were carefully examined by two independent observers, who were completely blinded with respect to clinical features of the patients. In each case, at least 500 cells within randomly selected and defined area sections on each slide were counted. For statistical analysis, cut-off points were chosen to classify tumors to be intensely or faintly positive for Gal-7 staining. A cut-off point of lower 1/3 of the intensity profile value (arbitrary units) between intensity of the background signal and the intensity profile value in corresponding non-malignant control epithelia within the tumor cell population was arbitrarily set to determine the range of faintly positive cells. Cut-offs were defined prior to relating clinical parameters to results of histochemical staining. Ki67-positive cells were counted per 1000 cells, an in this way percentages of Ki67-positive cells in different samples were determined. Mean average of Ki67-positive cells for tumors with identical Gal-7 cytoplasmatic staining profiles were calculated.

Statistical analysis

The Chi-squared test was used to set Gal-7 parameters in relation to the different clinicopathological parameters, except for the Ki67 status treated with the Mann-Whitney U test. Overall survival and disease-free survival were calculated using the standard method, data sets being analyzed by using the Gehan-generalised Wilcoxon test. Statistica 6.0 software (StatSoft, Prague, Czech Republic) was run in all statistical analyses. Overall survival was computed from the date of surgery to the documented date of the last follow-up or death, whereas disease-free survival was considered to cover the period from the date of surgery to the date of recurrence.

Results

Normal epithelia in situ

Application of the non-cross-reactive anti-Gal-7 antibody preparation to fixed sections of squamous cell epithelia of the epidermis and mucosal coverings (oral cavity, oropharynx, larynx and hypopharynx) detected lectin presence from the basal region to the most superficial layer (Fig. 1). Cytoplasmic and also nuclear presence, the latter most prominently in nucleoli, were seen in both basal and suprabasal layers. These observations extend the evidence for nuclear presence of galectins from proto-type Gal-1 and chimera-type Gal-3 (Smetana et al., 2005) and, most recently, Gal-2 (Dvořánková et al., 2008). As a measure of cell differentiation, cytokeratin-14 was present in cells of the basal layer in epithelia of all specimens, wheras cytokeratin-10 was encountered in keratinized epithelia of epidermis and tongue only. The typical nuclear expression of the proliferation marker Ki67 was observed in the basal layer and to a restricted extent in the surrounding suprabasal layers. A subpopulation of Ki67-positive cells represented about 5% of the cells in the basal layer. As assessed by monitoring collagen IV presence, the basal lamina was well established and continuous in the studied epithelia.

Basal cell carcinomas

As also shown in Fig. 1, a qualitative difference was seen for negative tumor cells compared to the positive surrounding non-transformed epithelium. Cytokeratin-10 was not detected in studied tumors, and the presence of nuclear Ki67 antigen was observed in about 5-15% of cells, predominantly in peripheral parts of tumor nodules. Continuous collagen IV staining appeared around the studied tumors (not shown).

Squamous cell carcinomas

The staining profile for Gal-7 was not uniform in the different specimens of primary and metastatic squamous cell carcinomas. Four different patterns could be discerned (Fig. 1). Intense staining with homogeneous distribution in all tumor cells (intense and homogeneous) was present in 32% of primary tumors and 12% of regional lymph node metastases. Gal-7 presence confined to the central parts of the tumor, mostly to regions of formation of keratin pearls (intense heterogeneous pattern), was observed in 25.5% of primary tumors and 32% of regional lymph node metastases. Faint but homogeneous staining throughout the entire tumor cell population applied to 25.5% of the primary tumors and 20% of the regional lymph node

metastases. 17% of primary tumors and 36% of regional lymph node metastases did not show immunohistochemical positivity. A clear-cut difference between primary tumors and corresponding lymph node metastases could not reliably be described. Nuclear positivity of tumor cells concerned only cases with intense staining, irrespective of presenting homogeneous or heterogeneous profiles. Regarding the markers for cytodifferentiation, cytokeratin-14 was spotted in all primary carcinomas and regional lymph node metastases, cytokeratin-10 in keratinized carcinomas only (Fig. 2). Gal-7 presence correlated with keratinization (P = 0.0105). Ki67-expressing cells were in the peripheral regions of tumor nodules. Studied tumors differed among each other in the size of the Ki67-positive cell population from 5 to 60% (Fig. 2, Table 2). There was no apparent correlation of the proliferation status with Gal-7 presence (P = 0.1376). In general, peritumoral basement membranes were covered with a continuous layer of collagen IV in the tumors with intense and homogeneous staining for Gal-7. The other Gal-7 staining profiles showed variability for the appearance of the collagen IV layer, ranging from major defects to even complete absence in tumors lacking Gal-7 (P 0.0024) (Fig. 2, Table 2). In contrast, intense and homogeneous Gal-7 staining correlated with the level of differentiation (grading) (P = 0.0009). When Gal-7 staining was set in relation with other factors, i.e. gender (P = 0.3781), primary tumor site (P = 0.2703), T-stage (P = 0.6222), N-stage (P = 0.1065), clinical stage (P = 0.5127), extracapsular spread (P = 0.5998), angioinvasion (P = 0.6443) and lymphangioinvasion (P = 0.3781), perineural spread (P = 0.1306) and treatment outcome, no statistically significant association turned

Table 2. Comparison of clinical and histopathological parameters with Gal-7 presence.

PRIMARY CARCINOMAS		INTENSE SIGNAL 27	FAINT/NO SIGNAL 20	P value
Site	larynx/hypopharynx	13	7	0.2703
	oropharynx/oral cavity	14	13	
Gender	male	24	16	0.3781
	female	3	4	
T-stage	T 1+2	8	9	0.6222
	T 3+4	19	11	
N-stage	N 0	5	5	0.1065
	N 1-3	22	15	
Clinical stage	CS 1+2	1	1	0.5127
	CS 3+4	26	19	
Grading	G1+G2	22	8	0.0009
	G3+G4	5	12	
Keratinization	keratinized	15	4	0.0105
	non-keratinized	12	16	
Extracapsular spread (ECS)	ECS -	21	17	0.5998
	ECS +	6	3	
Lymphangioinvasion	lymphangioinvasion -	22	18	0.3781
	lymphangioinvasion +	5	2	
Angioinvasion	angioinvasion -	22	16	0.6443
	angioinvasion +	5	4	
Perineural spread	perin. spr. -	26	18	0.1306
	perin. spr. +	1	2	
Outcome	local recidive	2	2	
	regional recidive	3	2	
	distant metastases	3	2	
	no evidence of disease	20	16	
		7	4	
Basal lamina (Col IV) formation	well surrounded	19	4	0.0024
	poorly formed	8	16	
Ki 67-positive population	mean	30%	38%	0.1376

Clinical and histopathological parameters (TNM staging, extracapsular spread, grading, keratinization, angio- and lymphangioinvasion, perineural spread), pattern of basal lamina (collagen IV) formation and proliferation (Ki-67) vs. Gal-7 presence in primary head and neck squamous cell carcinomas. Intense signal corresponds to tumors with either intense homogeneous or intense inhomogeneous Gal-7-dependent staining. Faint/No signal corresponds to tumors with either weak homogeneous staining or no detectable Gal-7. Mean average of Ki-67-positive cells counted per 1000 tumor cells for tumors with identical staining pattern were calculated.

up (Table 2). Also, no statistically significant differences of survival among the studied patient groups suffering from squamous cell carcinoma were observed relative to Gal-7 (Fig. 3).

Discussion

Using stratification, a prognostic correlation has been reported for stage IV hypopharyngeal squamous cell carcinoma patients. In this group, Gal-7 therefore has the potential to identify patients at risk of recurrence and with dismal prognosis (Saussez et al., 2006). Of note, staining characteristics for Gal-7 differed from those of Gal-1 belonging to the same subgroup, a strong argument for non-overlapping functionalities in the galectin network (Saussez et al., 2008). Also, associated with a feature of tumor progression, Gal-7 presence was correlated to muscle-infiltrating growth in urothelial cancer (Langbein et al., 2007), whereas Gal-7 monitoring in progression of thyroid cancer appeared to reflect a dual role, with anti- and promalignant features at different stages (Rorive et al., 2002). These results, revealing a tumor-type- and also stage-of-tumorigenesis-related activity profile in the case of Gal-7, have a bearing on considerations to devise new treatment modalities based on modulating endogenous galectin expression.

Absence of the signal for Gal-7 in basal cell carcinoma was also observed earlier (Magnaldo et al., 1998; Chovanec et al., 2005). Explanation of this phenomenon is only hypothetical but it can be related to the low level of differentiation of tumor epithelial cells. They express keratin 19, marker typical for epidermal stem cells and α2,6-linked sialic acid, marker of poorly differentiated epithelial cells (Holíková et al., 2002; Dvofiánková et al., 2005). In harmony with these observations, cells of basal cell carcinoma were never recognized by labeled galectin-3, feature typical for suprabasal cells of squamous epithelia (Plzák et al., 2001).

Following its description as a marker associated with the normal keratinocyte phenotype and as p53-induced

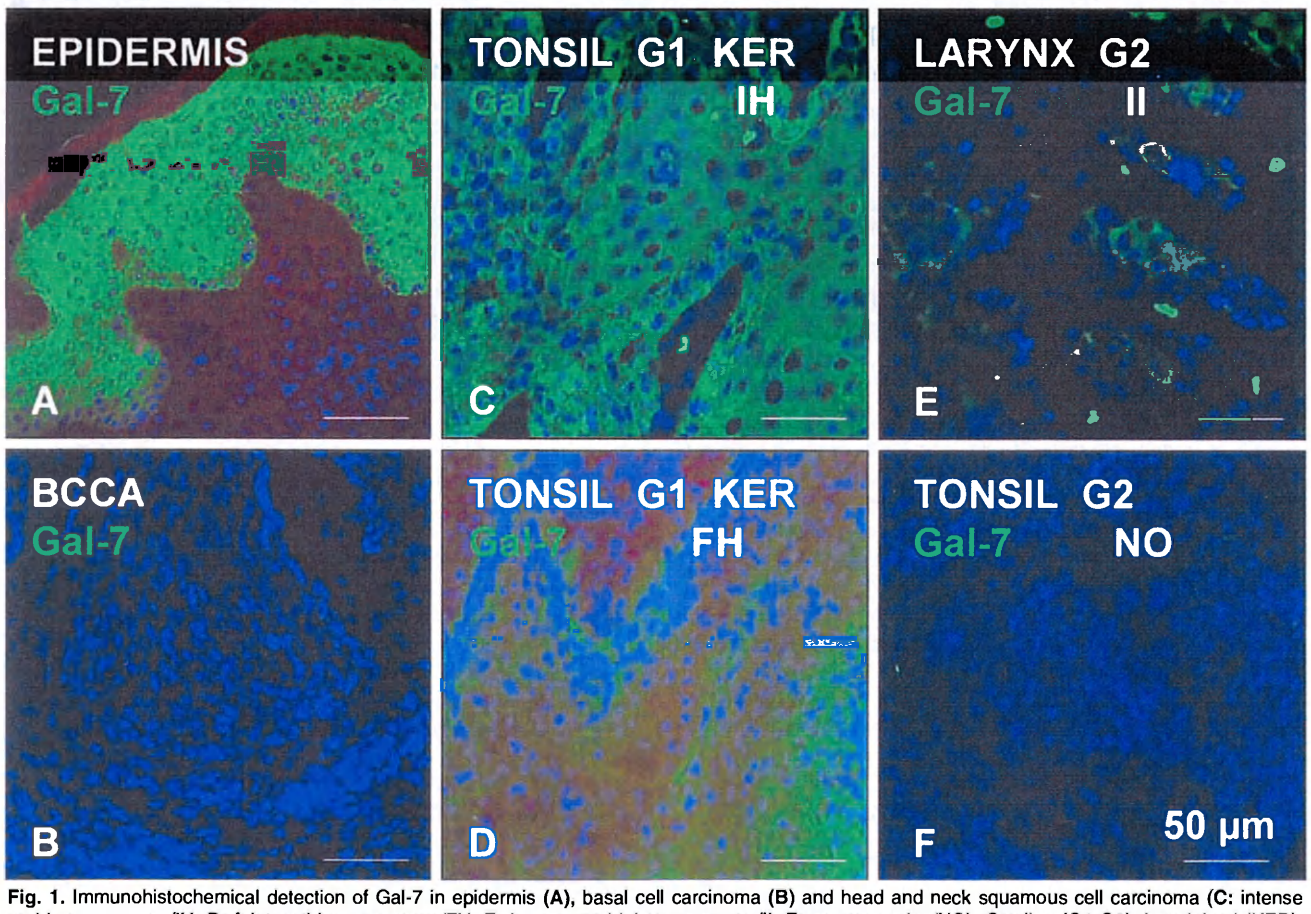


Fig. 1. Immunohistochemical detection of Gal-7 in epidermis **(A)**, basal cell carcinoma **(B)** and head and neck squamous cell carcinoma (**C:** intense and homogeneous/IH, **D:** faint and homogeneous/FH, **E:** intense and inhomogeneous/II, **F:** no expression/NO). Grading (G1-G3), keratinized (KER). Nuclei were counterstained by DAPI.

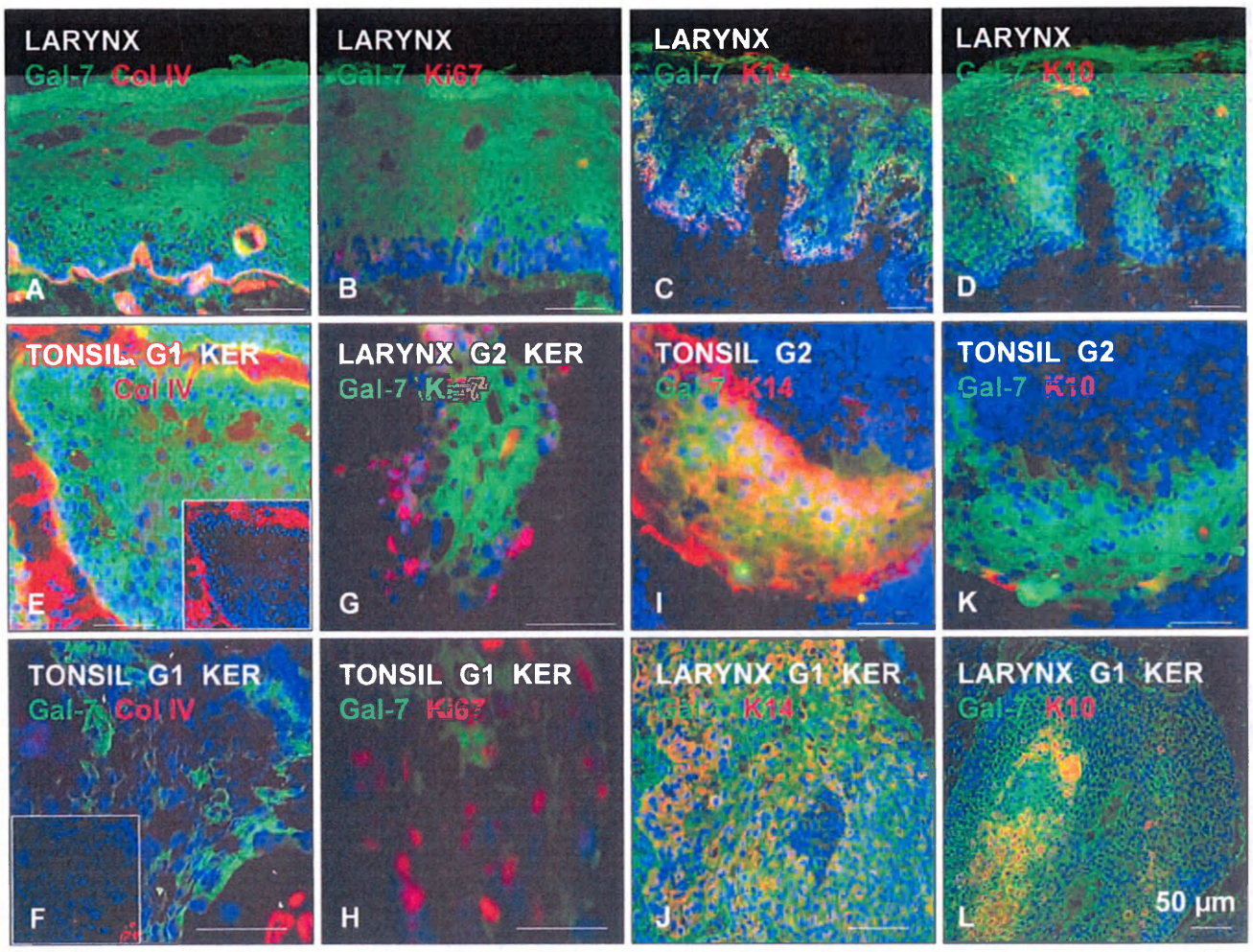


Fig. 2. Immunohistochemcial detection of Gal-7, a marker of proliferation (Ki67), collagen IV (basement membrane) and keratins (K10, K14) in squamous cell epithelia (**A-D**) and head and neck squamous cell carcinomas (**E- L**). Grading (G1-G3), keratinized (KER). Nuclei were counterstained by DAPI.

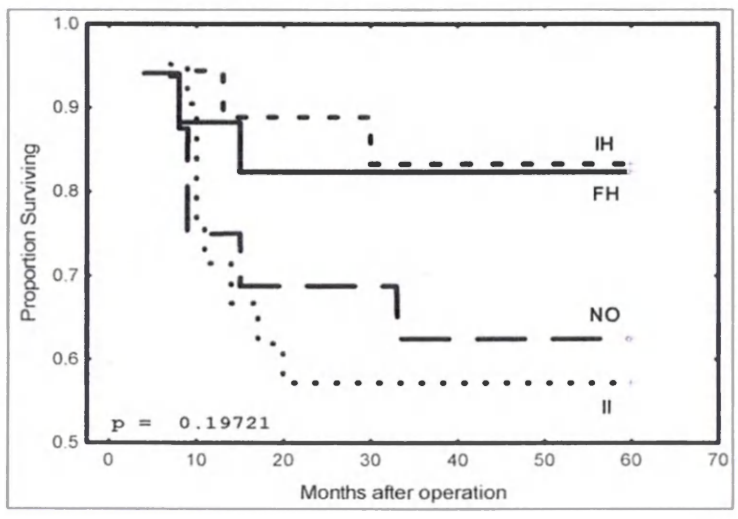


Fig. 3. Kaplan-Meier graph of overall survival of patients suffering from head and neck squamous carcinoma and Gal-7-associated parameters. Phenotype of Gal-7 localization: IH: intense and homogeneous, FH: faint and homogeneous, II: intense and inhomogeneous, NO: no expression.

gene product in DLD-1 colon cancer cells, cell biological data had indicated differential regulation of Gal-7 in squamous cell carcinomas of different origin, a differential response to chemical carcinogenesis in rat models, and anti- or pro-malignancy activities in different human tumor models. To decide on clinical correlations in patient material we studied tissue sections immunohistochemically and disclosed a correlation to increased status of differentiation and keratinization in head and neck squamous cell carcinomas.

Acknowledgements. The study was supported by the IGA MZ ČR, project No. NR 9049-3, by the Ministry of Education, Youth and Sport of the Czech Republic, project No. MSM 002160806, by the GAČR, project No 304/08/P175, by the research initiative LMUexcellent and by an EC Marie Curie Research Training Network grant (contract No. MRTN-CT-2005-019561). Authors are grateful to Dr. B. Friday and Prof. Dr. S. Namirha for valuable discussion, to Prof. Dr. D. Sedmera for critical reading as well as to Eva Vancová and Vít Hajdúch for excellent technical assistance.

References

André S., Kaltner H., Furuike T., Nishimura S.I. and Gabius H.J. (2004). Persubstituted cyclodextrin-based glycoclusters as inhibitors of protein-carbohydrate recognition using purified plant and mammalian lectins and wild-type and lectin-gene-transfected tumor cells as targets. Bioconjugate Chem. 15, 87-98.

André S., Kožár T., Schuberth R., Unverzagt C., Kojima S. and Gabius H.J. (2007a). Substitutions in the N glycan core as regulators of biorecognition: the case of core-fucose and bisecting GlcNAc moieties. Biochemistry 46, 6984-6995.

André S., Sanchez-Ruderisch H., Nakagawa H., Buchholz M., Kopitz J., Forberich P., Kemmner W., Böck C., Deguchi K., Detjen K.M., Wiedenmann B., von Knebel Doeberitz M., Gress T.M., Nishimura S.I., Rosewicz S. and Gabius H.J. (2007b). Tumor suppressor $p16^{INK4a}$ - modulator of glycomic profile and galectin-1 expression to increase susceptibility to carbohydrate-dependent induction of anoikis in pancreatic carcinoma cells. FEBS J. 274, 3233-3256.

Bernerd F., Sarasin A. and Magnaldo T. (1999). Galectin-7 overexpression is associated with the apoptotic process in UVB-induced sunburn keratinocytes. Proc. Natl. Acad. Sci. USA 96, 11329-11334.

Bryne M., Koppang H.S., Lilleng R., Stene T., Bang G. and Dabelsteen E. (1989). New malignancy grading is a better prognostic indicator than Broders' grading in oral squamous cell carcinomas. J. Oral. Pathol. Med. 18, 432-437.

Chen J., He Q.Y., Yuen A.P.W. and Chiu J.F. (2004). Proteomics of buccal squamous cell carcinoma: the involvement of multiple pathways in tumorigenesis. Proteomics 4, 2465-2475.

Chovanec M., Smetana K. Jr., Plzák J., Betka J., Plzáková Z., Štork J., Hrdlicková E., Kuwabara I., Dvoránková B., Liu F.T., Kaltner H., André S. and Gabius H.J. (2005). Detection of new diagnostic markers in pathology by focus on growth-regulatory endogenous lectins. The case study of galectin-7 in squamous epithelia. Prague Med. Rep. 106, 209-216.

Cooper D.N.W. (2002). Galectinomics: finding themes in complexity. Biochim. Biophys. Acta 1572, 209-231.

Demers M., Biron-Pain K., Hébert J., Lamarre A., Magnaldo T. and St-Pierre Y. (2007). Galectin-7 in lymphoma: elevated expression in human lymphoid malignancies and decreased lymphoma dissemination by antisense strategies in experimental model. Cancer Res. 67, 2824-2829.

Dvořánková B., Smetana K. Jr., Chovanec M., Lacina L., Štork J., Plzáková Z., Galoviãová M. and Gabius H.J. (2005). Transient expression of keratin K19 is induced in originally negative interfollicular epidermal cells by adhesion of suspended cells. Int. J. Mol. Med. 16: 525-531.

Dvořánková B., Lacina L., Smetana K. Jr., Lensch M., Manning J.C., André S. and Gabius H.J. (2008). Human galectin-2: nuclear presence in vitro and its modulation by quiescence/stress factors. Histol. Histopathol. 23, 167-178.

Gabius H.J. (1997a). Concepts of tumor lectinology. Cancer. Invest. 15, 454-464.

Gabius H.J. (1997b). Animal lectins. Eur. J. Biochem. 243, 543-576.

Gabius H.J. (2006). Cell surface glycans: the why and how of their functionality as biochemical signals in lectin-mediated information transfer. Crit. Rev. Immunol. 26, 43-79.

Holíková Z., Hrdliãková-Cela E., Plzák J., Smetana K. Jr., Betka J., Dvořánková B., Esner M., Wasano K., André S., Kaltner H., Motlík J., Hercogová J., Kodet R. and Gabius H.-J. (2002). Defining the glycophenotype of squamous epithelia by plant and mammalian lectins. Differentiation-dependent expression of α-2,6- and α-2,3-linked N-acetylneuraminic acid in squamous epithelia and carcinomas and its differential effect on binding of the endogenous lectins galectins-1 and -3. APMIS. 110, 845-856.

Kasai K.I. and Hirabayashi J. (1996). Galectins: a family of animal lectins that decipher glycocodes. J. Biochem. 119, 1-8.

Kayser K., Hoeft D., Hufnagl P., Caselitz J., Zick Y., André S., Kaltner H. and Gabius H.J. (2003). Combined analysis of tumor growth pattern and expression of endogenous lectins as prognostic tool in primary testicular cancer and its lung metastases. Histol. Histopathol. 18, 771-779.

Kopitz J., André S., von Reitzenstein C., Versluis K., Kaltner H., Pieters R.J., Wasano K., Kuwabara I., Liu F.T., Cantz M., Heck A.J.R. and Gabius H.J. (2003). Homodimeric galectin-7 (p53 induced gene 1) is a negative growth regulator for human neuroblastoma cells. Oncogene 22, 6277-6288.

Langbein S., Brade J., Badawi J.K., Hatzinger M., Kaltner H., Lensch M., Specht K., André S., Brinck U., Alken P. and Gabius H.J. (2007). Gene-expression signature of adhesion/growth-regulatory tissue lectins (galectins) in transitional cell cancer and its prognostic relevance. Histopathology 51, 681-690.

Lohr M., Lensch M., André S., Kaltner H., Siebert H.C., Smetana K. Jr., Sinowatz F. and Gabius H.J. (2007). Murine homodimeric adhesion/growth-regulatory galectins-1, -2, and -7: comparative profiling of gene/promoter sequences by database mining, of expression by RT-PCR/immunohistochemistry and of contact sites for carbohydrate ligands by computational chemistry. Folia Biol. (Praha). 53, 109-128.

Lu J., Pei H., Kaeck M. and Thompson H.J. (1997). Gene expression changes associated with chemically induced rat mammary carcinogenesis. Mol. Carcinogen. 20, 204-215.

Madsen P., Rasmussen H., Flint T., Gromov P., Kruse A., Honore B., Vorum H. and Celis J. (1995). Cloning, expression, and chromosome mapping of human galectin-7. J. Biol. Chem. 270, 5823-5829.

Magnaldo T., Bernerd F. and Darmon M. (1995). Galectin-7, a human 14-kDa S-lectin, specifically expressed in keratinocytes and sensitive to retinoic acid. Dev. Biol. 168, 259-271.

Magnaldo T., Fowlis D. and Darmon M. (1998) Galectin-7, a marker of all types of stratified epithelia. Differentiation 63, 159-168.

Moisa A., Fritz P., Eck A., Wehner H.D., Mürdter T., Simon W. and Gabius H.J. (2007). Growth/adhesion-regulatory tissue lectin galectin-3: stromal presence but not cytoplasmic/nuclear expression in tumor cells as negative prognostic factor in breast cancer. Anticancer Res. 27, 2131-2140.

Moisan S., Demers M., Mercier J., Magnaldo T., Potworowski E.F. and St-Pierre Y. (2003). Upregulation of galectin-7 in murine lymphoma cells is associated with progression toward an aggressive phenotype. Leukemia 17, 751-759.

Østergaard M., Rasmussen H.H., Nielsen H.V., Vorum H., Ømtoft T.F., Wolf H. and Celis J.E. (1997). Proteome profiling of bladder squamous cell carcinomas: identification of markers that define their degree of differentiation. Cancer Res. 57, 4111-4117.

Plzák J., Smetana K. Jr., Hrdliãková E., Kodet R., Holíková Z., Liu F.T, Dvofiánková B., Kaltner H., Betka J. and Gabius H.J. (2001). Expression of galectin-3-reactive ligands in squamous cancer and normal epithelial cells as a marker of differentiation. Int. J. Oncol. 19, 59-64.

Polyak K., Xia Y., Zweier J.L., Kinzler K.W. and Vogelstein B. (1997). A model for p53-induced apoptosis. Nature 389, 300-305.

Ravasz L.A., Hordijk G.J., Slootweg P.J., Smit F. and Tweel I.V. (1993). Uni- and multivariate analysis of eight indications for post-operative radiotherapy and their significance for local-regional cure in advanced head and neck cancer: J. Laryngol. Otol. 107, 437-440.

Rorive S., Eddafali B., Fernandez S., Decaestecker C., André S., Kaltner H., Kuwabara I., Liu F.T., Gabius H.J., Kiss R. and Salmon I. (2002). Changes in galectin-7 and cytokeratin-19 expression during the progression of malignancy in thyroid tumors: diagnostic and biological implications. Mod. Pathol. 15, 1294-1301.

Rotblat B., Niv H., André S., Kaltner H., Gabius H.J. and Kloog Y. (2004). Galectin-1(L11A) predicted from a computed galectin-1 farnesyl-binding pocket selectively inhibits Ras-GTP. Cancer Res. 64, 3112-3118.

Saussez S., Cucu D.R., Decaestecker C., Chevalier D., Kaltner H., André S., Wacreniez A., Toubeau G., Camby I., Gabius H.J. and Kiss R. (2006). Galectin 7 (p53-induced gene 1): a new prognostic predictor of recurrence and survival in stage IV hypopharyngeal cancer. Ann. Surg. Oncol. 13, 999-1009.

Saussez S. and Kiss R. (2006). Galectin-7. Cell. Mol. Life Sci. 63, 687-697.

Saussez S., Lorfevre F., Decaestecker C., Chevalier D., Kaltner H., André S., Toubeau G., Gabius H.J. and Leroy X. (2008). Increased expression and altered intracellular distribution of adhesion/growth-regulatory lectins galectins-1 and -7 during tumour progression in hypopharyngeal and laryngeal squamous cell carcinomas. Histopathology 52, 483-493.

Smetana K. Jr., Dvofiánková B., Chovanec M., Bouãek J., Klíma J., Motlík J., Lensch M., Kaltner H., André S. and Gabius H.J. (2005). Nuclear presence of adhesion/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin. Histochem. Cell Biol. 125, 171-182.

Sturm A., Lensch M., André S., Kaltner H., Wiedenmann B., Rosewicz S., Dignass A.U. and Gabius H.J. (2004). Human galectin-2: novel inducer of T cell apoptosis with distinct profile of caspase activation. J. Immunol. 173, 3825-3837.

Villalobo A., Nogales-González A. and Gabius H.J. (2006). A guide to signaling pathways connecting protein-glycan interaction with the emerging versatile effector functionality of mammalian lectins. Trends Glycosci. Glycotechnol. 18, 1-37.

Wang J.L., Gray R.M., Haudek K.C. and Patterson R.J. (2004). Nucleocytoplasmic lectins. Biochim. Biophys. Acta 1673, 75-93.

Wu A.M., Singh T., Wu J.H., Lensch M., André S. and Gabius H.J. (2006). Interaction profile of galectin-5 with free saccharides and mammalian glycoproteins: probing its fine specificity and the effect of naturally clustered ligand presentation. Glycobiology 16, 524-537.

Accepted July 11, 2008

Journal of Dermatological Science 55 (2009) 18–26

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.intl.elsevierhealth.com/journals/jods

Phenotypic characterization of human keratinocytes in coculture reveals differential effects of fibroblasts from benign fibrous histiocytoma (dermatofibroma) as compared to cells from its malignant form and to normal fibroblasts

L. Kideryová [a], L. Lacina [b,c,d], B. Dvořánková [b,c], J. Štork [d], Z. Čada [b,e], P. Szabo [b], S. André [f], H. Kaltner [f], H.-J. Gabius [f], K. Smetana Jr. [b,c,*]

[a] Charles University in Prague, 1st Faculty of Medicine, 1st Department of Internal Medicine, Prague, Czech Republic
[b] Charles University in Prague, 1st Faculty of Medicine, Institute of Anatomy, Prague, Czech Republic
[c] Charles University in Prague, 2nd Faculty of Medicine, Center of Cell Therapy and Tissue Repair, Prague, Czech Republic
[d] Charles University in Prague, 1st Faculty of Medicine, Department of Dermatovenereology, Prague, Czech Republic
[e] Charles University in Prague, 1st Faculty of Medicine, Department of Otorhinolaryngology, Head and Neck Surgery, Prague, Czech Republic
[f] Ludwig-Maximilians-University Munich, Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Munich, Germany

ARTICLE INFO

Article history:
Received 6 January 2009
Received in revised form 16 March 2009
Accepted 18 March 2009

Keywords:
Benign fibrous histiocytoma
Fibroblasts
Galectin
Keratin
Nucleostemin

ABSTRACT

Background: Benign and malignant fibrous histiocytoma present with a considerable difference concerning cellular organization in their vicinity.
Objective: Normally appearing epithelium covers the malignant form in contrast to hyperplastic epidermis for benign tumors. It is an open question as to whether the tumor-associated fibroblasts are capable to affect phenotypic features of normal keratinocytes, prompting this comparative analysis.
Methods: Fibroblasts were isolated from benign and malignant fibrous histiocytomas, respectively, and also from normal dermis. The resulting cell populations were thoroughly characterized immunocytochemically using a large panel of antibodies. The three fibroblast preparations were cocultured with normal interfollicular keratinocytes. Their phenotype was characterized for distinct properties including differentiation and proliferation.
Results: Fibroblasts prepared from both tumor types were phenotypically practically identical with normal dermal fibroblasts. Their activities on keratinocytes were different. Cells prepared from benign fibrous histiocytoma were capable to effect strong expression of keratin 19 and production of a galectin-1-rich extracellular matrix. Fibroblasts isolated from malignant fibrous histiocytoma led to a phenotype very similar to that when keratinocytes were cocultured with normal dermal fibroblasts.
Conclusion: Fibroblasts prepared from benign fibrous histiocytoma were biologically active on keratinocytes in a particular manner. Our results on fibroblast activity are suggested to be relevant for morphologic differences observed *in vivo* between normal epidermis and epidermis adjacent to the studied tumor types.

1. Introduction

The fibrohistiocytic tumors of the skin are a heterogeneous group of dermal/subcutaneous mesenchymal neoplasms. "Fibrohistiocytic" refers in this context to a morphologic similarity of the cells with fibroblasts and histiocytes. Indeed, the cells of such tumors show fibroblastic, myofibroblastic and histiocytic (macrophage-like) differentiation, often in the same tumor. The WHO classification (2005) includes benign types of tumors (e.g. fibrous histiocytoma, synonymous: dermatofibroma), tumors of intermediate nature (e.g. plexiform fibrohistiocytic tumor and dermatofibrosarcoma protuberans) and aggresive tumors (e.g. malignant fibrous histiocytoma) [1]. Benign fibrous histiocytoma (BFH) is a common cutaneous soft tissue tumor with a frequency of approximately 3% of the population [2]. Changes of the epidermis in contact with the tumor represent a characteristic diagnostic feature of this type of neoplasia. The epidermal morphology varies from simple acanthosis to pronounced basaloid hyperplasia, very

* Corresponding author at: Charles University in Prague, 1st Faculty of Medicine, Institute of Anatomy, U nemocnice 3, 128 00 Prague 2, Czech Republic
Tel.: +420 2 24965873; fax: +420 2 24965770.
E-mail address: Karel.Smetana@lf1.cuni.cz (K. Smetana Jr.).

0923-1811/$36.00
doi:10.1016/j.jdermsci.2009.03.009

similar in appearance to cell clusters in basal cell carcinoma [3–7]. Immunohistochemical analysis of the epidermis overlying the center of the dermatofibroma revealed distinct changes in proliferation and level of differentiation [8]. It is obvious that activation of epidermal keratinocytes may be due to factors originating from tumor and/or stromal cells acting on their environment. The precise origin of fibrohistiocytic tumors has been disputed for decades. Histiocytes, fibroblasts, or cells with intermediate features between fibroblasts and histiocytes and mesenchymal stem cells have all been proposed as origin of the tumor cells. At any rate, an exclusive histiocytic origin is, no longer considered [9].

Emerging insights into the modes of regulating progeny production of epidermal stem cells provide instructive suggestions in this respect [10]. Evidently, adult tissue stem cells acquire proper functionality within a very specialized microenvironment, the so-called niche [11]. Despite recent progress in understanding the complexity of this entity in skin [12], detailed characterization of this type of microenvironment continues to warrant efforts. Because it is generally accepted that mutual mesenchymal–epithelial interactions comprise salient mechanisms of morphogenesis, *in vitro* studies with tumor-derived fibroblasts are an attractive tool toward further progress. Of relevance in this context, cellular parameters of fibroblasts are significantly influenced by their site of localization with ability to maintain these features under physiological conditions [13], and they can be the source of modulatory effects as e.g. demonstrated by expression of distinct types of keratins in cocultured keratinocytes [14]. Following this line of evidence a regulatory role of cancer-associated stromal fibroblasts on the biology of neighboring cells including tumor cells was delineated [15]. Thus, when fibroblasts were prepared from basal or squamous cell carcinomas, they were active to influence phenotypic features of normal human keratinocytes [16,17]. At this stage, it is an open question as to whether stromal fibroblasts from a benign tumor have a similar activity, which may underlie establishment of morphological features of the tumor *in situ*.

In this study, we addressed this issue and answer the question on a possible role of fibroblasts from BFH on normal human keratinocytes using an *in vitro* cocultivation model. As internal standard we have run assays in parallel with cancer-associated stromal fibroblasts (CASF) from MFH. This tumor type is morphologically diverse including the presence of condensed, fibroblast-rich stroma (similar to BFH and basal cell carcinoma) but lacking appearance of strong hyperplasia with no hyperplastic changes in the adjacent epidermis [18,19]. The use of immunohistochemistry can be valuable in the diagnostic workup of any spindle-cell fibrohistiocytic tumors, diagnosis of MFH based on morphology alone not being reliable. There are no markers or combinations of markers that establish the diagnosis of malignant fibrous histiocytoma. The tumor cells of MFH can often show a "vimentin only" immunophenotype with no ability of other immunostains to discern any marked sign of differentiation. The lesional cells of MHF must be negative for cytokeratins and S100 protein; a small extent of expression of actin, indicating myofibroblastic differentiation, is acceptable. Fibrohistiocytic tumors usually contain nontumoral S100 protein-positive Langerhans cells, CD31-positive endothelial cells and macrophages, as well as factor XIIIa-positive dendritic cells. CD68 expression does not support or exclude the diagnosis of MHF in line with the other traditional histiocytic markers (α_1-antitrypsin, α_1-antichymotrypsin, and factor XIII) [20].

We isolated CASF from both types of tumor and cocultured them with normal interfollicular keratinocytes to probe into and to characterize biological effect(s) triggered by the studied fibroblasts. To establish an internal reference value we added experimental series with normal dermal fibroblasts (DF). Based on our previous studies with basal/squamous cell carcinoma [16,17], in which we demonstrated a shift of phenotype of normal keratinocytes cocultured with tumor stromal cells, we focused analysis on keratin 8 (that is present in squamous cell carcinoma and not in normal epidermis and basalioma), on keratin 19 (that is present in bulge epidermal stem cells and in a minimum of 50% of basalioma) and on vimentin. Its coexpression with keratins is indicative of epithelial–mesenchymal interaction. Expression of nucleostemin, binding sites for Gal-1 and Ki 67 can be related not only to proliferation but also to differentiation status of keratinocytes monitored (for details see [16,17]).

2. Material and methods

2.1. Tissue preparation and cell culture

Specimens of BFH and MFH (one specimen of each tumor) were obtained from the Department of Dermatovenereology of the 1st Faculty of Medicine (Charles University, Prague, Czech Republic), and tissue for control, i.e. normal skin, came from the Department of Aesthetic Surgery of the 3rd Faculty of Medicine of the Charles University in Prague, in all cases with written informed consent of the donors. The experiments were approved by local ethical committee and performed strictly according to the Declaration of Helsinki principles. A part of each tumor and of normal skin was fixed with paraformaldehyde, embedded in paraffin and used for routine pathologic characterization after hematoxilin and eosin staining and for processing by immunohistochemistry (panel of keratins, keratin 19, galectin-1; for details, please see below).

Normal DF and keratinocytes were prepared by mild trypsinization overnight and cultured by a modified Rheinwald and Green procedure [21], as described previously [16,17]. Fibroblasts present at the site of the tumor were isolated and cultured according to a routine protocol [22] with modifications given elsewhere [16,17]. Cells with normal fibroblastoid appearance were used from the seventh passage cultured for 53 days in the case of BFH and from the sixth passage cultured for 77 days for MFH, respectively. Their phenotype was repetitively examined by the detection of vimentin, keratins and CD68 (please see below). This procedure ensured to work with fibroblasts. Feeder cells were seeded on cover glass at the low density of 4,000 cells/cm^2 and cultured for 24 h, the suspension of keratinocytes (30,000 cells/cm^2) was then added, cells were then kept in culture in a keratinocyte medium (DMEM + F12, 3:1) at 37 °C and 5% CO_2 [16,17] for 5 days. This experiment was repeated up to five times independently to ascertain reproducibility.

2.2. FACScan analysis of fibroblasts

The cultured fibroblasts prepared from both types of tumor and from normal dermis were analysed after trypsinization using FACSCalibur™ equipment (BD Biosciences, Heidelberg, Germany) and data processing followed using the Summit™ V3.3, Build 1024 software (DakoCytomation, Fort Collins, CO, USA) [16,17]. Single-cell suspensions in phosphate-buffered saline (PBS) containing 2% fetal calf serum were characterized by probing for presence of the following markers: cluster of differentiation markers CD11b, CD18, CD29, CD44, CD45, CD49a, CD49d, CD63, CD90, CD106, and CD166 (all from Becton Dickinson, Prague, Czech Republic), CD11c, CD14, CD34, CD45, CD68, CD71, CD235a, CD105, HLA DR, DQ, DP and HLA-A, -B, and -C (all from Dako, Brno, Czech Republic), CXCR4, and alkaline phosphatase (R&D Systems, Minneapolis, MN, USA) as well as CD19e and CD49c (Chemicon, Temecula, CA, USA). Isotype immunoglobulins were used as negative controls in all experiments.

2.3. Immunocytochemistry

Samples of normal epidermis, and epidermis surrounding the tumors (BFH and MFH) were paraffinized and routinely stained for presence of a panel of keratins, keratin 19 and galectin-1 after the retrieval of antigen (Antigen Unmasking Solution; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instruction- for the description of staining procedure, please see below).

Fibroblasts prepared from both types of tumors and keratinocytes kept in coculture, as was also the case with normal DF, were characterized immunocytochemically by multiple labelling at the single-cell level as described in detail elsewhere [16,17]. Fibroblasts were processed to detect presence of the macrophage tandem-repeat-type mannose receptor (Gaubius Laboratory, TNO Prevention and Health, Leiden, The Netherlands), CD14 (MEM 15 antibody; generous gift of Prof. V. Hořejší, Institute of Molecular Genetics of the Academy of Science, vvi., Prague, Czech Republic), CD45 (Sigma–Aldrich, Prague, Czech Republic), CD34, CD68, CD71, vimentin, smooth muscle actin, Ki67 (DAKO Cytomation, Brno, Czech Republic) and nucleostemin (Neuromics, Bloomington, MN, USA). Keratinocytes were characterized by determining presence of a panel of keratins using a rabbit polyclonal antibody (Abcam, Cambridge, UK). Keratin 8 was detected by a mouse monoclonal antibody (DAKOCytomation, Brno, Czech Republic) and keratin 19 by a mouse monoclonal (Sigma–Aldrich, Prague, Czech Republic). Epithelial–mesenchymal transition zone was defined immunocytochemically by the occurrence of coexpression of keratins with vimentin (see above). Nucleostemin and Ki67 were also detected (as mentioned above). As a common marker of tumor stroma the endogenous lectin galectin-1 was visualized using a home-made polyclonal rabbit anti-human galectin-1 antibody, rigorously checked for absence of cross-reactivity with other galectins [23–25], in coculture of keratinocytes with experimental fibroblasts. Western blotting of cell extracts comparing mock-treated and galectin-1-overexpressing transfected cells with strong ectopic expression was performed as further control, running highly sensitive signal visualization by chemiluminescence [26,27].

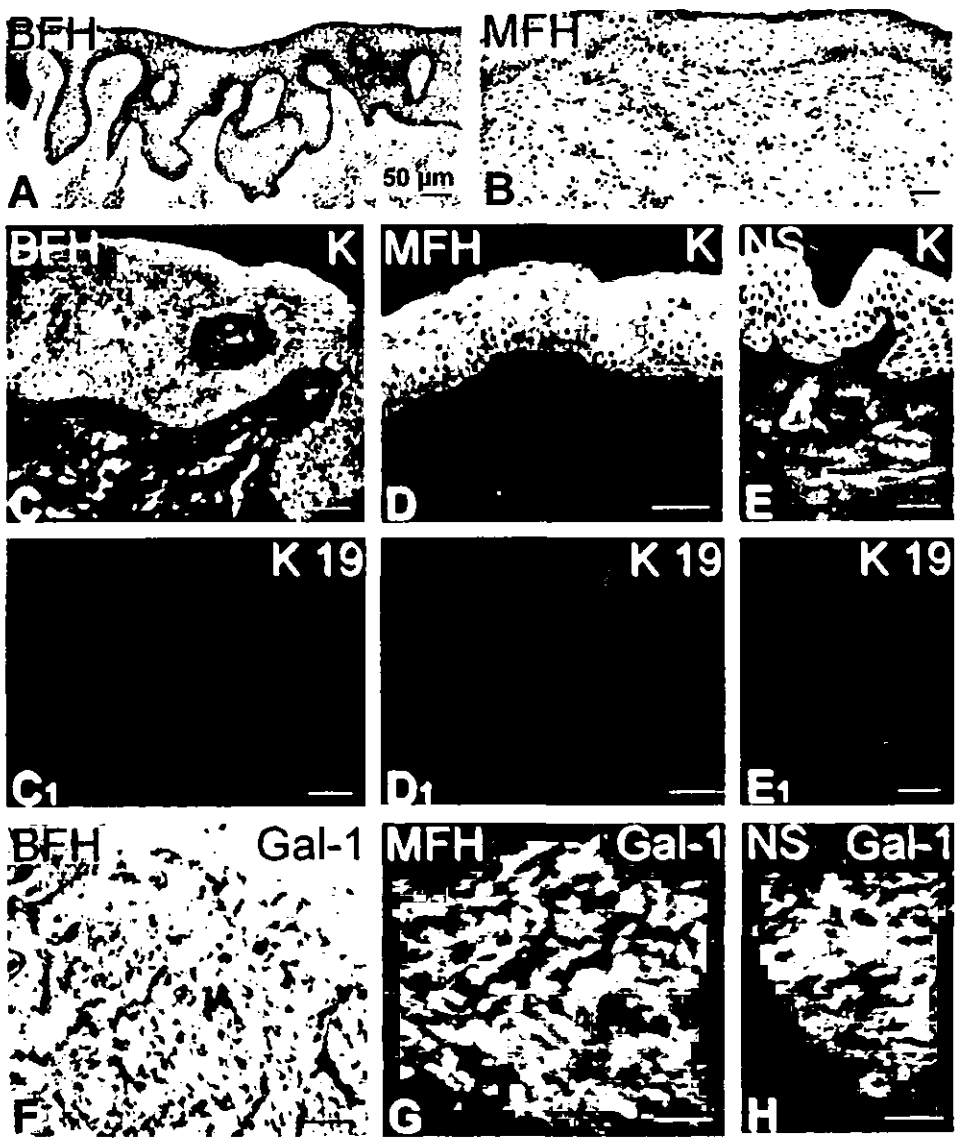


Fig. 1. Benign fibrous histiocytoma (BFH) with extensive hyperplastic epidermis (A) in contrast to malignant fibrous histiocytoma (MFH) covered by epidermis with normal appearance (B). Epidermis above both types of tumors (C and D) resembled epidermis of normal skin (NS), (E) in terms of absence of keratin 19 (C_1–E_1). Stroma of BFH contains a high level of the endogenous lectin galectin-1 (Gal-1) (F) whose extent of expression is comparatively low in MFH (G) and in dermis of normal skin (H). Bar is 50 μm.

Purification and biotinylation, controls for purity and binding activity as well as measuring degree of labelling of this human lectin were described in detail elsewhere [24,28,29]. Fibronectin as extracellular matrix component, a glycoprotein ligand for galectin-1, was also detected in cultured cells with rabbit polyclonal antibody (DAKOCytomation, Brno, Czech Republic). Fixation procedure and dilution of primary antibodies were set according to the recommendation of the corresponding suppliers. FITC-labeled swine anti-mouse serum (AlSeVa, Prague, Czech Republic) was the second-step reagent in the cases of CD14, CD34, CD45, CD68, CD71 and vimentin, FITC-labeled swine anti-rabbit serum (AlSeVa, Prague, Czech Republic) for processing to detect the macrophage tandem-repeat-type mannose receptor, galectin-1 and the panel of keratins, respectively. TRITC-labeled goat anti-mouse serum (Sigma–Aldrich, Prague, Czech Republic) facilitated visualization of signals for vimentin, keratin 8, keratin 19 and smooth muscle actin, TRITC-labeled donkey ant-goat serum (Jackson Laboratories, West Growe, PA, USA) for nucleostemin. Control experiments were performed by replacement of specific antibodies by mono- or polyclonal antibodies with specificity that is irrelevant in the studied cells and tissues (in the case of monoclonals of the same isotype). DNA visualization by DAPI (4′,6′-diamidino-2-phenyindole dilactate; Sigma–Aldrich, Prague, Czech Republic) provided a signal for the cell nucleus. Specimens were then mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and inspected using an Eclipse 90i (Nikon, Prague, Czech Republic) fluorescence microscope

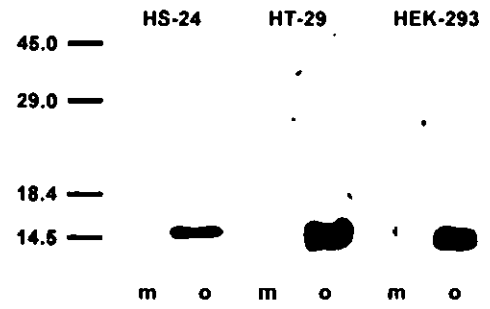


Fig. 2. Western blot analysis of extracts of mock-treated (m) and galectin-1-overexpressing (o) cells (50 μg protein per lane; molecular weight markers designated by mass) to illustrate specificity of the anti-galectin-1 immunoglobulin G preparation. Extracts from human HS-24 non-small cell lung cancer (left), HT-29 colorectal adenocarcinoma (center) and HEK 293 embryonic kidney (right) cells were processed.

equipped with the suited filterblocks, a high-resolution CCD camera (Vosskühler Cool-1300Q; Vosskühler, Osnabrück, Germany) and a computer-assisted image analyzer (LUCIA 5.10; Laboratory Imaging, Prague, Czech Republic). In addition to routine documentation microscopy was also performed for acquiring quantitative data on populations of 500 cells per specimen. The statistical significance was tested using the Student *t*-test. Any difference with a level lower than 0.05 was considered to be statistically significant.

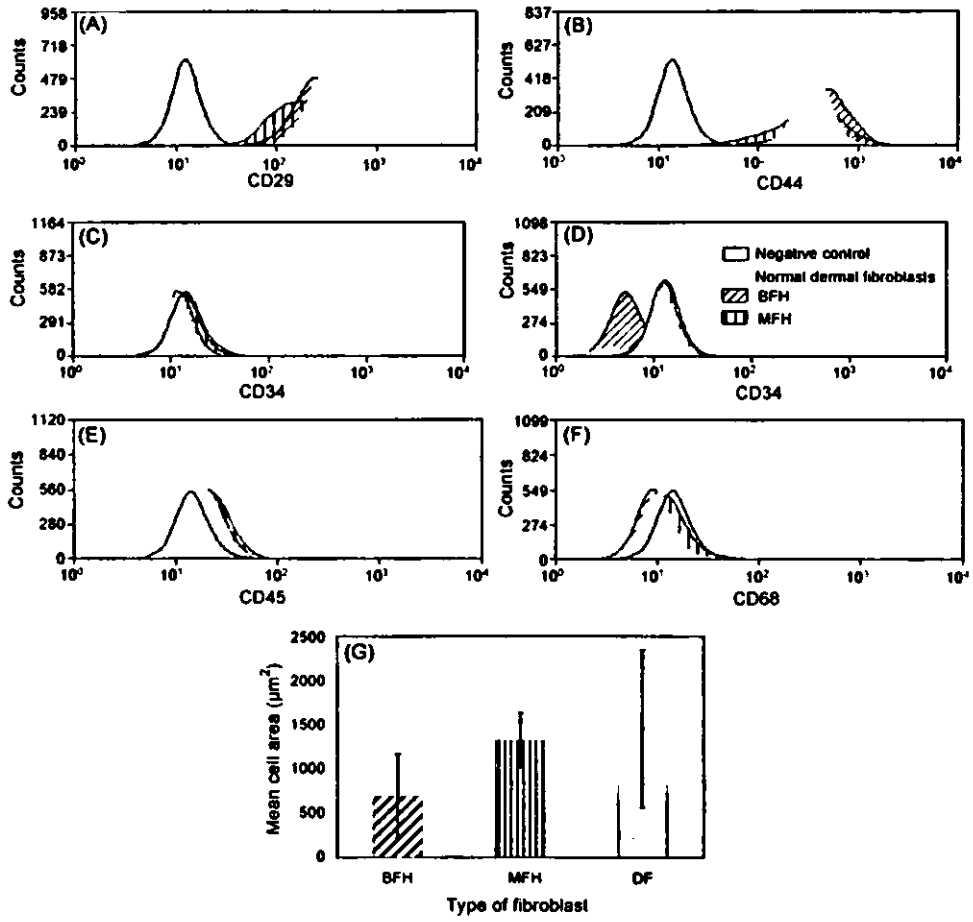


Fig. 3. Representative documentation of intensity of immunodetection of selected markers in normal dermal fibroblasts and fibroblasts prepared from BFH and MFH (A–F). While all three types of cells are positive for CD29 (A) and CD44 (B), they were negative for CD14 (C), CD34 (D), CD45 (E) and CD68 (F). The size of cells when measured after adhesion and spreading was lower in the case of fibroblasts prepared from BFH than that of cells originating from MFH and of normal dermal fibroblasts, the difference not reaching the level of statistical significance (G).

3. Results

3.1. Characterization of epidermis

Epidermis overlying BFH showed the characteristic hyperplasia in contrast to a rather normal appearance of epidermis when monitoring MFH (Fig. 1A and B). Epidermis above both types of tumor expressed keratins, as seen in normal epidermis (positive control of accessibility of antigen for antibody) (Fig. 1C–E). Keratin 19 was not detected in both normal interfollicular epidermis and in the epidermis in contact with both studied tumors (Fig. 1C_1–E_1). In contrast to normal skin and MFH, BFH exhibited an intense signal for presence of galectin-1 in stromal component (Fig. 1F–H). The antibody preparation did not cross-react with other members of the family of human galectins and its specificity was further ascertained by Western blotting with human cell extracts (Fig. 2).

3.2. Characterization of fibroblasts

Fibroblasts prepared from both types of tumor presented a similar phenotype without major deviation from appearance of normal DF (Fig. 3A–F; Fig. 4 A–H; Table 1), with several notable exceptions. They express no markers typical for leukocytes/histiocytes (CD11, CD14, CD18, CD45, CD49, CD63, CD68, CD71, CD90, CD105, CD166, CD235, CXCR4), for hemopoietic precursor and endothelial cells (CD34, CD105, CD106, CD166) and for mesenchymal stem cells (alkaline phosphatase). Also, the expression profile of both HLA-I/II determinants was identical with

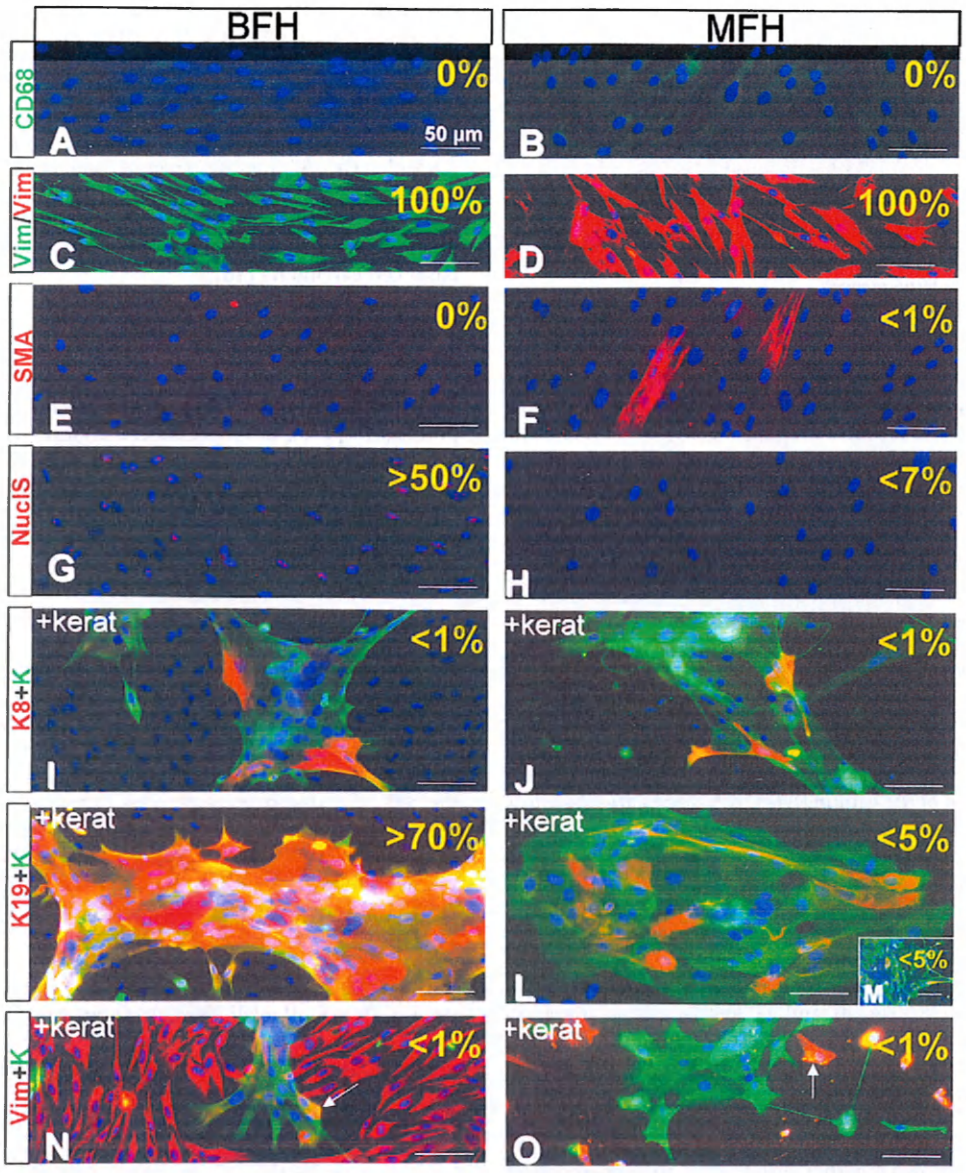


Fig. 4. Fibroblasts prepared from both types of tumor are negative for CD68 (A and B) and highly positive for vimentin (Vim) (C and D). Whereas no smooth muscle actin (SMA)-containing myofibroblasts were present among the fibroblast population prepared from BFH (E), these cells, albeit at very low frequency, were detected in the pool of fibroblasts prepared from MFH (F). A high proportion of nuclei of fibroblasts from BFH is positive for nucleostemin (NuclS) (G). No nucleostemin was present in nucleoli among fibroblasts prepared from MFH (H). Keratin 8 (K8) was detected in a very low number of keratinocytes cocultured with fibroblasts prepared from BFH (I) and MFH (J). Coculture of BFH-derived fibroblasts with keratinocytes led to a strong staining for keratin 19 (K19) in these cells (K). Very low level of presence of K19 was observed in keratinocytes cocultured with fibroblasts from MFH (L) and with normal dermal fibroblasts (M). Coexpression of keratins with vimentin (Vim, arrow) was negligible in keratinocytes cocultured with BFH- (N) and MFH-derived fibroblasts (O). Bar is 50 µm.

Table 1
Phenotypic characterization of studied fibroblast populations by FACScan analysis.

Marker	NDF	BFHF	MFHF
CD11b	–	–	–
CD11c	–	–	–
CD14	–[a]	–[a]	–[a]
CD18	–	–	–
CD29	+	+	+
CD34	–[a]	–[a]	–[a]
CD44	+	+	+
CD45	–[a]	–[a]	–[a]
CD49a	–	–	–
CD49c	–	–	–
CD49d	–	–	–
CD49e	–	–	–
CD63	–	–	–
CD68	–[a]	–[a]	–[a]
CD71	–[a]	–[a]	–[a]
CD90	–	–	–
CD105	–	–	–
CD106	–	–	–
CD166	–	–	–
CD235a	–	–	–
CXCR4	–	–	–
HLA-I	+	+	+
HLA-II	–	–	–
Alkaline phosphatase	–	–	–

NDF: normal dermal fibroblasts BFHF; fibroblasts from benign fibrous histiocytoma
MFHF: malignant fibrous histiocytoma
[a] Assessment was ascertained immunocytochemically

normal fibroblasts. CD29 and CD44 positivity indicates that all three types of evaluated cells have the same ability to interact with components of the extracellular matrix. In contrast to fibroblasts prepared from BFH and to normal DF, myofibroblasts were present in the pool of cells prepared from MFH (Fig. 4E and F). Of further note, the number of nucleostemin-positive cells was significantly increased ($p < 0.001$) in fibroblasts originating from BFH (Fig. 4G and H). Overall, these fibroblasts were somewhat smaller than those prepared from MFH and from normal dermis. However, the differences did not reach the level of statistical significance (Fig. 3G, $p = 0.08$). Summarizing the immunophenotyping carried out with the three cell preparations (Table 1), the cells used for further *in vitro* coculture experiments with normal keratinocytes were fibroblasts, with no other elements being present in the population of cultured stromal cells of BFH and MFH.

3.3. Characterization of the keratinocytes cocultured with fibroblasts

First examining morphology, keratinocyte colonies cocultured with fibroblasts prepared from both types of tumor and normal dermis had a rather similar appearance (Fig. 4I–O, Fig. 5A–K). Next, the status of differentiation was monitored based on keratin immunocytochemistry. While only very few keratinocytes cultured on all three types of fibroblasts (MFH, BFH, normal skin) were positive for keratin 8 (Fig. 4I and J), keratinocytes grown in coculture with fibroblasts prepared from BFH significantly ($p < 0.02$) expressed keratin 19, a definitively distinctive feature (Fig. 4K and L). Expression of this type of keratin in keratinocytes cocultured with normal dermal fibroblasts (Fig. 4M) was practically identical with appearance in those cultures where keratinocytes were grown together with fibroblasts prepared from MFH (Fig. 4L). Presence of keratinocytes coexpressing both keratins and vimentin was negligible in coculture with all types of fibroblasts (Fig. 1N and O). This observation excludes a significant extent of epithelial–mesenchymal transition in any of the tested systems. The adhesion/growth-regulatory endogenous lectin galectin-1, known to be expressed in the stroma of various tumors including basal cell carcinoma *in situ* and *in vitro* [25,30], was part of deposits of the extracellular matrix produced by fibroblasts from BFH cocultured with keratinocytes (Fig. 5A–C). These deposits also contained the glycoprotein fibronectin, a ligand of this lectin (not shown). The majority of nuclei of keratinocytes cocultured with all three types of fibroblasts harbored presence of galectin-1-binding sites (Fig. 5D and E). In comparison, the signal was rather strong in nuclei of keratinocytes cocultured with fibroblasts from BFH, a situation also encountered when keratinocytes were cocultured with normal dermal fibroblasts, this result being quantitatively substantiated by measuring the profile of fluorescence intensity (Fig. 5D–G).

Next, the proliferation status of the keratinocytes was determined by monitoring Ki67 presence. Its extent was apparently higher for keratinocytes in coculture with fibroblasts prepared from BFH, this difference yet not passing the threshold for statistical significance ($p = 0.09$) (Fig. 5H and I). A further difference concerned expression of nucleostemin. Interfollicular keratinocytes in coculture with DF revealed no signal for nucleostemin (not shown), practically all cells cocultured with both types of tumor fibroblasts contained nucleostemin-positive nucleoli (Fig. 5J and K). Overall, the characterization of the phenotype of interfollicular keratinocytes cocultured with fibroblasts prepared from BFH revealed an influence akin to the effect on keratinocytes when cocultured with fibroblasts prepared from basal cell carcinoma [16].

4. Discussion

Although the marked hyperplasia of epidermis overlying BFH is well known as morphological feature and used in diagnostic procedures [3–8], the mechanisms underlying this phenomenon are yet to be defined. Rather likely, stromal fibroblasts may play a role in this cascade of processes, because fibroblasts prepared from both basal and squamous cell carcinoma, respectively, are able to significantly influence the phenotype of keratinocytes in coculture [16,17]. Our current experiments were designed to address this issue. As an essential prerequisite due to the heterogeneous nature of tumor stroma in these cases, thorough and extensive characterization of experimental fibroblasts was mandatory [18,19,31]. Fibroblasts prepared from the both types of tumors, i.e. BFH and MFH, were phenotypically identical with normal dermal fibroblasts except for the presence of rare cases of cells exhibiting a signal for smooth muscle actin in cultured cells prepared from MFH. The comparative phenotypic investigation of the cells prepared from both types of tumor indicated that these cells can reliably be considered as fibroblasts. High level of expression of nucleostemin in nuclei of cells prepared from MFH can be related to their enhanced proliferation potential and low differentiaton status [32], and myofibroblasts are frequently present in malignant tumor stroma [33].

When grown in contact to fibroblasts prepared from BFH, keratinocytes were found to strongly express of keratin 19. A similar effect on the keratinocyte population had previously been observed, in coculture with stromal cells prepared from basal cell carcinoma [16]. Keratin 19 is present in epidermal stem cells under physiological conditions [34], and this cytoskeletal protein is also detectable in cells of basal cell carcinoma [35]. In our previous study, we had observed that keratin 19 could also be transiently induced in a fraction of the population of basal interfollicular keratinocytes after a suspension regimen [36]. In contrast, no substantial presence of keratin 8 was seen in keratinocytes under the influence of BFH-derived fibroblasts, a feature common for normal keratinocyte coculture with fibroblasts from squamous cell carcinoma [17]. Because this keratin protein is normally not present in postnatal squamous epithelia and is typical for malignant cells of squamous cell carcinoma of the head and neck

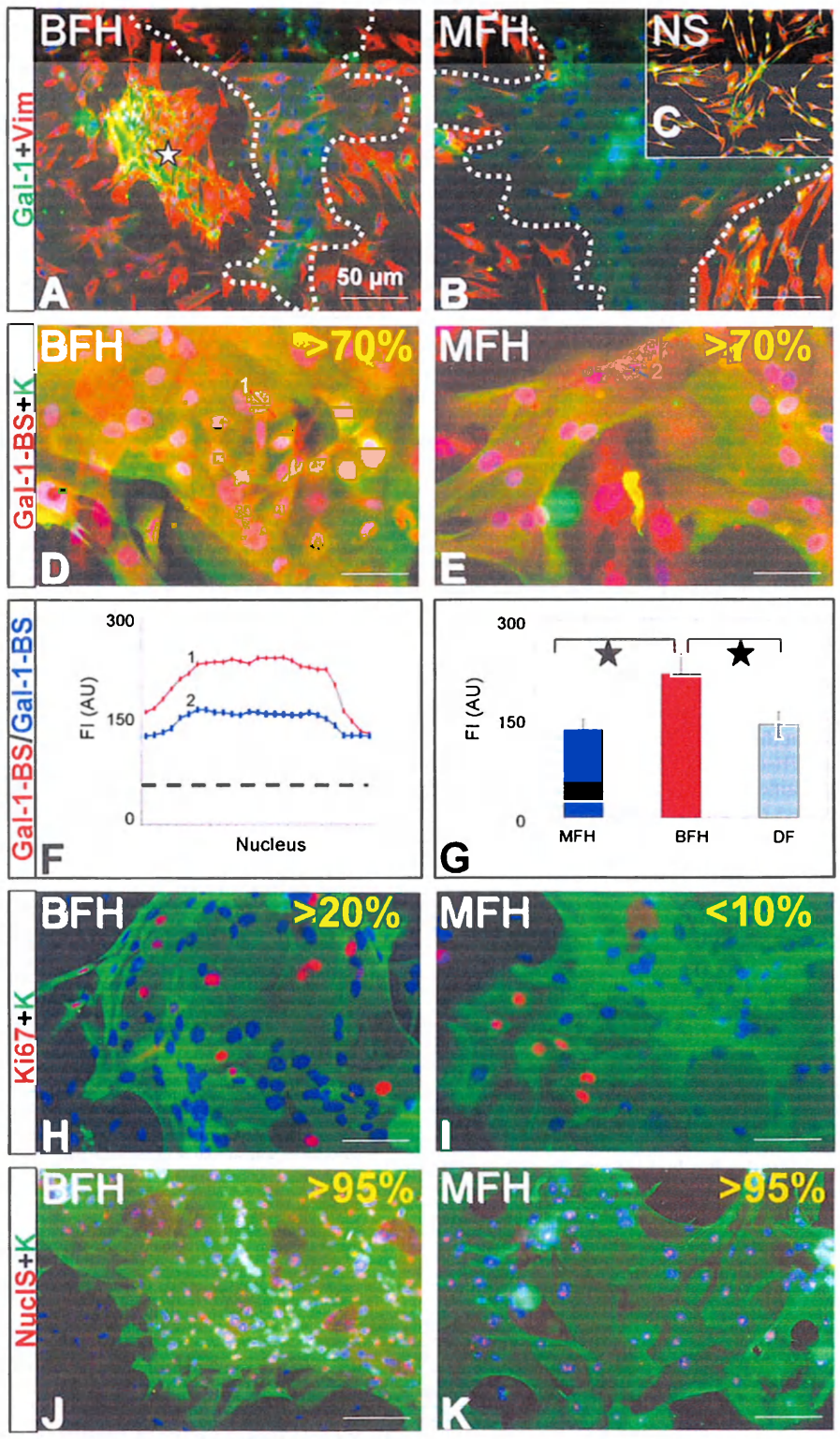


Fig. 5. Fibroblasts prepared from BFH cocultured with interfollicular keratinocytes characteristically produce galectin-1 (Gal-1)-rich extracellular matrices (white star, A) that are not seen in parallel experiments with either cells from MFH (B) or from normal dermis (C). Majority of keratinocyte nuclei were reactive with Gal-1 in both cell populations cocultured with fibroblasts prepared from BFH (C) and MFH (D). Measuring the fluorescence intensity profile of Gal-1 binding, the intensity of reactivity was significantly increased in the cell system starting from BFH and from normal dermal fibroblasts (DF) (at the significance level of $p = 0.01$ in the case of MFH and $p = 0.03$ in the case of DF) (F and G). Representative profiles are marked by numbers 1 and 2 (D and E). Presence of the proliferation marker Ki67 could apparently be observed more frequently (not reaching the $p < 0.05$ threshold) in the system containing BFH (H) than in that from MFH (I). Also, the expression of nucleostemin was not significantly influenced by the origin of cells from BFH and MFH (J and K). The non-paired Student *t*-test was applied to process data statistically, differences with $p < 0.05$ being considered as statistically significant.

of patients with poor prognosis [37], the influence of BFH-associated fibroblasts appears distinct and separate from the effect of fibroblasts originating from squamous cell epithelia. This observation is corroborated by a practically absent coexpression of keratins with vimentin, a protein that defines the epithelial–mesenchymal transition [17,38,39]. The difference between absence of keratin 19 in epidermis positioned over the tumor in BFH *in situ* and the positive signal from keratinocytes cocultured with fibroblasts prepared from this tumor can be due to differences in the environmental conditions *in vitro* and *in situ*, to which fibroblasts from MFH can contribute, similar to fibroblasts from the basal cell carcinoma [16].

Another example of an effect of the BFH-derived fibroblasts on the normal keratinocytes was provided by increased binding activity of the endogenous lectin galectin-1 to nuclei of keratinocytes under their influence. The expression of this lectin, a potent mediator of cell adhesion and tissue invasion as well as growth regulator by outside–inside signaling and intracellular target selection, e.g. to oncogenic H-ras [40–45], was also upregulated. To draw comparisons the stroma sections of basal cell carcinomas [16] and squamous cell carcinomas [46–48] as well as, interestingly, of psoriatic plaque [49] also have abundant presence of this lectin, and similar galectin-1-containing deposits are produced by stromal fibroblasts of basal cell carcinoma *in vitro* [16]. Thus, the production of this lectin and of sites with galectin reactivity are targets for factor(s) originating from stromal fibroblasts. This finding will aid the establishment of assays to define the nature of the effector molecules. When looking at the epidermal hyperplasia *in situ*, our results on the impact of BFH-derived fibroblasts in coculture, set in relation to results obtained in this system using basal cell carcinoma, appear to reflect the similar morphological status in the epidermis. Thus, the merit of the tested model appears to be underscored by the revealed similarities, warranting further work in this system.

Conflict of Interest Statement

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within that could inappropriately influence their work.

Acknowledgements

Authors are grateful to Mrs. Iva Burdová for excellent technical assistance. This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project Nos. MSM 0021620806 and 1M0538 as well as an EC Marie Curie Research Training Network (Contract No. 2005-019561).

References

[1] Hügel H. Fibrohistiocytic skin tumors. J Dtsch Dermatol Ges 2006;4:544–55.
[2] Rahbari H, Mehregan AH. Adnexal displacement and regression in association with histiocytoma. J Cutaneous Pathol 1985;12:94–102.
[3] Biberstein H. Fibrome mit atypischer Epithelwucherung. Arch Dermatol Syphilol 1931;164:69–81.
[4] Halpryn HJ, Allen AC. Epidermal changes associated with sclerosing hemangiomas. Arch Dermatol 1959;80:160–6.
[5] Schoenfeld RJ. Epidermal proliferations overlying histiocytomas. Arch Dermatol 1964;90:266–70.
[6] Dalziel K, Marks R. Hair follicle-like changes over histiocytomas. Am J Dermatopathol 1986;8:462–6.
[7] Cheng L, Amini SB, Zaim MT. Follicular basal cell hyperplasia overlying dermatofibroma. Am J Surg Pathol 1997;21:711–8.
[8] Han K-H, Huh C-H, Cho K-H. Proliferation and differentiation of the keratinocytes in hyperplastic epidermis overlying dermatofibroma. Immunohistochemical characterization. Am J Dermatopathol 2001;23:90–8.
[9] Folpe AL, Cooper K. Best practices in diagnostic immunohistochemistry: pleomorphic cutaneous spindle cell tumors. Arch Pathol Lab Med 2007;131:1517–24.
[10] Blanpain C, Fuchs E. Epidermal stem cells of the skin. Annu Rev Cell Dev Biol 2006;22:339–73.
[11] Watt FM, Hogan BL. Out of Eden: stem cells and their niches. Science 2000;287:1427–30.
[12] Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, et al. Defining the epithelial stem cell niche in skin. Science 2004;303:359–63.
[13] Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, et al. Diversity, topographic differentiation, and positional memory in human fibroblasts. PNAS 2002;99:12877–82.
[14] Yamaguchi Y, Itami S, Tarutani M, Hosokawa K, Miura H, Yoshikawa K, et al. Regulation of keratin 9 in nonpalmoplantarkeratinocytes by palmoplantar fibroblasts through epithelial-mesenchymal interactions. J Invest Dermatol 1999;112:483–8.
[15] Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature 2004;432:332–7.
[16] Lacina L, Smetana Jr K, Dvořánková B, Pytlík R, Kideryová L, Kučerová L, et al. Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. Brit J Dermatol 2007;156:819–29.
[17] Lacina L, Dvořánkova B, Smetana Jr K, Chovanec M, Plzák J, Tachezy R, et al. Marker profiling of normal keratinocytes identifies the stroma from squamous cell carcinoma of the oral cavity as a modulatory microenvironment in coculture. Int J Radiat Biol 2007;83:837–48.
[18] Rosenberg AE. Malignant fibrous histiocytoma: past, present, and future. Skeletal Radiol 2003;32:613–8.
[19] Taher A, Pushpanathan C. Plexiform fibrohistiocytic tumor. Arch Pathol Lab Med 2007;131:1135–7.
[20] Al-Agha OM, Igbokwe AA. Malignant fibrous histiocytoma: between the past and the present. Arch Pathol Lab Med 2008;132:1030–5.
[21] Matoušková E, Veselý P, Königová R. Modified method of in vitro cultivation of human keratinocytes suitable for grafting. Folia Biol (Praha) 1989;35:118–23.
[22] Grando SA, Schofield O, Skubitz APN, Kist DA, Zelickson BD, Zachary CB, et al. Nodular basal cell carcinoma *in vivo* vs *in vitro*. Arch Dermatol 1996;132:1185–93.
[23] Gabius HJ, Wosgien B, Hendrys M, Bardosi A. Lectin localization in human nerve by biochemically defined lectin-binding glycoproteins, neoglycoproteins and lectin-specific antibody. Histochemistry 1991;95:269–77.
[24] Purkrábková T, Smetana Jr K, Dvořánková B, Holíková Z, Böck C, Lensch M, et al. New aspects of galectin functionality in nuclei of cultured bone marrow stromal and epidermal cells: biotinylated galectins as tool to detect specific binding sites. Biol Cell 2003;95:535–45.
[25] Smetana KJ, Dvořánková B, Chovanec M, Bouček J, Klíma J, Motlík J, et al. Nuclear presence of adhesion/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin. Histochem Cell Biol 2006;125:171–82.
[26] André S, Kaltner H, Furuike T, Nishimura SI, Gabius HJ. Persubstituted cyclodextrin-based glycoclusters as inhibitors of protein-carbohydrate recognition using purified plant and mammalian lectins and wild-type and lectin-gene-transfected tumor cells as targets. Bioconjugate Chem 2004;15:87–98.
[27] Lohr M, Lensch M, André S, Kaltner H, Siebert H-C, Smetana Jr K, et al. Murine homodimeric adhesion/growth-regulatory galectins-1, -2 and -7: comparative profiling of gene/promoter sequences by database mining, of expression by RT-PCR/immunohistochemistry and of contact sites for carbohydrate ligands by computational chemistry. Folia Biol (Praha) 2007;53:109–28.
[28] Siebert HC, André S, Lu SY, Frank M, Kaltner H, van Kuik JA, et al. Unique conformer selection of human growth-regulatory lectin galectin-1 for ganglioside GM1 versus bacterial toxins. Biochemistry 2003;42:14762–73.
[29] André S, Pei Z, Siebert HC, Ramström O, Gabius HJ. Glycosyldisulfides from dynamic combinatorial libraries as O-glycoside mimetics for plant and endogenous lectins: their reactivities in solid-phase and cell assays and conformational analysis by molecular dynamics simulations. Bioorg Med Chem 2006;14:6314–26.
[30] Lahm H, André S, Hoeflich A, Kaltner H, Siebert H-C, Sordat B, et al. Tumor galectinology: insights into the network of a family of endogenous lectins. Glycoconjugate J 2004;20:227–38.
[31] Zelger B, Zelger BG, Burgdorf WHC. Dermatofibroma–a critical evaluation. Int J Surg Pathol 2004;12:333–4.
[32] Beekman C, Nichane M, De Clercq S, Maetens M, Floss T, Wurst W, et al. Evolutionarily conserved role of nucleostemin: Controlling proliferation of stem/progenitor cells during early vertebrate development. Mol Cell Biol 2006;26:9291–301.
[33] De Wever O, Demetter P, Mareel M, Bradle M. Stromal myofibroblasts are drivers of invasive cancer growthInt. J Cancer 2008;123:2229–38.
[34] Michel M, Török N, Godbout MJ, Lussier M, Gaudreau P, Royal A, et al. Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially located in function of anatomic sites, and their number varies with donor age and culture stage. J Cell Sci 1996;109:1017–28.
[35] Habets JM, Tank B, Vuzevski VD, Brevé J, Stolz E, van Joost T, et al. Absence of cytokeratin 8 and inconsistent expression of cytokeratin 7 and 19 in human basal cell carcinoma. Anticancer Res 1988;8:611–6.
[36] Dvořánková B, Smetana KJ, Chovanec M, Lacina L, Stork J, Plzáková Z, et al. Transient expression of keratin K19 is induced in originally negative interfollicular epidermal cells by adhesion of suspended cells. Int J Mol Med 2005;16:525–31.

[37] Gires O, Münz M, Schaffrik M, Kleu C, Rauch J, Ahlemann M, et al. Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics based technology. Cell Mol Life Sci 2004;61:1198–207.
[38] Barcellos-Hoff MH, Ravani SA. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. Cancer Res 2000;60:1254–60.
[39] Smetana Jr K, Dvořánková B, Lacina L, Čada Z, Vonka V. Human hair follicle and interfollicular keratinocyte reactivity to mouse HPV16-transformed cells: an *in vitro* study. Oncol Rep 2008;20:75–80.
[40] Gabius HJ. Probing the cons and pros of lectin-induced immunomodulation: case studies for the mistletoe lectin and galectin-1. Biochimie 2001;83:659–66.
[41] Rappl G, Abken H, Muche JM, Sterry W, Tilgen W, André S, et al. CD4$^+$ CD7$^-$ leukemic T-cells from patients with Sézary syndrome are protected from galectin-1-triggered T-cell death. Leukemia 2002;16:840–5.
[42] André S, Kojima S, Yamazaki N, Fink C, Kaltner H, Kayser K, et al. Galectins-1 and -3 and their ligands in tumor biology. J Cancer Res Clin Oncol 1999;125:461–74.
43] Rotblat B, Niv H, André S, Kaltner H, Gabius HJ, Kloog Y, et al. Galectin-1(L11A) predicted from a computed galectin-1 farnesyl-binding pocket selectively inhibits Ras-GTP. Cancer Res 2004;64:3112–8.
[44] Villalobo A, Nogales-González A. Gabius HJ. A guide to signaling pathways connecting protein-glycan interaction with the emerging versatile effector functionality of mammalian lectins. Trend Glycosci Glycotechnol 2006;18:1–37.
[45] André S, Sanchez-Ruderisch H, Nakagawa H, Buchholz M, Kopitz J, Forberich P, et al. Tumor suppressor p16^{INK4a}: modulator of glycomic profile and galectin-1 expression to increase susceptibility to carbohydrate-dependent induction of anoikis in pancreatic carcinoma cells. FEBS J 2007;272:3233–56.
[46] Saussez S, Decaestecker C, Lorfevre F, Chevalier D, Mortuaire G, Kaltner H, et al. Increased expression and altered intracellular distribution of adhesion/growth-regulatory lectins galectins-1 and -7 during tumour progression in hypopharyngeal and laryngeal squamous cell carcinoma. Histopathology 2008;52:483–93.
[47] Čada Z, Bouček J, Dvořánková B, Chovanec M, Plzák J, Kodet R, et al. Nucleostemin expression in squamous cell carcinoma of the head and neck. Anticancer Res 2007;27:3279–84.
[48] Saussez S, Decaestecker C, Cludts S, Ernoux P, Chevalier D, Smetana Jr K, et al. Adhesion/growth-regulatory tissue lectin galectin-1 in relation to angiogenesis/lymphocyte infiltration and prognostic relevance of stromal upregulation in laryngeal carcinomas. Anticancer Res 2009;29:59–66.
[49] Lacina L, Plzáková Z, Smetana Jr K, Štork J, Kaltner H, André S, et al. Glycophenotype of psoriatic skin. Folia Biol 2006;52:10–5.

FOLIA BIOLOGICA (PRAHA)
Editor-in-Chief: Professor Tomáš Zima, MD., DSc., MBA
Executive Editors: Professor Jan Bubeník, MD., DSc., Zdeněk Kostrouch, MD., PhD., Professor Karel Smetana, MD., DSc., *Reductor:* Mgr. Jiri Fruhauf.
Office Address: U Nemocnice 4, 128 52 Prague, Phone: (+420) 224 964 570, Phone/Fax: (+420) 224 964 574, e-mail: iri.fruhanf@lf1.cuni.cz

Manuscript Ref.:

- **title:** Immunohistochemical fingerprinting of the network of seven adhesion/growth-regulatory lectins in human skin and detection of distinct tumor-associated alterations
- **author:** Zdeněk Čada
- **number:** FB-2009-244M
- **submitted by:** Smetana Karel
- **submitted on:** 16.04.2009 - 15:22
- **last modification:** 16.04.2009 - 15:22
- **status:** Accepted
- **editor:** Zdenek Kostrouch

Corresponding author:

Professor Karel Smetana Jr., MD., PhD

Charles University, 1st Faculty of Medicine, Institute of Anatomy,

U nemocnice 3, 128 00 Prague 2, Czech Republic

E-mail: karel.smetana@lf1.cuni.cz Phone: +00420 2 2496 5873

May 4, 2009

Dear Professor Smetana,

Thank you very much for contributing your interesting article to Folia Biologica. Your manuscript was evaluated by two reviewers who recommended the manuscript for publication.

Based on that, your manuscript is now accepted for publication in Folia Biologica.

With best regards,

sincerely,

Zdenek Kostrouch

Immunohistochemical fingerprinting of the network of seven adhesion/growth-regulatory lectins in human skin and detection of distinct tumor-associated alterations

Zdeněk Čada[1,2], Karel Smetana Jr.[1,3*], Lukáš Lacina[1,3,4], Zuzana Plzáková[1,3], Jiří Štork[4], Herbert Kaltner[5], Roland Russwurm[5], Martin Lensch[5], Sabine André[5], and Hans-Joachim Gabius[5]

[1]Charles University, 1st Faculty of Medicine, Institute of Anatomy, U nemocnice 3, 128 00 Prague, Czech Republic

[2]Charles University, 1st Faculty of Medicine, Department of Otorhinolaryngology and Head & Neck Surgery, V Úvalu 84, 150 06 Prague, Czech Republic

[3]Charles University, 2nd Faculty of Medicine, Center of Cell Therapy and Tissue Repair, V Úvalu 84, 150 06 Prague, Czech Republic

[4]Charles University, 1st Faculty of Medicine, Department of Dermatovenerology, U nemocnice 2, 128 21 Prague, Czech Republic

[5]Ludwig-Maximilians-University Munich, Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Veterinärstrasse 13, D-80539 Munich, Germany

Running title: Galectin profiling in human skin

* To whom correspondence should be addressed:

Karel Smetana Jr.

Charles University, 1st Faculty of Medicine, Institute of Anatomy,

U nemocnice 3, 128 00 Prague 2, Czech Republic

E-mail: karel.smetana@lf1.cuni.cz Phone: +00420 2 2496 5873

Summary

Glycans of natural glycoconjugates are considered as source of biological information relevant for cell adhesion or growth. Sugar-based messages are decoded and translated into responses by endogenous lectins. This mechanism assigns a functional dimension to tumor-associated changes of glycosylation. Consequently, it calls for mapping lectin presence in tumors. Such an analysis has so far commonly been performed with the scope to determine expression of few distinct proteins, e.g. from the effector family of galectins with focus on galectins-1 and -3. Due to the emerging evidence for functional divergence among galectins it is timely to address the challenge to evaluate their presence beyond these few family members. Having raised a panel of non-cross-reactive antibodies against seven human galectins covering all three subfamilies, we herein describe their expression profiles in human skin. Comparing normal and malignant tissues enabled us to define galectin-type-dependent alterations, arguing in favor of distinct functionalities. It is concluded that comprehensive monitoring to define the different aspects of the galectin network, as documented in this pilot study, is advisable for future histopathologic studies aimed at delineating clinical correlations.

Key words: apoptosis, galectin, glycohistochemistry, glycoprotein, integrin, stroma, sugar code

Introduction

Biological information transfer relevant for malignancy is mainly monitored on the level of nucleic acids and proteins. Of note, a third class of biomolecules is receiving increasing attention based on the emerging concept of the sugar code (Gabius, 2009). Due to the accumulated evidence on aberrations of glycosylation, the most frequent co- and posttranslational modification of proteins, in tumor cells, essential functions of glycans within the course of the disease are rendered likely (Caselitz, 1987; Hakomori, 1996; Gabius et al., 2002, 2004). As a marked conceptual advance from the initial phenomenologic monitoring of structural glycan modifications a direct link of this aspect to its decoding by tissue receptors (lectins) and ensuing cellular responses has been drawn (Gabius, 2008, 2009). In detail, changes in glycosylation are thus not simply viewed as random events establishing disease-associated parameters but they are assumed to carry a distinct sugar-encoded message. Its information is then converted by protein(lectin)-carbohydrate interactions to eventually trigger specific signaling and glycan-dependent cell activities (Villalobo et al., 2006). As a consequence, detection of lectins as part of tumor characterization offers the perspective to define relevant molecular pathways of information flow. Toward this aim, we herein focus on a family of adhesion/growth-regulatory lectins in a suitable model system.

Squamous epithelium forming the epidermis is morphologically and functionally stratified. Of relevance regarding cell growth, only cells of the basal layer anchored to the basement membrane have the potential to proliferate (Watt, 2002). The cells of the upper layers are terminally differentiated which makes them prone to desquamation (Kanitakis, 2002). The stratification predestines this type of epithelium to serve as suitable model for the study of cell parameters at different levels of cell maturation, as

illustrated by delineating finely tuned glycosylation previously (Holíková et al., 2002). Especially carbohydrate epitopes at branch ends of glycan chains are subject to versatile structural modifications, and these readily accessible β-galactoside determinants can bind to - among others - members of the galectin family. This lectin group is further subdivided into three subclasses (proto-, chimera- and tandem-repeat-type proteins), the individual proteins apparently capable to exert diverse and cell-type-specific roles in the control of cell adhesion, apoptosis, growth and migration via homing in on distinct glycans and also peptide motifs (Kasai and Hirabayashi, 1996; Gabius, 2001; Lahm et al., 2004; Smetana et al., 2006). Their remarkable selectivity for cell surface glycans fulfills the requirement for eliciting distinct responses. For example, cross-linking galectins can modulate growth of different cell types by binding either ganglioside GM1 (neuroblastoma cells), $\alpha_5\beta_1$-integrin (colon and pancreatic carcinoma cells) or the T_H1-specific cell surface molecule Tim-3 (galectins-1, -7 and -9), apical membrane trafficking by binding distinct N-glycans and sulfatide with 2'-hydroxylated long-chain fatty acid (galectin-4) or adhesion (HeLa cells) and cell activity (superoxide production of neutrophils) by binding α_M- (of the $\alpha_M\beta_2$-complex) or $\alpha_3\beta_1$-integrins (galectin-8) (Kopitz et al., 2001, 2003; Levy et al., 2001; Nishi et al., 2003; Delacour et al., 2005; Fischer et al., 2005; Zhu et al., 2005; Stechly et al., 2009). As evident from these cases, galectins appear to be capable to fulfil distinct assignments by virtue of non-identical ligand selection. Thus, they will likely not be functionally redundant, as e.g. shown for activated T cells and induction of different routes of caspase-dependent apoptosis or for neuroblastoma cells and a functional divergence toward inhibition of proliferation (Kopitz et al., 2001; Sturm et al., 2004; André et al., 2005a; Stillman et al., 2006). These results intimate to draw an analogy for galectins to the complexities of

integrin expression and functionality.

It is therefore a key step on the way to understand the operativity of the assumed galectin network to define the expression profiles of different family members in tumor tissues. The development of non-cross-reactive antibodies will make it possible to move from initial biochemical and RT-PCR profiling of galectin expression in tumor tissues and cell lines (Gabius et al., 1984, 1986; Lahm et al., 2001) to immunohistochemical analysis not restricted to only one or two members of this family, commonly galectins-1 and/or -3. The extended monitoring should ideally comprise members of all three galectin categories. We herein report results of a pilot study on immunohistochemical analysis of expression of human galectins-1, -2, -3, -4, -7, -8 and -9 (for classification into subfamilies, please see Table 1; of note, presence of genes for galectins-5 and -6 is restricted to the rat or mouse, respectively (Cooper, 2002)). Specimen of normal skin and basal cell carcinomas were processed with a respective panel of non-cross-reactive antibodies under identical conditions.

Materials and methods

Six specimens of normal skin from breast and seventeen samples of basal cell carcinoma of the skin were obtained with informed consent of donors either from the Department of Plastic and Reconstructive Surgery (Charles University, 3rd Faculty of Medicine, Prague) or from the Department of Dermatovenerology. Tissue samples were immediately protected by Tissue-Tek (Sakkura, Zoeterwoude, The Netherlands), frozen in liquid nitrogen and stored at -80 °C until further processing. Seven-µm-thin sections were obtained using a Cryocut-E microtome (Reicher-Jung, Vienna, Austria). The

sections were mounted onto poly-L-lysine (Sigma-Aldrich, Prague, Czech Republic)-coated glass slides, rehydrated with 20 mM phosphate-buffered saline (PBS, pH 7.3) and fixed with 2 % (w/v) paraformaldehyde in PBS for five minutes. Washed sections were first processed with albumin-containing solution to block any sites with non-specific protein-binding capacity to minimize protein adsorption during the next steps. The applied galectin-type-specific polyclonal antibodies had been systematically tested for specificity and lack of cross-reactivity, with affinity depletion being performed by affinity chromatography in each positive case followed by another round of controls by ELISA (Kayser et al., 2003; Nagy et al., 2003; Saal et al., 2005; Lensch et al., 2006; Langbein et al., 2007; Dvoránková et al., 2008). They were used at the constant concentration of 20 µg/ml. After extensive washing with PBS to remove unbound antibody, FITC-labeled swine-anti-rabbit antibody (SwAR-FITC; AlSeVa, Prague, Czech Republic) diluted as recommended by the producer was used as second-step reagent. 4',6'-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Prague, Czech Republic) facilitated to stain nuclei in the sections. To ascertain absence of antigen-independent binding of the tested immunoglobulin G fractions, for example via binding of the F_c part of the antibody to F_c receptors in the tissue, galectin-type-specific antibodies were replaced by a rabbit poly-clonal antibody raised against the tandem-repeat-type mannose receptor, which is not present in this epithelial tissue. Further controls to spot any antigen-independent staining reaction were performed by omitting the incubation with first-step reagent from the protocol. After finishing routine processing under conditions carefully kept constant throughout this study specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent the fluorescent signal from bleaching by UV-light. A fluorescence microscope (Nikon Eclipse 90i; Nikon, Prague, Czech Republic) equipped

with filterblocks specific for the optical properties of FITC and DAPI and a high-resolution cooled CCD camera (Cool-1300Q CCD camera; Vosskühler, Osnabrück, Germany) and a computer-assisted image analyzer (LUCIA 5.10; Laboratory Imaging, Prague, Czech Republic).were employed for image analysis and data storage. Data were further processed to visualize quantitative aspects of the immunofluorescence staining (Dubový et al., 2002; Purkrábková et al., 2003; Cada et al., 2009), and calculations using the Student´s non-paired t-test led to assessment of significance levels for differences between cell types.

Results

The parallel monitoring of frozen sections of human epidermis after processing with the seven antibody preparations was performed to address the issue on extent of complexity of galectin presence. The results which were obtained attested the validity of the concept to extend the range of galectin detection to more than one or two proteins. In fact, our antibody panel enabled to detect the presence of all seven different galectins tested immunohistochemically, as exemplarily illustrated in Figs. 1-3. Under the given experimental conditions, which were rigorously kept constant throughout this comparative study, reactivity was mostly cytoplasmic and present at cell surfaces, except for nuclear staining of cells of the basal and spinous layers with the anti-galectin-7-specific antibody fraction (Fig. 1G). Monitoring of staining intensity revealed the intensity pattern listed in Table 1, galectin-1 being an exception when examining normal skin epithelium (Fig. 1). In this instance, a strong signal was recorded in the extracellular matrix of the dermis (Fig. 1A). An obvious difference regarding zonal cell positioning was noted in the case of galectin-9 and basal/suprabasal cells (Fig. 3, Table 1). Having

hereby provided results on the normal tissue, we proceeded to analyze a malignant counterpart.

The manifestation of basal cell carcinomas led to a conspicuous and rather uniform decrease of galectin presence, except for galectin-1 (Figs. 1-3, Table 1). However, stromal reactivity was still detectable, and its extent was higher in tumor tissue than in normal skin for galectin-1 (Fig. 1, Fig. 2). Underscoring operativity of differential regulatory mechanisms, the opposite situation was measured for galectins-4, -7 and -8 (Fig. 2). Not exceeding background values, monitoring presence of galectins-2, -3 and -9 practically led to no evidence for stromal expression in this tumor type, further clear evidence for disparate regulation.

Discussion

This study focused on endogenous lectins of the galectin family, because i) they are emerging effectors in the regulation of diverse cell activities, with a range of functions reaching the clinical level, as e.g. documented for clonal selection of $CD4^+CD7^-$ vs. $CD4^+CD7^+$ leukemic T cells in patients during progression of the Sézary syndrome by galectin-1 and relation of tumor suppressor $p16^{INK4a}$ to this lectin (Rappl et al., 2002; André et al., 2007a) and ii) they can sense changes in glycan structure at branch ends or the core region, which can accompany malignancy (Ahmad et al., 2002; Hirabayashi et al., 2002; André et al., 2004; 2005b, 2007b). By using a panel of non-cross-reactive antibodies against seven galectins covering all three subfamilies we moved beyond the scope of previous studies in this area (Smetana et al., 2006). Our panel enabled us to answer the pertinent questions on the extent of complexity of expression profiles and of tumor-associated changes. As model system, we tested human epidermis and basal cell

carcinomas.

It is now clear that the galectin network, at least in these cell types, is not restricted to only few activities. Our strategy therefore makes a strong case for introducing the profiling, as carried out here, to further investigations and hereby gives research in this field a clear direction. Correlation of expression patterns to particular cellular properties may then aid in delineating functional aspects for certain galectins undercovering new clinical implications. In this respect, the relationship between galectin-9 presence in basal/suprabasal cells and proliferation may indicate a distinct role of this protein. Interestingly, galectin-9 is strongly expressed in nasopharyngeal carcinomas, down-regulated in oral and cervical squamous cell carcinoma cases/lines and an inducer of apoptosis of MM-RU melanoma cells (Kageshita et al., 2002; Kasamatsu et al., 2005; Pioche-Durieu et al., 2005; Liang et al., 2008). Tumor-associated changes detected in our study pertain to dermal and stromal cells in a galectin-type-dependent manner. These results extend previous observations in the cases of basal and squamous cell carcinomas (Lacina et al., 2007; Saussez et al., 2009a,b). Of note, inverse shifts between nuclear and cytoplasmic localization had even been noted for galectins from the same subgroup upon tumor progression (Saussez et al., 2006, 2008).

These results argue in favor of at least partial functional non-redundancy among galectins. This interpretation is supported by previous immunohistochemical observations on colon carcinomas, especially for the tandem-repeat-type galectins -4 and -8 and monitoring expression of proto- and chimera-type and tandem-repeat-type galectins in murine tissue (Nagy et al., 2002, 2003; Lohr et al., 2007, 2008; Nio-Kobayashi et al., 2009). Evidently, even members of the same subfamily can reveal

functional divergence, a finding warranting thorough analysis of tissue sections or arrays. Such studies appear to harbor a noteworthy advantage for figuring out exploitable correlations compared to work with engineered cell systems: the manipulation of levels of galectin expression *in vitro*, a common tool to track down clues for functions, can entail alterations of expression levels of diverse other proteins such as integrins or cadherins, as seen in respective studies with galectins-1 and -3 (Warfield et al., 1997; Matarrese et al., 2000; Camby et al., 2005; Mourad-Zeidan et al., 2008). This detected effect confounds to reach an unambiguous conclusion on galectin functionality from such models, making immunohistochemical monitoring of clinical specimen indispensable.

Because lectin activity is governed by the protein's binding to ligands (glycoconjugates or peptide motifs), it is tempting to add a methodological aspect, which complements this research line. Explicitly, galectins can not only be detected immunohistochemically but also be employed as histochemical tools. This study design with a labeled tissue lectin, performed recently in the case of galectin-3 and skin, has already also provided clinical correlations by the analysis of fixed sections of tumors from head and neck cancer patients (Delorge et al., 2000; Plzák et al., 2002, 2004; Chovanec et al., 2005; Szabo et al., 2009). The results presented herein, together with this perspective, underscore the potential of comprehensive mapping of lectin-related parameters in the quest to define new molecular aspects relevant for the course of the disease.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sport of the Czech

Republic (project No. MSM 0021620806), the Mizutani Foundation for Glycoscience (Tokyo, Japan) and an EC Marie Curie Research Training Network grant (contract No. HRTN-CT-2005-019561). Authors are grateful to Eva Vancová and Iva Burdová for excellent technical assistance and Dr. R. Hase for helpful discussion.

References

Ahmad N., Gabius H.-J., Kaltner H., André S., Kuwabara I., Liu F.-T., Oscarson S., Norberg T., Brewer C.F. (2002) Thermodynamic binding studies of cell surface carbohydrate epitopes to galectins-1, -3, and -7: evidence for differential binding specificities. *Can J Chem.* **80,** 1096-1104.

André S., Unverzagt C., Kojima S., Frank M., Seifert J., Fink C., Kayser K., von der Lieth C.-W., Gabius H.-J. (2004) Determination of modulation of ligand properties of synthetic complex-type biantennary N-glycans by introduction of bisecting GlcNAc *in silico, in vitro* and *in vivo*. *Eur J Biochem.* **271,** 118-134.

André S., Kaltner H., Lensch M., Russwurm R., Siebert H.-C., Fallsehr C., Tajkhorshid E., Heck A.J.R., von Knebel-Doeberitz M., Gabius H.-J., Kopitz J. (2005a) Determination of structural and functional overlap/divergence of five proto-type galectins by analysis of the growth-regulatory interaction with ganglioside GM_1 *in silico* and *in vitro* on human neuroblastoma cells. *Int J Cancer.* **114,** 46-57.

André S., Kojima S., Prahl I., Lensch M., Unverzagt C., Gabius H.-J. (2005b) Introduction of extended LEC14-type branching into core-fucosylated biantennary N-

glycan. Glycoengineering for enhanced cell binding and serum clearance of the neoglycoprotein. *FEBS J.* **272,** 1986-1998.

André S., Kozár T., Schuberth R., Unverzagt C., Kojima S., Gabius H.-J. (2007a) Substitutions in the N-glycan core as regulators of biorecognition: the case of core-fucose and bisecting GlcNAc moieties. *Biochemistry* **46,** 6984-6995.

André S., Sanchez-Ruderisch H., Nakagawa H., Buchholz M., Kopitz J., Forberich P., Kemmner W., Böck C., Deguchi K., Detjen K.M., Wiedenmann B., von Knebel-Döberitz M., Gress T.M., Nishimura S.-I., Rosewicz S., Gabius H.-J. (2007b) Tumor suppressor $p16^{INK4a}$: modulator of glycomic profile and galectin-1 expression to increase susceptibility to carbohydrate-dependent induction of anoikis in pancreatic carcinoma cells. *FEBS J.* **274,** 3233-3256.

Cada, Z., Chovanec M., Smetana K., Betka J., Lacina L., Plzak J., Kodet R., Stork J., Lensch M., Kaltner H., Andre S., Gabius H.J. (2009) Galectin-7: will the lectin's activity establish clinical correlations in head and neck squamous cell and basal cell carcinomas? *Histol. Histopathol.* **24,** 41-48.

Camby I., Decaestecker C., Lefranc F., Kaltner H., Gabius H.-J., Kiss R. (2005) Galectin-1 knocking down in human U87 glioblastoma cells alters their gene expression pattern. *Biochem Biophys Res Commun.* **335,** 27-35.

Caselitz J. (1987) Lectins and blood group substances as "tumor markers". *Curr Top Pathol.* **77,** 245-278.

Chovanec M., Smetana K. Jr., Betka J., Plzák J., Brabec J., Moya-Álvarez V., André S., Kodet R., Gabius H.-J. (2005) Correlation of expression of nuclear proteins pKi67 and p63 with lectin histochemical features in head and neck cancer. *Int J Oncol.* **27,** 409-415.

Cooper D.N.W. (2002) Galectinomics: finding themes in complexity. *Biochim Biophys Acta.* **1572,** 209-231.

Delacour D., Gouyer V., Zanetta J.-P., Drobecq H., Leteurtre E., Grard G., Moreau-Hannedouche O., Maes E., Pons A., André S., Le Bivic A., Gabius H.-J., Manninen A., Simons K., Huet G. (2005) Galectin-4 and sulfatides in apical membrane trafficking in enterocyte-like cells. *J Cell Biol.* **169,** 491-501.

Delorge S., Saussez S., Pelc P., Devroede B., Marchant H., Burchert M., Zeng F.-Y., Danguy A., Salmon I., Gabius H.-J., Kiss R., Hassid S. (2000) Correlation of galectin-3/galectin-3-binding sites with low differentiation status in head and neck squamous cell carcinomas. Otolaryngol Head Neck Surg. **122,** 834-841.

Dubový P., Svíženská I., Klusáková I. (2002) Computer-assisted quantitative analysis of immunofluorescence staining of the extracellular matrix in rat dorsal and ventral spinal roots. *Acta Histochem.* **104,** 371-374.

Dvoránková B., Lacina L., Smetana K. Jr., Lensch M., Manning J.C., André S., Gabius H.-J. (2008) Human galectin-2: nuclear presence *in vitro* and its modulation by quiescence/stress factors. *Histol. Histopathol.* **23,** 167-178.

Fischer C., Sanchez-Ruderisch H., Welzel M., Wiedenmann B., Sakai T., André S., Gabius H.-J., Khachigian L., Detjen K.M., Rosewicz S. (2005) Galectin-1 interacts with the $\alpha_5\beta_1$ fibronectin receptor to restrict carcinoma cell growth via induction of p21 and p27. *J Biol Chem.* **280,** 37266-37277.

Gabius H.-J. (2001) Glycohistochemistry: the why and how of detection and localization of endogenous lectins. *Anat Histol Embryol.* **30,** 3-31.

Gabius H.J. (2008) Glycans: bioactive signals decoded by lectins. *Biochem. Soc. Trans.* **36,** 1491-1496.

Gabius H.-J. (2009) The Sugar Code. Fundamentals of glycosciences pp. in press. Wiley-VCH: Weinheim, Germany.

Gabius H.-J., Engelhardt R., Rehm S., Cramer F. (1984) Biochemical characterization of endogenous carbohydrate-binding proteins from spontaneous murine rhabdomyosarcoma, mammary adenocarcinoma, and ovarian teratoma. *J. Natl. Cancer Inst.* **73,** 1349-1357.

Gabius H.-J., Engelhardt R., Sartoris D.J., Cramer F. (1986) Pattern of endogenous lectins of a human sarcoma (Ewing´s sarcoma) reveals differences to human normal tissues and tumors of epithelial and germ cell origin. *Cancer Lett.* **31,** 139-145.

Gabius H.-J., André S., Kaltner H., Siebert H.-C. (2002) The sugar code: functional lectinomics. *Biochim Biophys Acta.* **1572,** 165-177.

Gabius H.-J., Siebert H.-C., André S., Jiménez-Barbero J., Rüdiger H. (2004) Chemical biology of the sugar code. *ChemBioChem.* **5,** 740-764.

Hakomori S.-I. (1996) Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res.* **56,** 5309-5318.

Hirabayashi J., Hashidate T., Arata Y., Nishi N., Nakamura T., Hirashima M., Urashima T., Oka T., Futai M., Müller W.E.G., Yagi F., Kasai K.-I. (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim Biophys Acta.* **1572,** 232-254.

Holíková Z., Hrdlicková-Cela E., Plzák J., Smetana K. Jr., Betka J., Dvoránková B., Esner M., Wasano K., André S., Kaltner H., Motlík J., Hercogová J., Kodet R., Gabius H.-J. (2002) Defining the glycophenotype of squamous epithelia using plant and mammalian lectins. Differentiation-dependent expression of α2,6- and α2,3-linked N-acetylneuraminic acid in squamous epithelia and carcinomas, and its differential effect on binding of the endogenous lectins galectins-1 and -3. *APMIS* **110,** 845-856.

Kageshita T., Kashio Y., Yamauchi A., Seki M., Abedin M.J., Nishi N., Shoji H., Nakamura T., Ono T., Hirashima M. (2002) Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. *Int J Cancer.* **99,** 809-816.

Kanitakis J. (2002) Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol.* **12,** 390-399.

Kasai K.-I., Hirabayashi J. (1996) Galectins: a family of animal lectins that decipher glycocodes. *J Biochem (Tokyo).* **119,** 1-8.

Kasamatsu A., Uzawa K., Nakashima D., Koike H., Shiiba M., Bukawa H., Yokoe H., Tanzawa H. (2005) Galectin-9 as a regulator of cellular adhesion in human oral squamous cell carcinoma cell lines. *Int J Mol Med.* **16,** 269-273.

Kayser K., Hoeft D., Hufnagl P., Caselitz J., Zick Y., André S., Kaltner H., Gabius H.-J. (2003) Combined analysis of tumor growth pattern and expression of endogenous lectins as a prognostic tool in primary testicular cancer and its lung metastases. *Histol Histopathol.* **18,** 771-779.

Kopitz J., von Reitzenstein C., André S., Kaltner H., Uhl J., Ehemann V., Cantz M., Gabius H.-J. (2001) Negative regulation of neuroblastoma cell growth by carbohydrate-dependent surface binding of galectin-1 and functional divergence from galectin-3. *J Biol Chem.* **276,** 35917-35923.

Kopitz J., André S., von Reitzenstein C., Versluis K., Kaltner H., Pieters R.J., Wasano K., Kuwabara I., Liu F.-T., Cantz M., Heck A.J.R., Gabius H.-J. (2003) Homodimeric galectin-7 (p53-induced gene 1) is a negative growth regulator for human neuroblastoma cells. *Oncogene.* **22,** 6277-6288.

Lacina L., Smetana K. Jr., Dvořánková B., Pytlík R., Kideryová L., Kučerová, L., Plzáková Z., Štork J., Gabius H.-J., André S. (2007) Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. *Brit J Dermatol.* **156,** 819-829,

2007.

Lahm H., André S., Hoeflich A., Fischer J.R., Sordat B., Kaltner H., Wolf E., Gabius H.-J. (2001) Comprehensive galectin fingerprinting in a panel of 61 human tumor cell lines by RT-PCR and its implications for diagnostic and therapeutic procedures. *J Cancer Res Clin Oncol.* **127,** 375-386.

Lahm H., André S., Hoeflich A., Kaltner H., Siebert H.-C., Sordat B., von der Lieth C.-W., Wolf E., Gabius H.-J. (2004) Tumor galectinology: insights into the complex network of a family of endogenous lectins. *Glycoconj J.* **20,** 227-238.

Langbein S., Brade J., Badawi J.K., Hatzinger M., Kaltner H., Lensch M., Specht K., André S., Brinck U., Aiken P., Gabius H.-J. (2007) Gene-expression signature of adhesion/growth-regulatory tissue lectins (galectins) in transitional cell cancer and its prognostic relevance. *Histopathology* **51,** 681-690.

Lensch M., Lohr M., Russwurm R., Vidal M., Kaltner H., André S., Gabius H.-J. (2006) Unique sequence and expression profiles of rat galectins-5 and -9 as a result of species-specific gene divergence. *Int. J. Biochem. Cell Biol.* **38,** 1741-1758.

Levy Y., Arbel-Goren R., Hadari Y.R., Eshhar S., Ronen D., Elhanany E., Geiger B., Zick Y. (2001) Galectin-8 functions as a matricellular modulator of cell adhesion. *J Biol Chem.* **276,** 31285-31295.

Liang M., Ueno M., Oomizu S., Arikawa T., Shinonaga R., Zhang S., Yamauchi A., Hirashima M. (2008) Galectin-9 expression links to malignant potential of cervical

squamous cell carcinoma. *J. Cancer Res. Clin. Oncol.* **134,** 899-907.

Lohr M., Lensch M., Andr S., Kaltner H., Siebert H.-C., Smetana K. Jr., Sinowatz F., Gabius H.-J. (2007) Murine homodimeric adhesion/growth-regulatory galectins-1, -2, and -7: comparative profiling of gene/promoter sequences by database mining, of expression by RT-PCR/immunohistochemistry and of contact sites for carbohydrate ligands by computational chemistry. *Folia Biol.* **53,** 109-128.

Lohr M., Kaltner, H., Lensch M., André S., Sinowatz F., Gabius H.-J. (2008) Cell-type-specific expression of murine multifunctional galectin-3 and its association with follicular atresia/luteolysis in contrast to pro-apoptotic galectins-1 and -7. *Histochem. Cell Biol.* **130,** 567-581.

Matarrese P., Fusco O., Tinari N., Natoli C., Liu F.-T., Semeraro M.L., Malorni W., Iacobelli S. (2000) Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties. *Int J Cancer.* **85,** 545-554.

Mourad-Zeidan A.A., Melnikova V.O., Wang H., Raz A., Bar-Eli M. (2008) Expression profiling of galectin-3-depleted melanoma cells reveals its major role in melanoma plasticity and vasculogenic mimicry. *Am. J. Pathol.* **173,** 1839-1852.

Nagy N., Bronckart Y., Camby I., Legendre H., Lahm H., Kaltner H., Hadari Y., Van Ham P., Yeaton P., Pector J.-C., Zick Y., Salmon I., Danguy A., Kiss R., Gabius H.-J. (2002) Galectin-8 expression decreases in cancer compared with normal and dysplastic human colon tissue and acts significantly on human colon cancer cell migration as a suppressor. *Gut.* **50,** 392-401.

Nagy N., Legendre H., Engels O., André S., Kaltner H., Wasano K., Zick Y., Pector J.-C., Decaestecker C., Gabius H.-J., Salmon I., Kiss R. (2003) Refined prognostic evaluation in colon carcinoma using immunohistochemical galectin fingerprinting. *Cancer.* **97,** 1849-1858.

Nio-Kobayashi J., Takahashi-Iwanaga H., Iwanaga T. (2009) Immunohistochemical localization of six galectin subtypes in the mouse digestive tract. *J. Histochem. Cytochem.* **57,** 41-50.

Nishi N., Shoji H., Seki M., Itoh A., Miyanaka H., Yuube K., Hirashima M., Nakamura T. (2003) Galectin-8 modulates neutrophil function via interaction with integrin α_M. *Glycobiology.* **13,** 755-763.

Pioche-Durieu C., Keryer C., Souquere S., Bosq J., Faigle W., Loew D., Hirashima M., Nishi N., Middeldorp J., Busson P. (2005) In nasopharyngeal carcinoma cells, Epstein-Barr virus LMP1 interacts with galectin-9 in membrane raft elements resistant to simvastatin. *J Virol.* **79,** 13326-13337.

Plzák J., Holíková Z., Smetana K. Jr., Dvořánková B., Hercogová J., Kaltner H., Motlík J., Gabius H.-J. (2002) Differentiation-dependent glycosylation of cells in squamous epithelia detected by a mammalian lectin. *Cells Tissues Organs.* **171,** 135-144.

Plzák J., Betka J., Smetana K. Jr., Chovanec M., Kaltner H., André S., Kodet R., Gabius H.-J. (2004) Galectin-3: an emerging prognostic indicator in advanced head and neck carcinoma. *Eur J Cancer.* **40,** 2324-2330.

Purkrábková T., Smetana K. Jr., Dvořánková B., Holíková Z., Böck C., Lensch M., André S., Pytlík R., Liu F.-T., Klíma J., Smetana K., Motlík J., Gabius H.-J. (2003) New aspects of galectin functionality in nuclei of cultured bone marrow of stromal and epidermal cells: biotinylated galectins as tool to detect specific binding sites. *Biol Cell.* **95,** 535-545.

Rappl G., Abken H., Muche J.M., Sterry W., Tilgen W., André S., Kaltner H., Ugurel S., Gabius H.-J., Reinhold U. (2002) $CD4^{+}CD7^{-}$ leukemic T cells from patients with Sézary syndrome are protected from galectin-1-triggered T cell death, *Leukemia.* **16,** 840-845.

Saal I., Nagy N., Lensch M., Lohr M., Manning J.C., Decaestecker C., André S., Kiss R., Salmon I., Gabius H.-J. (2005) Human galectin-2: expression profiling by RT-PCR/immunohistochemistry and its introduction as a histochemical tool for ligand localization. *Histol Histopathol.* **20,** 1191-1208.

Saussez S., Cucu D.-R., Decaestecker C., Chevalier D., Kaltner H., André S., Wacreniez A., Toubeau G., Camby I., Gabius H.-J., Kiss R. (2006) Galectin-7 (p53-induced gene-1): a new prognostic predictor of recurrence and survival in stage IV hypopharyngeal cancer. *Ann. Surg. Oncol.* **13,** 999-1009.

Saussez S., Decaestecker C., Lorfevre F., Chevalier D., Mortuaire G., Kaltner H., André S., Toubeau G., Gabius H.-J., Leroy X. (2008) Increased expression and altered intracellular distribution of adhesion/growth-regulatory lectins galectins-1 and -7 during tumour progression in hypopharyngeal and laryngeal squamous cell carcinomas. *Histopathology* **52,** 483-493.

Saussez S., Cludts S., Capouillez A., Mortuaire G., Smetana K. Jr., Kaltner H., Andre S., Leroy X., Gabius H.J., Decaestecker C. (2009a) Identification of matrix metalloproteinase-9 as an independent prognostic marker in laryngeal and hypopharyngeal cancer with opposite correlations to adhesion/growth-regulatory galectins-1 and -7. *Int. J. Oncol.* **34,** 433-439.

Saussez S., Decaestecker C., Cludts S., Ernoux P., Chevalier D., Smetana K. Jr., André S., Leroy X., Gabius H.-J. (2009b) Adhesion/growth-regulatory tissue lectin galectin-1 in relation to angiogenesis/lymphocyte infiltration and prognostic relevance of stromal up-regulation in laryngeal carcinomas. *Anticancer Res.* **29,** 59-66.

Smetana K. Jr., Dvoránková B., Chovanec M., Boucek J., Klima J., Motlík J., Lensch M., Kaltner H., André S., Gabius H.-J. (2006) Nuclear presence of adhesion/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin. *Histochem. Cell Biol.* **125,** 171-182.

Stechly L., Morelle W., Dessein A.F., Andre S., Grard G., Trinel D., Dejonghe M.J., Leteurtre E., Drobecq H., Trugnan G., Gabius H.J., Huet G. (2009) Galectin-4-regulated delivery of glycoproteins to the brush border membrane of enterocyte-like cells. *Traffic* **10,** 438-450.

Stillman B.N., Hsu D.K., Pang M., Brewer C.F., Johnson P., Liu F.-T., Baum L.G. (2006) Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J Immunol.* **176,** 778-789, 2006.

Sturm A., Lensch M., André S., Kaltner H., Wiedenmann B., Rosewicz S., Dignass A.U., Gabius H.-J. (2004) Human galectin-2: novel inducer of T cell apoptosis with distinct profile of caspase activation. *J Immunol.* **173,** 3825-3837.

Szabo P., Dam T.K., Smetana K. Jr., Dvoránková B., Kübler D., Brewer C.F., Gabius H.-J. (2009) Phosphorylated human lectin galectin-3: analysis of ligand binding by histochemical monitoring of normal/malignant squamous epithelia and by isothermal titration calorimetry. *Anat. Histol. Embryol.* **38,** 68-75.

Villalobo A., Nogales-González A., Gabius H.-J. (2006) A guide to signaling pathways connecting protein-glycan interaction with the emerging versatile effector functionality of mammalian lectins. *Trends Glycosci Glycotechnol.* **18,** 1-37.

Warfield P.R., Makker P.-N., Raz A., Ochieng J. (1997) Adhesion of human breast carcinoma to extracellular matrix proteins is modulated by galectin-3. *Invasion Metastasis.* **17,** 101-112.

Watt F.M. (2002) The stem cell compartment in human interfollicular epidermis. *J Dermatol Sci.* **28,** 173-180.

Zhu C., Anderson A.C., Schubart A., Xiong H., Imitola J., Khoury S.J., Zheng X.X., Strom T.B., Kuchroo V.K. (2005) The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol.* **6,** 1245-1252.

e 1. Overview on galectin fingerprinting in normal and transformed squamous epithelium

	Epidermis		
	basal cells	suprabasal cells	
ctin-1 (proto-type)	-	-	-[a]/+*
ctin-2 (proto-type)	++	++	-*
ctin-3 (chimera-type)	++	++	-*
ctin-4 (tandem-repeat)	+++	+++	-*
ctin-7 (proto-type)	+++	+++	-[a]/+*
ctin-8 (tandem-repeat)	+++	+++	-[a]/+*
ctin-9 (tandem-repeat)	+++	+*	+/++[a]*

signal, +: weak but significant positivity, ++: medium positivity, +++: strong positivity, ++++: very strong positivity; [a]predomina
ory in cases with inter- and intraindividual variability, *statistically significant decrease/increase in comparison betwe
ent types of normal cell and between normal and malignant cells

Legends to figures

Fig. 1. Immunohistochemical detection of different galectins from the three subfamilies, i.e. galectins-1 (A, B), -3 (C, D), -4 (E, F) and -7 (G, H), in frozen sections of normal human epidermis (Ep; A, C, E, G) and basal cell carcinoma (BCC; B, D, F, H). Insert in G shows nucleolar positivity for galectin-7, nucleoli being indicated by two black arrows. Epithelial knots of basal cell carcinoma in D, F and H are encircled. Bar: 100 µm.

Fig. 2. Quantitation of fluorescence intensity in immunohistochemical detection for the different galectins from the three subfamilies, i.e. galectins-1 (A), -2 (B), -3 (C), -4 (D), -7 (E), -8 (F) and -9 (G), in cells of normal epidermis (termed Cells, white column) and of basal cell carcinomas (termed BCC Cells, black column) as well as in dermis (termed Dermis, white column) and the stroma of tumors (termed BCC Stroma, black column). Statistical significance of differences between signal characteristics of normal and malignant tissues was evaluated by the Student's non-paired t-test. Results are given at significance levels of $p<0.01$, $p<0.02$ and $p<0.05$, respectively, a cross denoting an increase and asterisks the decrease of extent of signal intensity. The background level measured in each case is either marked by a bold line (Cells/BCC Cells) or a dashed line (Dermis/BCC Stroma) in each panel.

Fig. 3. Immunohistochemical detection of galectin-9 in frozen sections of epidermis (Ep; A, B) and a basal cell carcinoma (BCC; C). The white arrows in panels A denote the length of the analyzed tissue sector and the direction of quantitative

fluorescence profiling illustrated in panel B. For further clarity on the regions under scrutiny, two sets of white (A) and black (C) arrows mark corresponding positions in the sections and the profiles. The background level is depicted by a bold line in panel B. The tumor area is encircled by a dashed line in panel C. Bar: 100 µm.

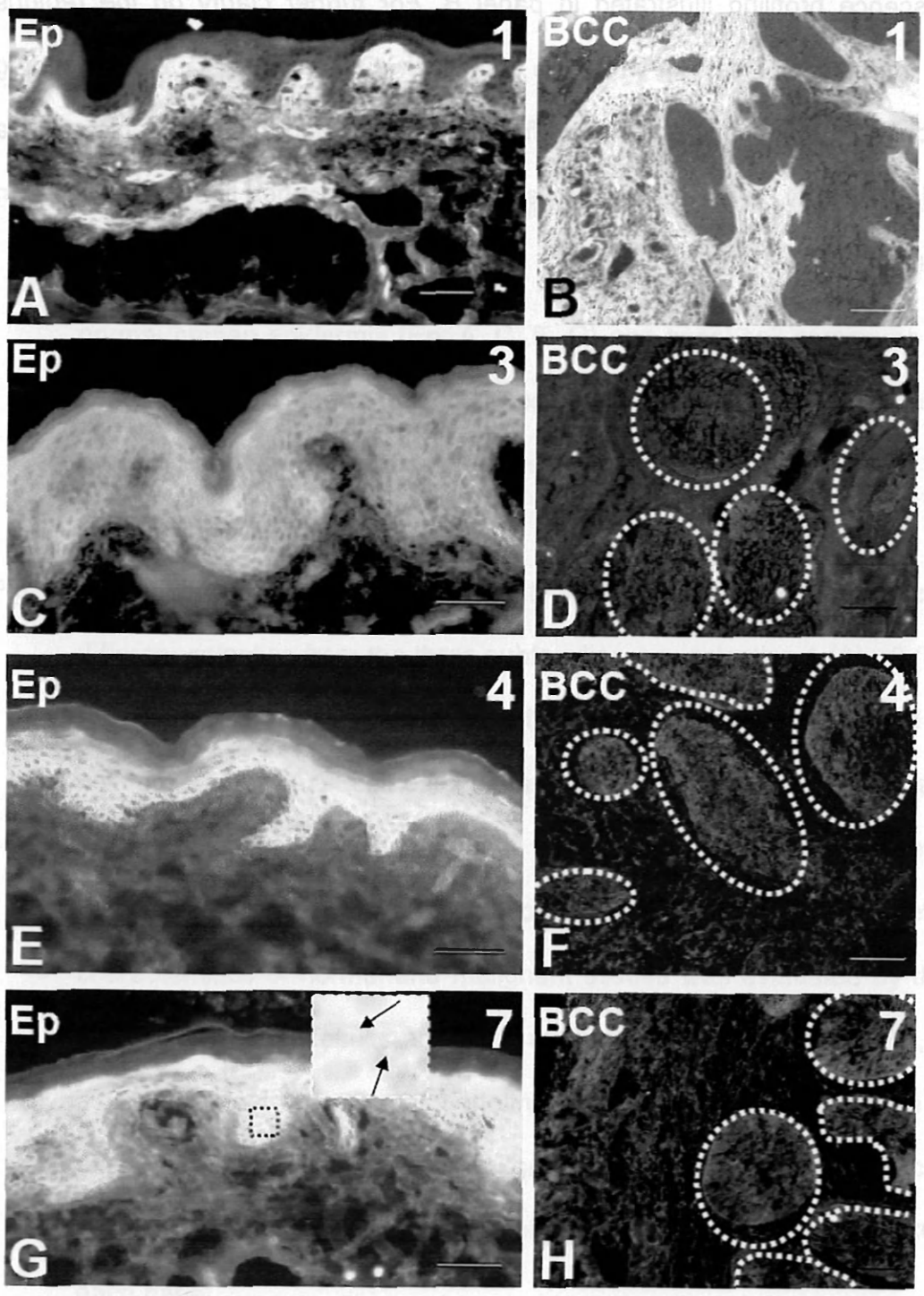


Fig.1

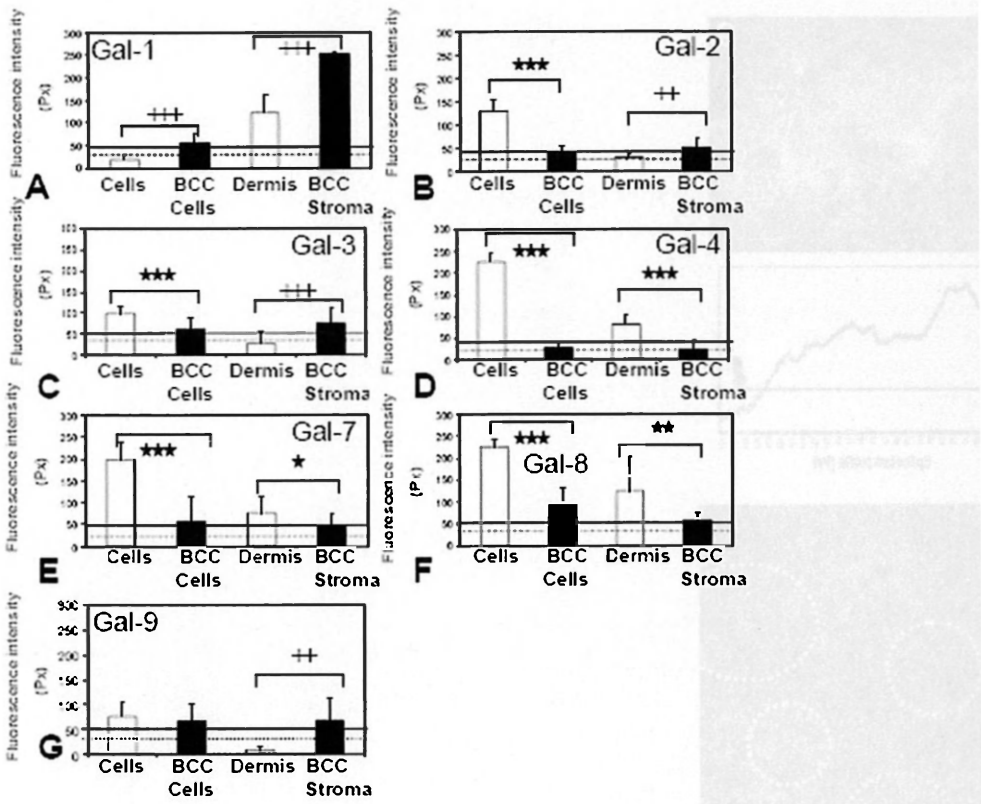

Gal-1
Gal-2
Gal-3
Gal-4
Gal-7
Gal-8
Gal-9
Fluorescence intensity (Px)
Cells
BCC Cells
Dermis
BCC Stroma
A
B
C
D
E
F
G

Fig 2

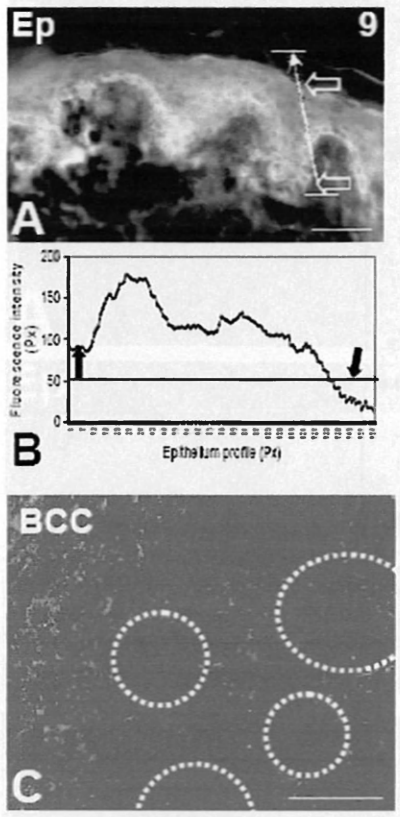

Ep
9
A
200
150
100
50
0
Fluorescence intensity (Px)
Epithelium profile (Px)
B
BCC
C

Fig 3