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DIZERTAČNÍ PRÁCE

SPECIALIZACE: MOLEKULÁRNÍ A BUNĚČNÁ BIOLOGIE, GENETIKA A VIROLOGIE

Geneticky modifikované buněčné vakcíny proti *bcr-abl*-transformovaným buňkám

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Prohlášení

Čestně prohlašuji, že tuto dizertační práci jsem vypracovala samostatně s použitím uvedené literatury. Dále prohlašuji, že tato práce ani její jednotlivé části nebyly použity k získání stejného nebo podobného akademického titulu.

Praha 2012

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Na závěr chci vyjádřit vděčnost mé rodině za jejich podporu, pomoc a lásku.

Seznam použitých zkratek

AAV - Adeno Associated Virus
AICD – Activation-Induced Cell Death
ABL - Abelson murine leukemia viral oncogene homolog
ADCC – Antibody-dependent Cell-mediated Cytotoxicity
ALL - Acute Lymphoblastic Leukemia
APC - Antigen Presenting Cells
ATP – Adenosine Triphosphate
BCG – Bacillus Calmete-Guérin
BCR - Breakpoint Cluster Region
BrdU - Bromdeoxyuridine
CAF – Carcinoma-Associated Fibroblast
CAR – Chimeric Antigen Receptor
CCL – C-C motif Chemokine Ligand
CD – Cluster of Differentiation
COX - Cyclooxygenase
CML - Chronic Myeloid Leukemia
cTK – cellular Thymidine Kinase
CTL – Cytotoxic T-Lymphocyte
CTLA – Cytotoxic T-Lymphocyte Antigen
CXCL – C-X-C motif Chemokine Ligand
Cy - Cyclophosphamide
DAMP – Damage-Associated Molecular Pattern
DC - Dendritic Cell
DNA – Deoxyribonucleid Acid
DTH – Delayed-Type Hypersensitivity
EGF – Epidermal Growth Factor
Fas – apoptosis antigen 1
FasL – Fas Ligand
FCS – Fetal Calf Serum
FLT3 – Fms related tyrosine kinase 3
FOXP3 – Forkhead box P3
FSP1 – Fibroblast Secreted Protein 1

G250 - Carbonic anhydrase IX

G-CSF – Granulocyte Colony-Stimulating Factor

GCV - Ganciclovir

GFP - Green Fluorescent Protein

GM-CSF – Granulocyte-Macrophage Colony-Stimulating Factor

GMO – Genetically Modified Organism

GVAX – GM-CSF gene-transduced tumor vaccine

GvHD – Graft versus Host Disease

HAT – Hypoxanthine – Aminopterin - Thymidine

HLA – Human Leukocyte Antigen

HSP – Heat Shock Protein

HSV TK – Herpes Simplex Virus Thymidine Kinase

hTERT - human Telomerase Reverse Transcriptase

IDO – Indoleamine 2,3-dioxygenase

IFN - Interferon

IL – Interleukin

iNOS – inducible Nitric Oxide Synthase

IM – Imatinib Mesylate

IP-10 - Interferon gamma-induced Protein 10

IRES – Internal Ribosome Entry Site

LAA – Leukemia-Associated Antigen

LAG-3 – Lymphocyte Activation Gene 3

MHC – Major Histocompability Complex

MDSC – Myeloid Derived Suppressor Cells

MFG-E8 – Milk Fat Globule-Epidermal Growth Factor protein 8

MRD – Minimal Residual Disease

MTD – Maximum Tolerated Dose

MMP – Matrix MetalloProteinase

NK – Natural Killer

NKT – Natural Killer T lymphocyte

Nor-NOHA – N(omega)-hydroxy-nor-L-arginine

PAMP – Pathogen-Associated Molecular Pattern

PD-1 – Programmed cell Death receptor 1

PD-L – Programmed cell Death Ligand

Pr3 – Proteinase 3

PRAME - Preferentially Expressed Antigen in Melanoma

RHAMM - Receptor of Hyaluronic Acid-Mediated cell Motility

RNA – Ribonucleid Acid

ROS – Reactive Oxygen Species

RPMI – Roswell Park Memorial Institute medium

RT-PCR - Reverse Transcription-Polymerase Chain Reaction

SDF-1 – Stromal cell-Derived Factor 1

siRNA – small interfering RNA

STAT - Signal Transducer and Activator of Transcription

TAA - Tumor Associated Antigen

TAM - Tumor Associated Macrophages

TCR – T-Cell Receptor

TGF – Transforming Growth Factor

Th – T helper lymphocytes

TIL – Tumor Infiltrating Lymphocytes

TKI – Tyrosine Kinase Inhibitor

TLR – Toll-Like Receptor

TNF – Tumor Necrosis Factor

TRAIL – TNF-Related Apoptosis-Inducing Ligand

Treg – regulatory T lymphocytes

TSA – Tumor Specific Antigen

VEGF – Vascular Endothelial Growth Factor

VLP – Virus Like Particles

WT-1 - Wilms´ Tumor 1

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1. ÚVOD

Ústav hematologie a krevní transfúze se specializuje na hematologická onemocnění. Poskytuje lékařskou péči pacientům a má k dispozici laboratoře, které umožňují diagnostiku nemocí a monitorování jejich průběhu na buněčné a molekulární úrovni a laboratoře, které se zabývají základním i aplikovaným výzkumem těchto onemocnění.

V naší laboratoři se již několik let věnujeme studiu chronické myeloidní leukémie (CML) a možnosti její léčby pomocí imunoterapie. Tuto problematiku zkoumáme na myších modelech BALB/c s použitím syngenních myších buněčných linií B210 a 12B1 transformovaných fúzním genem bcr-abl. Navrženo a testováno bylo několik typů experimentálních vakcín zaměřených proti proteinu BCR-ABL: (a) DNA vakcíny (Lucansky *et al*, 2009), (b) rekombinantní vakcíny na bázi viru vakcinie (Němečková *et al*, publikace v přípravě), (c) hybridní, viru podobné částice (VLP) (Hruskova *et al*, 2009), (d) vakcíny z dendritických buněk (Němečková *et al*, publikace v přípravě). Nejúčinnější se jevily DNA vakcíny nesoucí celý gen pro BCR-ABL. Fúzí genů bcr a abl vzniká sekvence aminokyselin, která se nevyskytuje v žádném jiném proteinu a je považována za specifický nádorový antigen. Ukázalo se však, že imunita proti nádorovým buňkám není vyvolána fúzní zónou hybridního proteinu, ale epitopy lokalizovanými v jiných jeho částech (Lucansky *et al*, 2009). Dalším cílem bylo připravit nádorové buněčné vakcíny, které nejsou namířeny proti určitému antigenu, ale mohou vyvolat imunitní odpověď proti celému komplexu specifických nádorových antigenů (TSA) a s nádorem asociovaných antigenů (TAA). Pro zvýšení jejich protinádorového účinku lze do buněk vnést gen, jehož produkt posiluje imunostimulační účinky vakcíny.

Tato dizertační práce mapuje přípravu a testování buněčných vakcín založených na buňkách B210 a 12B1 exprimujících gen pro vybraný cytokin a popisuje i některé nežádoucí změny, které mohou genově modifikované buňky v organizmu vyvolat.

Cíle práce:

- Najít metodu transfekce vhodnou pro lymfoblastoidní buňky B210 a 12B1
- Vpravit do buněk plazmid nesoucí gen pro cytokin IL-2, GM-CSF nebo IL-12 a získat klony modifikovaných buněk se stabilní expresí cytokinu
- Charakterizovat vybrané klony modifikovaných buněk B210 a 12B1 *in vitro*
- Zjistit onkogenní potenciál genově modifikovaných buněk B210 a 12B1
- Stanovit imunogenní potenciál genově modifikovaných buněk B210 a 12B1
- Popsat případné nežádoucí změny, vyvolané inokulací genově modifikovaných buněk myším

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2. LITERÁRNÍ PŘEHLED

2.1 Vznik nádoru

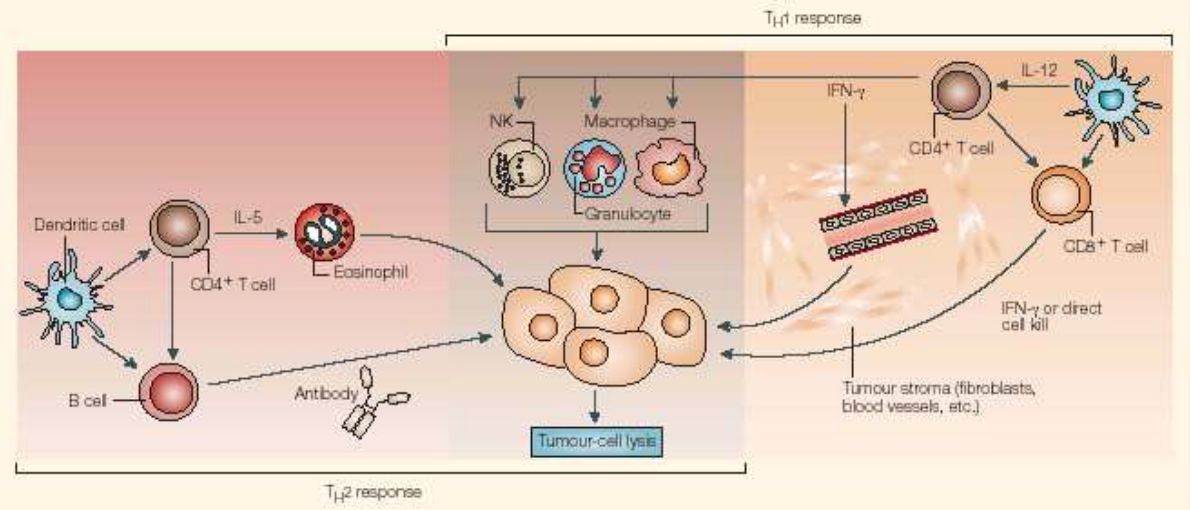
Nádorová buňka vzniká postupným hromaděním genetických změn, jejichž následkem se stane odolnější k apoptóze, nekontrolovaně se začne množit, sníží se její závislost na růstových faktorech z vnějšího prostředí a ztratí závislost na buněčné adhezi. Základem nádorové transformace je aktivace buněčných protoonkogenů na onkogeny a ztráta funkčnosti genů potlačujících nádor, označovaných jako antionkogeny.

Děje probíhající v organizmu, a tedy i vznik nádorů, jsou neustále pod imunitním dozorem. Z toho hlediska můžeme rozlišit tři fáze ve vývoji nádorů: *eliminaci, ekvilibrium a únik* (Dunn *et al*, 2002). Ve fázi *eliminace* rozpozná imunitní systém nádorovou buňku a zahájí protinádorovou odpověď vedoucí k její likvidaci. Pokud některé nádorové buňky přežijí, dostává se nádor do fáze *ekvilibria*, ve které imunitní dozor nedokáže nádorové buňky úplně zlikvidovat a jen je udržuje v omezeném množství. Nádorové buňky pod imunitním tlakem díky své genetické nestabilitě mění své vlastnosti, až se některým podaří uniknout imunitnímu systému. Začnou se nekontrolovaně množit, takže ve třetí fázi, *úniku*, dojde k rozvoji a růstu nádoru. Nádor se pak skládá z heterogenní populace nádorových buněk různě geneticky pozměněných, které jsou různě vnímavé k imunitnímu systému i k onkologické léčbě. Kromě toho nádorové buňky působí na okolní buňky v nádoru a přeměňují jejich vlastnosti „k obrazu svému“. Vytvoří si tak nádorové mikroprostředí, které je schopné, kromě jiného, aktivně bránit nádorové buňky proti imunitnímu systému (přehled (Zamarron & Chen, 2011)).

2.2 Protinádorová odpověď

Proti nádoru se imunitní systém brání jednak mechanizmy specifické imunity prostřednictvím T-lymfocytů a protilátek a jednak nespecifické imunity prostřednictvím makrofágů, NK buněk, NKT buněk a neutrofilních granulocytů (Obrázek 1).

Obrázek 1. Protinádorová odpověď imunitního systému



Převzato z (Gilboa & Vieweg, 2004)

Za alfu a omegu protinádorové odpovědi jsou považovány dendritické buňky a T-lymfocyty. Aby mohlo dojít k pomnožení nádorově specifických efektorových a paměťových T-lymfocytů, musí dojít k aktivaci a maturaci dendritických buněk a vystavení nádorových antigenů na jejich povrchu.

Profesionální antigen prezentující buňky (APC; dendritické buňky, monocyty, makrofágy, B-lymfocyty) rozeznávají určité molekulární struktury na povrchu mikroorganizmů (PAMP) nebo molekulární signály asociované s poškozením organizmu (DAMP) pomocí tzv. *toll-like* receptorů (TLR). Další stimuly k aktivaci získají prostřednictvím receptoru CD40 a receptorů pro cytokiny jako je GM-CSF (faktor stimulující tvorbu kolonií granulocytů a monocytů). APC vystavují na svém povrchu jednak antigeny endogenní a jednak exogenní, které získaly pohlcením a zpracováním buněk, virů a bakterií ze svého okolí. Exogenní antigeny bývají prezentovány prostřednictvím molekul MHCII třídy, ale pokud jsou pohlceny a následně uvolněny z endozómu do cytosolu, jsou zpracovány jako endogenní antigeny a vystaveny prostřednictvím molekul MHCI třídy. Tento jev se nazývá zkřížená prezentace antigenu. Aktivované DC vystavující i nádorové antigeny migrují do lymfatických uzlin, v kterých stimulují protinádorové CD4+ a CD8+ T-buňky.

T-buňky rozpoznají nádorové antigeny prezentované na povrchu APC pomocí svých T-buněčných receptorů (TCR). K aktivaci potřebují dostat ještě druhý signál prostřednictvím kostimulačních molekul CD80 (B7.1) a CD86 (B7.2), které se vážou na receptor CD28. Svou roli při aktivaci můžou hrát i molekuly rodiny TNF (4-1BBL nebo

OX40L). Aktivace pomocných CD4+T-lymfocytů a jejich polarizace směrem k Th1 nebo Th2 odpovědi závisí na cytokinovém prostředí. Působením IL-12 se naivní Th0-buňky diferencují na Th1-buňky, zatímco pro diferenciaci Th0 na Th2-buňky je třeba IL-4. Th1-buňky produkují hlavně IL-2 a IFN-γ a stimulují buněčnou imunitní odpověď. Th2-buňky sekretují především IL-4, IL-5 a IL-10 a stimulují protilátkovou odpověď. Aktivované CD8+T-lymfocyty (CTL) jsou roznášeny krevním oběhem až do míst, kde se setkají s nádorovými buňkami. K jejich rozpoznání a následnému usmrcení jim stačí vazba TCR s nádorovým antigenem prezentovaným pomocí molekul MHCI třídy na povrchu nádorových buněk.

T-buňky, které jsou chronicky vystavené určitému antigenu, se můžou vyčerpat a nejsou schopny aktivace (přehled (Wherry, 2011)). Mají velmi nízkou produkci cytokinů a zvýšené množství inhibičních receptorů jako je PD-1, CTLA-4, TIM-3 a LAG-3 (Blank & Mackensen, 2007;Sakuishi *et al*, 2010;Ahmadzadeh *et al*, 2009;Richter *et al*, 2010). Jejich utlumení je reverzibilní, aktivace lze dosáhnout blokádou inhibičních receptorů. Vyčerpané lymfocyty jsou i mezi lymfocyty infiltrujícími nádor (TIL), takže s ním nejsou schopny dostatečně účinně bojovat (Whiteside, 2006).

2.3 Inhibiční mechanizmy protinádorové imunity

Nádorové buňky uplatňují ve střetu s imunitním systémem dvě strategie přežití (a) zneviditelnění se imunitnímu systému, (b) zneškodnění buněk imunitního systému (Whiteside, 2006).

Ad (a). Zneviditelnění dosáhnou buňky sníženou nebo pozastavenou expresí TAA, molekul MHC, kostimulačních molekul a molekul podílejících se na zpracování antigenu. Nádorové buňky také sníží expresi molekul indukujících apoptózu nebo naopak zvýší expresi genů pro neklasické MHC a genů inhibujících apoptózu, aby byly rezistentní k útoku imunitního systému.

Ad (b). Mezi nejdůležitější buněčné populace, které rozhodují o tom, zda bude nádorová buňka tolerována nebo zneškodněna, patří T-regulační buňky (Treg). Jejich fyziologickou funkcí je zabránit aktivaci efektorových buněk, které by reagovaly s vlastními antigeny. Navození tolerance chrání organizmus před autoimunitními nemocemi. Na nádorovou buňku reagují Treg jako na buňku tělu vlastní a blokují protinádorovou imunitní odpověď. Děje se to jak přímým kontaktem s CTL, tak sekrecí inhibičních

cytokinů jako je IL-10 a TGF-β (Serafini *et al*, 2006). Během růstu nádoru dochází k zmnožení populace Treg, které jsou $CD4^{+}CD25^{high}FOXP3^{+}$, a to jak v periferní krvi, tak v místě nádoru, což koreluje se špatnou prognózou u mnoha typů zhoubných nádorů (Perrone *et al*, 2008). Opakovaná imunizace může zvýšit populaci Treg a snížit účinnost vakcíny (Zhou *et al*, 2006). Pokud se současně s podáním vakcíny depletují Treg, protinádorová imunitní odpověď bývá účinnější (Dannull *et al*, 2005).

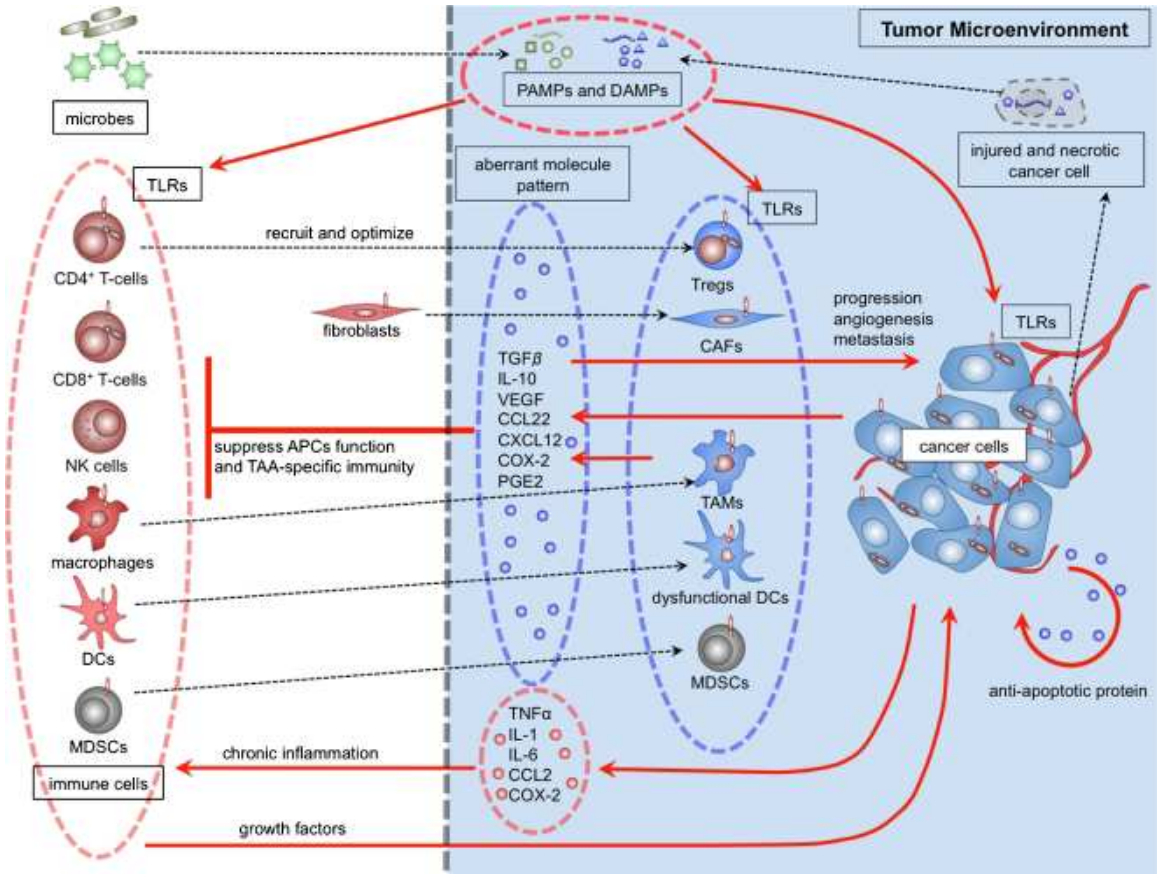
Negativně se při protinádorových imunitních reakcích uplatňují také myeloidní supresorové buňky (MDSC). Jde o morfologicky, fenotypově i funkčně heterogenní populaci nezralých myeloidních buněk, která se zmnožuje v případě patologických změn jako je zánět a zhoubné bujení. Rekrutují se z kostní dřeně vlivem nádorových rozpustných faktorů, jako jsou GM-CSF, VEGF a IL-10 a akumulují se v nádoru a sekundárních lymfoidních orgánech. U myší se MDSC identifikují na základě přítomnosti znaků $Gr\text{-}1^{+}$ a $CD11b^{+}$. U lidí jsou to znaky $CD33^{+}$ a $CD11b^{+}$ a MDSC se rozlišují na monocytární $CD14^{+}$ $HLA\text{-}DR^{-/low}$ $CD66b^{+}$ a granulocytární $CD14^{-}CD15^{+}HLA\text{-}DR^{-}$ (Tadmor *et al*, 2011). MDSC jsou schopny různými mechanizmy inhibovat vrozenou i získanou imunitu. T-buněčnou odpověď snižují produkcí syntetázy oxidu dusnatého (iNOS), reaktivních forem kyslíku (ROS), arginázy I, indoleamin-2,3-dioxygenázy (IDO), TGF-β, zabráněním příjmu cysteinu T-lymfocyty a snížením L-selektinů na jejich povrchu. Sekrecí cyklooxigenázy-2 (COX2), jejímž produktem je prostaglandinE2, a dalších substancí jako jsou např. metaloproteinázy (MMP) a IL-6, podporují MDSC angiogenezi, invazivitu a metastázování nádorových buněk. Jsou schopny indukovat Treg i vývoj tumor-asociovaných makrofágů (TAM). Eliminace MDSC dramaticky zlepší protinádorovou odpověď jak u myší, tak i u lidí. Někdy dokonce umožní odhojení nádoru (přehled (Ostrand-Rosenberg & Sinha, 2009), (Gabrilovich & Nagaraj, 2009)).

Nádorové mikroprostředí se skládá z nádorových fibroblastů (CAF), endotelií, buněk imunitního systému, extracelulární matrix a rozpustných faktorů, které se podílejí na růstu a agresivitě nádorových buněk (Obrázek 2).

Nádorové fibroblasty podporují proliferaci okolní nádorové tkáně pomocí růstových faktorů jako je metastazin (FSP1) nebo chemokinů jako je SDF-1α (CXCL12), který přitahuje endoteliální progenitorové buňky do nádoru a napomáhá tak angiogenezi. CAF produkují také velké množství aktinu, extracelulární matrix a MMP, které štěpením proteinů jako je kolagen ulehčují prorůstání nádoru do okolní tkáně. Nádorové fibroblasty

jsou schopné přilákat pomocí prozánětlivých cytokinů a chemokinů do nádoru makrofágy, neutrofily a lymfocyty (Xing *et al*, 2010).

Obrázek 2. Nádorové mikroprostředí



Převzato z (Sato *et al*, 2009)

Monocyty se v nádorovém mikroprostředí diferencují na TAM typu M2, které nejsou schopné aktivovat T-buňky a uvolňují pronádorové a proangiogenní faktory jako jsou TGF-β, IL-10, VEGF, CCL2, IL-1β, TNF-α a MMP (Whiteside, 2010).

Progrese nádoru koreluje i s akumulací nezralých dendritických buněk v nádoru, které nejsou schopny prezentovat antigen, ale aktivují Treg v lymfatických uzlinách, které infiltrují (Melief, 2008). Plasmocytoidní DC přitahované do nádoru chemokinem SDF-1α produkují TNF-α a IL-8 a podporují tak vaskularizaci nádoru (Curiel *et al*, 2004).

Z nádorových buněk se můžou uvolňovat mikrovezikulární organely zvané exozómy, které prostřednictvím molekul FasL a TRAIL indukují apoptózu protinádorových T- lymfocytů a přesměrují vývoj monocytů na MDSC (Valenti *et al*, 2007).

2.4 Léčba nádorů

V onkologii dochází v posledních desetiletích k úspěšnému vývoji v diagnostice i v léčbě. Daří se výrazně prodloužit život pacienta a přibývá případů, kdy je pacient vyléčen úplně. Výsledky se liší podle typu onkologického onemocnění a podle toho, v jakém stádiu je choroba podchycena. Onkologičtí pacienti jsou stále léčeni zejména pomocí klasických léčebných postupů - chirurgie, chemoterapie a radioterapie. Těmito metodami se dosáhne redukce nádorové masy a u mnoha pacientů úplného vyléčení. Nicméně u některých nádorových onemocnění, zvlášť v pokročilejším stádiu, je tato léčba méně úspěšná a má řadu vedlejších nežádoucích účinků. Proto se hledají další léčebné přístupy, které by léčbu zkvalitnily. Díky novým poznatkům v oblasti imunologie a molekulární biologie o tom, jak imunitní systém funguje, a jak interaguje s nádorovými buňkami, uplatňuje se v léčbě rakoviny ve vzrůstající míře imunoterapie.

2.4.1 Imunoterapie nádorů

Hlavním cílem imunoterapie nádorů je posílit pacientův imunitní systém tak, aby se dovedl účinně bránit proti nádorovým buňkám. Nádorová buňka je odvozena od buňky normální a většina antigenů, které vystavuje na svém povrchu, je imunitním systémem tolerována. Nicméně prezentuje i antigeny (TAA), které se v normální buňce netvoří buď vůbec, nebo jen v malém množství. Jsou většinu málo imunogenní a odpověď imunitního systému není tak silná, aby byla schopná nádorové buňky eliminovat. Imunoterapie se snaží prolomit tuto toleranci, aktivovat protinádorovou odpověď organizmu, potlačit imunosupresivní reakce nádorových buněk a zviditelnit nádorové buňky imunitnímu systému.

Ideální by bylo léčit pomocí imunoterapie pacienty v úplném počátku nádorového onemocnění, ale to zatím není realizovatelné. Velkou naději má imunoterapie při léčbě minimální reziduální nemoci (MRD), většinou v kombinaci s klasickými léčebnými postupy. Jako MRD se označuje stav, kdy část nádorových buněk odolá klasické terapii, ale pacient nemá klinické symptomy nemoci. Většinou se tyto buňky stávají k léčbě rezistentní a dříve či později vyvolají u pacienta relaps onemocnění.

Imunoterapie se dělí podle mechanizmu účinku na pasivní a aktivní (Tabulka1).

Tabulka 1. Současné možnosti imunoterapie nádorů

IMUNOTERAPIE PASIVNÍ		
Humorální	**Monoklonální protilátky**	• Samotné, zaměřené proti nádorovému antigenu • Konjugované s radioizotopovým nuklidem nebo toxinem • Bispecifické protilátky vázající se na nádorovou buňku a zároveň na T- lymfocyt
Buněčná	**TIL**	Lymfocyty infiltrující nádor se vyizolují z nádoru, ex *vivo* se namnoží a podají zpět pacientovi
	Nádorově specifické T-lymfocyty	Pacientovy lymfocyty se *ex vivo* inkubují s DC prezentujícími nádorový antigen, namnoží se a podají zpět pacientovi
	Geneticky modifikované T-lymfocyty	Do T-lymfocytů se vnese gen pro TCR, který je specifický pro nádorový antigen, takto modifikované T-lymfocyty se *ex vivo* namnoží a podají zpět pacientovi
	LAK buňky	Pacientovy T- a NK buňky se namnoží *ex vivo* a vrátí se zpět pacientovi
IMUNOTERAPIE AKTIVNÍ		
Specifická	**Peptidové a proteinové vakcíny**	Peptidové segmenty nebo proteiny odvozené od nádorového antigenu nesoucí T- a/nebo B-epitop
	DNA vakcíny	Plazmidová DNA kódující nádorový antigen nebo jeho část
	Rekombinantní vakcíny	Do virového, bakteriálního, kvasinkového nebo rostlinného genomu je vnesen gen pro nádorový antigen, ev. gen pro imunostimulační faktor
	Buněčné vakcíny	• **Dendritické buňky** - izolované z pacienta, inkubované s nádorovým antigenem či s lyzátem nádorových buněk nebo transfekované s RNA nesoucí nádorový antigen, ev. další geny
		• **Buněčné nádorové vakcíny** - inaktivované nebo lyzované autologní nebo alogenní nádorové buňky, zpravidla modifikované vnesením genu pro imunostimulační faktor
Nespecifická	**Rekombinantní cytokiny**	IFN-α, IL-2, GM-CSF, IL-12 aj. podané intratumorálně nebo intravenózně
	Bakteriální extrakty	*Mycobacterium bovis*, *Corynebacterium parvum*, *Listeria monocytogenes* aj.
	Chemoterapie a radioterapie	V suboptimálních dávkách, které nemají cytotoxický, ale imunomodulační efekt
	Imunomodulační látky	Levamisol, imiquimod aj.

Při pasivní imunoterapii jsou pacientovi podávány komponenty imunitního systému, jako jsou protilátky či aktivované lymfocyty, které mají přímo působit proti nádorovým buňkám, nezávisle na stavu imunitního systému pacienta. V klinické praxi se uplatňují hlavně monoklonální protilátky, které dokážou nádorové buňky opsonizovat, indukovat protilátkami zprostředkovanou buněčnou imunitu (ADCC), aktivovat komplement a/nebo donesou k nádorové buňce léčivo nebo radioizotop, které buňku zabijí. V případě expanze T-buněk *ex vivo* a jejich reimplantace jde o příklad personalizované medicíny, která je technicky velice náročná a nákladná. To zatím limituje její širší použití v klinické praxi.

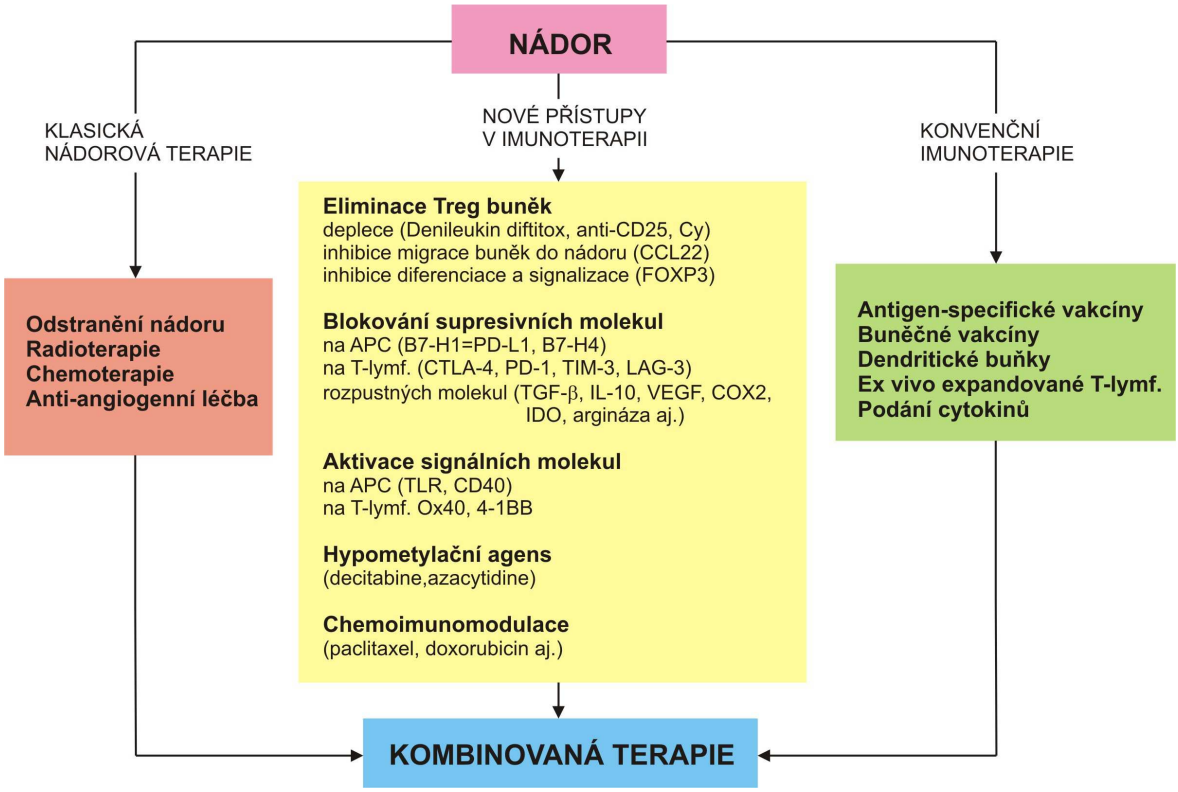
Cílem aktivní imunoterapie je zmobilizovat vlastní protinádorové mechanizmy imunitního systému. K tomuto účelu se používají různé typy vakcín. Zkouší se peptidové nebo proteinové vakcíny, DNA vakcíny a rekombinantní virové a bakteriální vakcíny. Dále se prověřují buněčné vakcíny založené na dendritických buňkách opracovaných tak, aby prezentovaly nádorové antigeny a buněčné vakcíny založené na nádorových buňkách, zpravidla geneticky modifikovaných.

Imunitní systém se dá při aktivní imunoterapii povzbudit také nespecificky pomocí rekombinantních cytokinů nebo bakteriálních extraktů. Poslední dobou se zkoumá imunomodulační účinek chemoterapeutik v ultra nízkých dávkách (1/5 – 1/20 maximální tolerované dávky MTD), které nemají cytotoxický a cytostatický efekt (Shurin *et al*, 2012). Prokázalo se, že zvyšují maturaci DC a prezentaci antigenu těmito buňkami. V nádorových buňkách mohou vyvolat expresi proteinů, které se účastní zpracování antigenů a jejich vystavení prostřednictvím molekul MHC na povrchu buňky, čímž se tyto buňky stanou snazším terčem pro CTL. Kromě toho některá cytostatika jako je např. cyklofosfamid dokážou redukovat populaci Treg (Kaneno *et al*, 2009;Emens, 2010).

Účinky aktivní imunoterapie by měly být dlouhodobé. V ideálním případě by měl být pacient vyléčen a chráněn tak před relapsem choroby. Dosavadní výsledky imunoterapie v klinických studiích jsou povzbudivé, i když prozatím nejsou dostatečně výmluvné. U značné části pacientů byla po vakcinaci prokázána specifická buněčná odpověď, která však neznamenala vždy zlepšení klinického stavu či prodloužení doby přežití. Trvalé remise se podařilo dosáhnout jen u poměrně malé části pacientů. Pozornost imunoterapie se v posledních letech zacílila na prolomení obranných mechanizmů nádoru, což by mělo zvýšit účinnost imunoterapeutických vakcín. Jde především o eliminaci Treg a MDSC, dysfunkčních a supresorových APC. Zdá se být zřejmé, že k dosažení vyléčení pacienta je

třeba kombinovat několik léčebných strategií a) zredukovat nádorovou masu b) podpořit imunitní systém pacienta c) zacílit terapii na supresorové buněčné populace d) pomocí imunoterapie dosáhnout vyléčení MRD (Melief, 2008;Mellman *et al*, 2011). Současné přístupy k léčbě nádorů jsou znázorněny na Obrázku 3.

Obrázek 3. Léčba nádorů



Převzato a upraveno (Zou, 2006)

2.6 Genová terapie

Genovou terapií rozumíme vnesení genetického materiálu (DNA nebo RNA), který má léčebný účinek, do buňky. Buňka buď získá určitou funkci, nebo je její patogenní funkce potlačena. Tato metoda byla primárně určena k léčbě monogenních vrozených nemocí, ale široké pole působnosti našla v onkologii. V tomto kontextu je hlavní snahou genové terapie buď přímo zničit nádorovou buňku, nebo pozměnit buňky imunitního systému, tak aby byly efektivnější v protinádorové odpovědi. Genetický materiál můžeme vnášet buď přímo do nádoru, nebo do buněk izolovaných z pacienta, které jsou mu po genetické modifikaci aplikovány zpět. Do genové terapie nádorů jsou řazeny DNA

vakcíny, geneticky modifikované virové, bakteriální, rostlinné a buněčné vakcíny, pokud jsou používány k terapeutickým účelům.

Pro přenos genetického materiálu, tzv. transfekci, se používá vektorů, které zajišťují jeho expresi v cílové buňce. Přenášený gen, tzv. transgen, se do buněk dostává pomocí fyzikálních, chemických nebo biologických metod. Mezi fyzikální metody patří metoda mikroinjekce, elektroporace, tetování, balistická metoda pomocí genové pistole a jiné. Nejčastěji používanými chemickými metodami transfekce jsou kalcium-fosfátová precipitace, lipofekce a kationické polymery. Nejvíce se však v dosavadních klinických studiích uplatnily biologické metody, které využívají ke vstupu do buňky virové vektory upravené tak, aby nebyly schopné se replikovat a byly pokud možno málo imunogenní. Jde hlavně o vektory odvozené od retrovirů, adenovirů, adeno-asociovaných virů a virů herpes simplex. Naděje jsou vkládány do geneticky upravených onkolytických virů, které jsou replikačně kompetentní, ale množí se jen anebo přednostně v nádorových buňkách, které tím zabíjí (Eager & Nemunaitis, 2011).

Modifikace nádorových buněk se provádí (a) vnesením funkčních tumor supresorových genů jako je p53, což způsobuje ztrátu maligního fenotypu, (b) inhibicí funkce aktivovaného onkogenu (pomocí antisense oligonukleotidů, ribozymů nebo siRNA) (c) vnesením sebevražedných genů (např. genů pro herpesvirovou tymidinkinázu nebo bakteriální cytozindeaminázu) a apoptických genů (d) vnesením genů, které činí buňky vnímavějšími k chemoterapii a radioterapii (e) vnesením imunomodulačních genů, které napomáhají rozpoznání nádorové buňky imunitním systémem (cytokiny, kostimulační molekuly) (f) vnesením genů, které inhibují angiogenezi (angiostatin, endostatin) (Scanlon, 2004), (Vonka 2010, http://www.zdn.cz/clanek/postgradualni-medicina/genova-terapie-nadoru-451654).

Vakcíny založené na dendritických buňkách, transfekovaných DNA nebo RNA nesoucí gen pro nádorový antigen, stimulují dobře protinádorovou odpověď (Boudreau *et al*, 2011;Frankenberger & Schendel, 2012). Jejich imunnogenita může být také zvýšena vnesením genu pro cytokiny, chemokiny, které pomáhají putování DC do lymfatických uzlin, nebo genu pro kostimulační a adhezivní molekuly (Chen *et al*, 2010).

V současnosti se věnuje velká pozornost genové modifikaci T-lymfocytů, vnesením genu pro žádoucí TCR nebo genu pro tzv. chimerický receptor pro antigen (CAR), který rozpoznává specifický nádorový antigen. Geneticky modifikované T-lymfocyty se pak pomnoží *in vitro* a podají zpět pacientovi, u něhož se podílejí na cytotoxické protinádorové odpovědi (Jena *et al*, 2010).

2.7 Buněčné vakcíny odvozené od nádorových buněk

Jelikož nádorové buňky nebývají dostatečně imunogenní, většinou se pro vakcinaci geneticky upravují vnesením genu, jehož produkt má imunostimulační účinky. Nejčastěji se buňky modifikují pomocí genů pro cytokiny a kostimulační molekuly (přehled (Parmiani *et al*, 2000)). Výhodou buněčných vakcín je, že nevyžadují znalost nádorového antigenu pro stimulaci protinádorových imunitních reakcí. Zároveň dovolují vyvolat imunitu proti celému komplexu antigenů, což je jejich veliká výhoda oproti peptidovým, rekombinantním a DNA vakcínám. Ty jsou cíleny proti předpokládanému imunodominantnímu nádorovému antigenu nebo jeho epitopu. Nádorové buňky ale můžou pod imunitním tlakem snížit nebo ztratit expresi daného antigenu a tak uniknout útoku antigen-specifických CTL (Pawelec, 2004).

V humánní medicíně jsou vyvíjené buněčné vakcíny buď autologní, tj. připravené přímo z vlastních nádorových buněk pacienta nebo alogenní, což jsou lidské nádorové linie odvozené od jiných pacientů se stejným typem nádoru. Pacientovi se aplikují lyzované nebo v inaktivovaném stavu, a to ozářené nebo ošetřené cytostatiky. Takto upravené buňky nejsou schopné proliferace, ale po podání pacientovi aktivují buňky jeho imunitního systému.

Nevýhodou buněčných autologních vakcín je složitost přípravy. Odvodit z nádoru linii kontinuálně rostoucích buněk je časově i finančně náročné a ne vždy se to podaří. V současnosti se upřednostňují spíše alogenní buněčné vakcíny. Příprava takových vakcín se dá lépe standardizovat a je to relativně levný a kontrolovatelný proces. Kromě toho je zdroj buněk víceméně neomezený a kdykoliv dostupný. Alogenní vakcína však nemusí být vhodná a účinná pro každého pacienta se stejným typem nádoru, protože jeho nádorové buňky můžou mít odlišné složení nádorových antigenů. Jednou z možností, jak problém vyřešit, by byla vakcína složená z několika nádorových linií se vzájemně se lišícím antigenním repertoárem. Určitým rizikem buněčných vakcín je možnost navození autoimunitních reakcí.

Buněčné vakcíny byly testovány na různých myších modelech. Z valné většiny byly buňky upraveny vnesením plazmidu nesoucího gen pro některý z následujících cytokinů: IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, G-CSF, GM-CSF, FLT3 ligand, IFN-α a IFN-γ (přehled (Allione *et al*, 1994;Mach & Dranoff, 2000;Dranoff, 2004)).

V naší laboratoři byly v nedávné minulosti zkoušeny buněčné vakcíny na myším modelu s buňkami transformovanými virem HPV-16, částečně ve spolupráci se skupinou Prof. Bubeníka z Ústavu molekulární genetiky AV (Rossner *et al*, 1999;Indrova *et al*, 2002;Bubenik *et al*, 2003;Mikyskova *et al*, 2004;Indrova *et al*, 2006;Mikyskova *et al*, 2011;Jinoch *et al*, 2003;Rittich *et al*, 2005) a na modelu myších buněk transformovaných fúzním genem bcr-abl (Sobotkova *et al*, 2004;Petrackova *et al*, 2009;Sobotkova *et al*, 2009). Výsledky těchto experimentálních studií ukázaly, že některé nádorové buňky exprimující IL-2, IL-12 nebo GM-CSF ztratily zcela nebo částečně svůj onkogenní potenciál, při současném zvýšení imunogenního potenciálu.

Vakcíny tvořené nemodifikovanými nádorovými buňkami se podávaly většinou spolu s oslabeným *Mycobacterium bovis* (Bacillus Calmette-Guerin - BCG). BCG aktivuje TLR na dendritických buňkách a podporuje odpověď typu Th1 (přehled (Copier *et al*, 2007;Le *et al*, 2010;Parmiani *et al*, 2011)). Modifikované buněčné vakcíny se v klinických studiích zkoušely hlavně v léčbě melanomu, nádoru prostaty, ledvin a slinivky. Přitom nejvíce klinických studií bylo provedeno s buněčnými vakcínami produkujícími GM-CSF. Přehled klinických studií, ve kterých byly použity modifikované nádorové buňky, je v tabulce 2.

Ve své práci jsem se zaměřila na cytokiny IL-2, IL-12 a GM-CSF. Stručnou informaci o použití těchto cytokinů při imunoterapii uvádím v následujícím textu.

Tabulka 2. Přehled klinických studií s modifikovanými nádorovými buňkami

Transgen	Typ nádoru	Vakcína	Reference
GM-CSF	melanom	autologní	(Soiffer *et al*, 1998;Chang *et al*, 2000;Soiffer *et al*, 2003;Luiten *et al*, 2005)
	karcinom prostaty	autologní	(Simons *et al*, 1999)
		alogenní	(Simons *et al*, 2006;Small *et al*, 2007;Higano *et al*, 2008;Urba *et al*, 2008;van den Eertwegh *et al*, 2012)
	nemalobuněčný plicní karcinom	autologní	(Salgia *et al*, 2003), mix s alogenní vakcínou (Nemunaitis *et al*, 2006)
	nádor slinivky	alogenní	(Jaffee *et al*, 2001;Laheru *et al*, 2008)
	nádor ledvin	autologní	(Simons *et al*, 1997;Tani *et al*, 2004)
	leukémie	autologní	(Ho *et al*, 2009;Borrello *et al*, 2009)
		alogenní	(Smith *et al*, 2010)
	nádor prsu	alogenní	(Emens *et al*, 2009)
GM-CSF + CD80	nádor prsu	alogenní	(Dols *et al*, 2003)
IL-2	melanom	autologní	(Schreiber *et al*, 1999;Palmer *et al*, 1999)
		alogenní	(Arienti *et al*, 1996;Belli *et al*, 1997)
	neuroblastom	autologní	(Bowman *et al*, 1998a)
		alogenní	(Bowman *et al*, 1998b)
	kolorektální karcinom	alogenní	(Sobol *et al*, 1999)
IL-2 + CD80	nádor ledvin	alogenní	(Buchner *et al*, 2010)
IL-2 + IFN-γ	nádor prostaty	alogenní	(Brill *et al*, 2007)
IL-4	melanom	alogenní	(Arienti *et al*, 1999)
IL-6	melanom	alogenní	(Nawrocki *et al*, 2000)
IL-7	melanom	autologní	(Moller *et al*, 1998)
IL-12	melanom	autologní	(Sun *et al*, 1998)
IFN-γ	melanom	autologní	(Abdel-Wahab *et al*, 1997;Nemunaitis *et al*, 1998)
B7.1	nádor ledvin	autologní	(Fishman *et al*, 2008)
	nemalobuněčný plicní karcinom	alogenní	(Raez *et al*, 2004)

2.7.1 IL-2

IL-2 je produkován hlavně Th1-buňkami. Je růstovým faktorem pro T-buňky stimulované antigenem, takže v jeho přítomnosti dojde k jejich klonální expanzi. Jeho protinádorový efekt je založen na indukci proliferace T a NK buněk, ale indukuje i proliferaci B-buněk.

IL-2 je schválen jako léčivo pro pacienty s pokročilým melanomem a renálním karcinomem (Rosenberg *et al*, 1994;Tsao *et al*, 2004). Dávají se ho poměrně vysoké dávky, které sebou nesou i nežádoucí toxické účinky. Lépe tolerované nižší dávky IL-2 ale nemají velký léčebný efekt (Berinstein, 2007;Yoshimoto *et al*, 2009).

Gen pro IL-2 byl také jedním z prvních cytokinových genů, které se vpravovaly do nádorových buněk při přípravě buněčných vakcín. Výsledky byly velmi variabilní. V některých experimentálních modelech se podařilo vyvolat systémovou imunitní odpověď proti jinak velmi málo imunogenním nádorovým buňkám, pokud po genové modifikaci exprimovaly IL-2 (Fearon *et al*, 1990;Ley *et al*, 1991;Bubenik *et al*, 1999;Indrova *et al*, 2002;Bubenik *et al*, 2003;Mikyskova *et al*, 2004). Na myším modelu MC38 byly zkoušeny dendritické buňky geneticky modifikované tak, aby exprimovaly IL-2. Po jejich podání došlo k oddálení vzniku nádorů a prodloužila se doba přežití (Rossowska *et al*, 2011). Nádorové buňky exprimující lidský IL-2 se úspěšně testovaly na CB-17/SCID myších s hepatocelulárním karcinomem (Bui *et al*, 1997;He *et al*, 2000). U pacientů s karcinomem prostaty byla zkoušena alogenní buněčná vakcína exprimující IL-2 a IFN-γ. Vakcína byla dobře snášena, u části pacientů došlo k poklesu nádorového prostatického antigenu (PSA) (Brill *et al*, 2007). Farzaneh a kol. prováděli pokusy s modifikovanými buňkami AML, do nichž je vnesen gen pro IL-2 a gen pro B7.1, který jim chybí k tomu, aby mohly plnit roli APC. Argumentovali tím, že IL-2 podporuje odpověď CTL a také aktivuje anergické T-lymfocyty (Chan *et al*, 2005). *In vitro* dokázali jak aktivaci NK buněk tak i CD8+buněk, které napadají nemodifikované buňky AML (Hardwick *et al*, 2010). V běhu je klinická studie s nádory hlavy a krku, při které je do nádoru vpravován ve vazbě na kationické lipidy plazmid s genem pro IL-2 (O'Malley *et al*, 2005). IL-2 je poslední dobou používán v imunoterapii spíše jako adjuvans (Baek *et al*, 2011;Ellebaek *et al*, 2012).

Kromě žádoucích protinádorových účinků může IL-2 zasáhnout do imunologických dějů i negativně. Podporuje proliferaci Treg a indukuje apoptózu zralých T-lymfocytů

zprostředkovanou Fas receptorem. V poslední době je IL-2 zmiňován v souvislosti s navozením tolerance u nemocí jako je diabetes nebo alergie (Malek & Pugliese, 2011). Proto se začala zkoumat léčba nádorů pomocí cytokinů IL-7, IL-15 a IL-21, které sdílejí s IL-2 imunostimulační schopnosti, ale nepodílejí se negativně na regulaci protinádorové imunity (O'Shea *et al*, 2002).

2.7.2 IL-12

IL-12 patří mezi prozánětlivé heterodimerické cytokiny. Skládá se z podjednotek p40 a p35. Je produkován hlavně APC jako odpověď na stimulaci antigenem. Receptor pro IL-12 mají T, NK a NKT-buňky, IL-12 indukuje jejich proliferaci a produkci cytokinů. Nízkou hladinu receptorů pro IL-12 mají i monocyty, makrofágy a DC.

IL-12 je důležitým cytokinem protinádorové obrany, protože indukuje odpověď Th1 a NK buněk. Ty pak produkují IFN-γ, který jednak přímo působí inhibičně na nádorové buňky, jednak zvyšuje expresi MHC a adhezivních molekul na APC a dále inhibuje angiogenezi tím, že vyvolává produkcí chemokinu CXCL10. IL-12 také inhibuje VEGF a některé MMP. Použití IL-12 v léčbě nádorů může mít ale vedlejší toxické účinky způsobené extrémně velkým množstvím indukovaného IFN-γ (přehled (Trinchieri, 2003)).

Na myších nádorových modelech bylo prokázáno, že terapeuticky podaný IL-12 inhibuje růst nádorů a indukuje jejich regresi (Brunda *et al*, 1993;Trinchieri, 2003;Indrova *et al*, 2006;Nemeckova *et al*, 2003).

Pokud se však zkoumala aktivita rekombinantního IL-12 na pacientech se solidním nádorem nebo hematologickou malignitou, efektivita léčby byla minimální (Del Vecchio *et al*, 2007). Dále byly testovány autologní geneticky modifikované fibroblasty a nádorové buňky exprimující IL-12 a DNA vakcína kódující IL-12 nebo rekombinantní virus exprimující IL-12, které byly aplikovány do nádoru pacientů s pokročilým melanomem (Sun *et al*, 1998;Kang *et al*, 2001;Heinzerling *et al*, 2005;Triozzi *et al*, 2005;Daud *et al*, 2008). Po podání těchto vakcín došlo k lokální infiltraci imunitními buňkami do nádoru a k dlouhodobé buněčné protinádorové odpovědi.

IL-12 byl také použit k modifikaci dendritických buněk, u kterých se tím zvýšila schopnost migrace do lymfatických uzlin a prezentace nádorového antigenu (Jinushi & Tahara, 2009). Podáním takto upravených dendritických buněk do místa nádoru došlo v myším modelu k redukci nádorové masy a k delšímu přežívání zvířat (Tatsumi *et al*,

2007). Klinická studie s modifikovanými dendritickými buňkami produkujícími IL-12 byla provedena na pacientech s gastrointestnálním nádory. U některých pacientů došlo ke zvýšení buněčné i protilátkové protinádorové odpovědi (Mazzolini *et al*, 2005).

2.7.3 GM-CSF

GM-CSF je produkován aktivovanými T lymfocyty, makrofágy, endoteliálními buňkami a stromálními buňkami kostní dřeně. Je nezbytným proliferačním a diferenciačním faktorem buněk myeloidní řady (Berinstein, 2007). GM-CSF se proto používá po chemoterapii nebo po transplantaci kostní dřeně k navrácení hladiny neutrofilů na původní hodnoty.

GM-CSF podporuje buněčnou i protilátkovou imunitní odpověď. Má schopnost rekrutovat dendritické buňky, vyvolat jejich maturaci a zesilovat na nich expresi MHC II molekul, kostimulačních a adhezních molekul. GM-CSF aktivuje cytotoxickou schopnost buněk imunitního systému a zvyšuje ADCC (přehled (Jinushi *et al*, 2008)). Je prokázané, že po podání GM-CSF ve formě rekombinantního proteinu nebo produkovaného modifikovanými nádorovými buňkami se v místě vakcinace zvyšuje množství dendritických buněk, které jsou s to lépe zpracovat nádorové antigeny (Weber *et al*, 2003;Zarei *et al*, 2009;Le *et al*, 2010). Jako adjuvans při léčbě nádorů se GM-CSF zkoušel v několika klinických studiích, které zaznamenaly prodloužení přežívání pacientů (přehled (Cruz-Merino *et al*, 2008)).

Když se zkoušely na myších buněčné vakcíny exprimující různé cytokiny, nejlépe fungoval právě GM-CSF, který dokázal vyvolat silnou, dlouhodobou specifickou protinádorovou odpověď. (Dranoff *et al*, 1993;Dranoff, 2002).

Na základě výsledků na myších modelech se buněčné GM-CSF-vakcíny (GVAX) začaly zkoušet v klinických studiích na pacientech s metastatickým melanomem, nemalobuněčným plicním karcinomem, s nádory ledvin a slinivky a s leukémií (Tabulka 2). Jako vakcíny byly užity jednak autologní nádorové buňky transdukované retrovirovým nebo adenovirovým vektorem nesoucím gen pro GM-CSF a jednak standardizované alogenní nádorové linie se stabilní expresí GM-CSF. Další variantou byla směs autologních nádorových buněk s buňkami K562 exprimujícími GM-CSF. U leukemických pacientů byla kombinována buněčná vakcína s alogenní transplantací kostní dřeně (přehled (Eager & Nemunaitis, 2005;Gupta & Emens, 2010)). Ve všech těchto studiích byla

prokázána lokální infiltrace dendritickými buňkami, granulocyty, makrofágy a T-lymfocyty. U některých pacientů se objevila infiltrace eosinofily odpovídající reakci přecitlivělosti opožděného typu (DTH) (Jinushi & Tahara, 2009). Imunoterapie byla dobře tolerována, byla prokázána jak T-buněčná, tak i protilátková odpověď.

Vakcína nazvaná Sipuleucel-T je schválená pro léčbu pacientů s metastatickým karcinomem prostaty. Jde o autologní dendritické buňky pacienta, které jsou inkubovány s fúzním proteinem složeným z PSA a GM-CSF. Sipuleucel-T významně zvyšuje celkové přežívání pacientů (Kantoff *et al*, 2010).

V klinické studii fáze III na pacientech s karcinomem prostaty se nečekaně ukázalo, že u pacientů léčených docetaxelem a vakcínami GVAX došlo ke zvýšené progresi nádoru a mortalitě ve srovnání s pacienty léčenými jen docetaxelem. Podobné negativní výsledky získali i Morton a kol. u pacientů s melanomem (Faries *et al*, 2009). To vedlo k přehodnocení použití GVAX vakcín a nutnosti vysvětlit molekulární mechanizmy, které k tomu vedly. Uvažuje se o tom, že ve studii s karcinomem prostaty se uplatnil imunosupresivní efekt současně podávaného docetaxelu. Nabízejí se i další vysvětlení. Při použití GM-CSF v jakékoliv podobě je třeba brát v úvahu, že může mít i negativní roli v protinádorové odpovědi. Taková situace nastává, když je tohoto cytokinu v organizmu nadměrné množství, zvlášť pokud jde o jeho chronickou produkci. GM-CSF pak indukuje růst populace MDSC, angiogenezi a expresi MFG-E8 (milk fat globule factor E8). Molekula MFG-E8 indukuje Treg, které můžou snížit účinnost GVAX vakcín (Serafini *et al*, 2004;Jinushi *et al*, 2007). Některé nádorové buňky dokonce spontánně produkují GM-CSF využívajíce těchto jeho vlastností (Jinushi & Tahara, 2009;Mattei *et al*, 1994;Lang *et al*, 1994;Pisa *et al*, 1992;Sawyers *et al*, 1992).

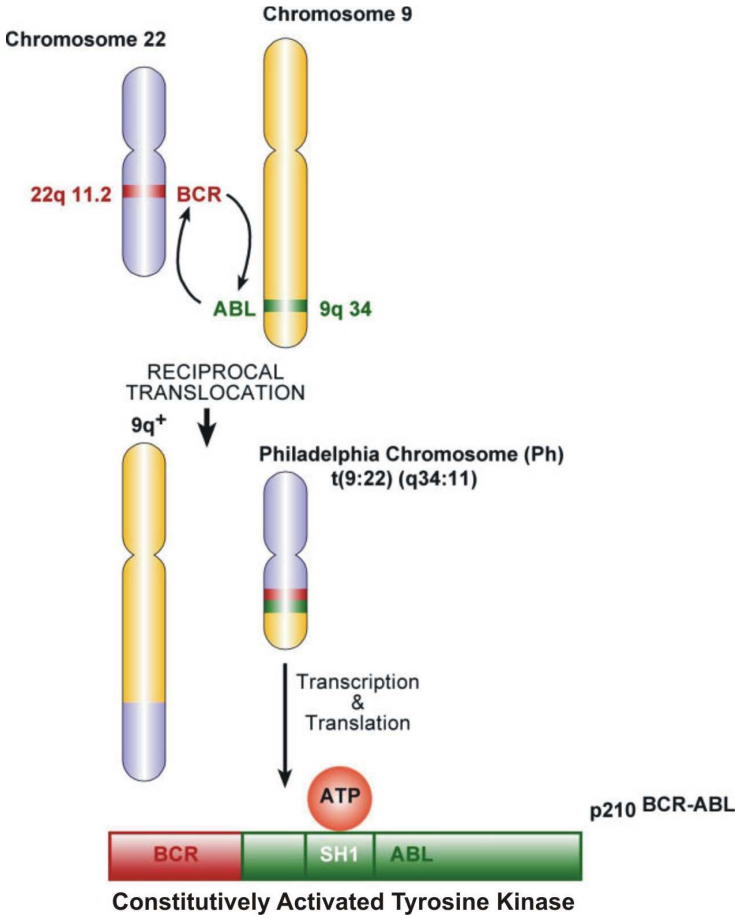
Současné podávání anti-CTLA4 protilátek a GVAX vakcín zvýšilo protinádorovou odpověď u pacientů s melanomem a nádorem prostaty (Hodi *et al*, 2003;Quezada *et al*, 2006;van den Eertwegh *et al*, 2012) a v myším melanomovém systému (van Elsas *et al*, 2001). Novější studie kombinují vakcínu GVAX s imunomodulačními dávkami cyklofosfamidu (Laheru *et al*, 2008) nebo cyklofosfamidu a doxorubicinu (Emens *et al*, 2009).

2.8 Charakteristika chronické myeloidní leukémie (CML)

CML je zhoubné hematologické onemocnění, které vzniká transformací kmenové hematopoetické buňky a její klonální expanzí. Charakteristickou genetickou mutací CML

je reciproká translokace mezi chromozómy 9 a 22 za vzniku tzv. filadelfského chromozómu a fúzního genu bcr-abl (Obrázek 4). Podle místa zlomu v genu pro bcr vznikají různě dlouhé produkty BCR-ABL o molekulové hmotnosti 190, 210 a 230 kDa.

Obrázek 4. Vznik filadelfského chromozómu



Převzato z (Frazer *et al*, 2007)

Nejčastějšími formami fúzního genu jsou b3a2 a b2a2 (podle novější nomenklatury e14a2 a e13a2), které generují BCR-ABL o hmotnosti 210 kDa. V místě spoje vzniká nová aminokyselina a vytvoří se tak unikátní sekvence, která se nevyskytuje v žádném jiném proteinu. BCR-ABL hraje klíčovou roli v patogenezi nemoci. Má podstatně zvýšenou tyrozinkinázovou aktivitu ve srovnání s produktem protoonkogenu abl. Svým působením na signální dráhy způsobuje jejich deregulaci, což vede k malignímu zvratu buňky. Ten se projeví sníženou adhezivitou k buňkám stromatu kostní dřeně, zvýšenou mitotickou aktivitou a zvýšenou odolností vůči apoptóze.

Klinický průběh nemoci se dá rozdělit do tří stádií: chronické fáze, akcelerované fáze a blastické krize. U většiny pacientů se nemoc zjistí během chronické fáze, která může trvat několik let. Její klinické příznaky jsou méně viditelné. Typickými projevy jsou únava, leukocytóza, bazofilie, krvácivost, mírná anémie, subfebrílie a splenomegálie. Nádorové leukocyty mají vyzrálejší charakter, a alespoň částečně si zachovávají funkci. Blastů je jen mizivé procento. Důsledkem dalších chromozomálních i genových mutací se stávají nádorové buňky agresivnějšími. Dochází k akcelerované fázi nemoci trvající několik měsíců až rok. Během tohoto období se zintenzivní všechny uvedené příznaky. Procento blastů mezi leukocyty se výrazně zvyšuje, ubývá normálně fungujících leukocytů. Konečným stádiem je blastická krize podobná akutní leukémii, která trvá jen několik týdnů či měsíců. Neléčená choroba končí smrtí. Smrtícími komplikacemi jsou obvykle krvácivé příhody a nezvládnutelné infekce.

CML je jednou ze čtyř základních forem leukémií. Incidence CML je 1-2 případy na 100 000 obyvatel za rok, což odpovídá 15% pacientů nemocných leukémií. Vyskytuje se spíše u dospělých, průměrný věk pacientů je 45-55 let. Nepatrně vyšší jsou procenta nemocných mužů než žen (Mauro & Druker, 2001).

2.9 Léčba CML

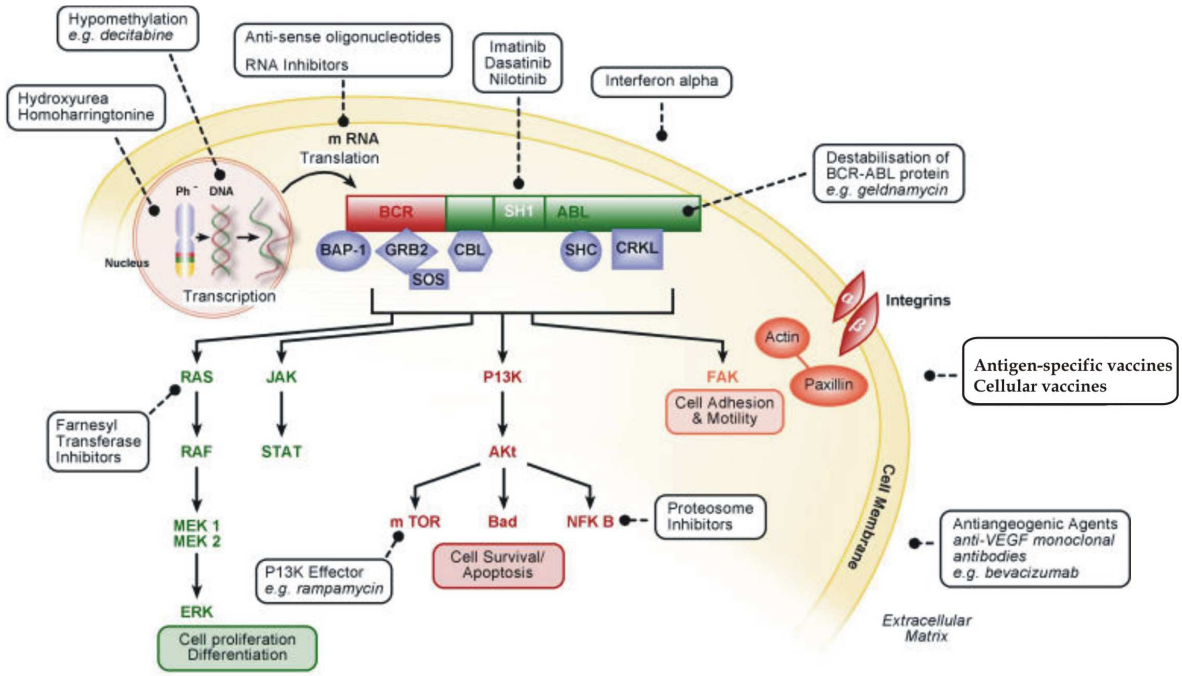
V současné době jedinou léčbou CML, která vede k úplnému vyléčení pacienta, je alogenní transplantace kostní dřeně. Stále je ale spojena s vysokou morbiditou a mortalitou, způsobenými toxicitou předtransplantačního režimu a reakcí štěpu proti hostiteli (GvHD). Není tak vhodná ani přístupná pro každého pacienta.

CML byla v minulosti léčena různými látkami včetně busulfanu, hydroxyurey a interferonu α (IFN-α), které dokázaly zmírnit symptomy nemoci a prodloužit dobu přežívání pacienta.

Dramatický obrat v léčbě nastal v roce 2001 zavedením cíleného terapeutika - imatinib mesylátu (IM = Gleevec, Glivec, STI 571). Je to inhibitor BCR-ABL tyrozinkinázy. Blokuje její vazebné místo pro ATP a zabraňuje tak vzniku aktivní konformace enzymu. Je poměrně vysoce specifický, dobře tolerovaný a vysoce účinný. Úspěšnost léčby se díky IM velmi zlepšila, zvlášť pokud byl podán v chronické fázi nemoci. Kompletní cytogenetické odpovědi bylo dosaženo u 90 % pacientů, pětileté přežívání bez rozvoje onemocnění vykazovalo 84 % pacientů (Druker *et al*, 2006).

Nicméně pacienti musí tento lék brát pravidelně a bez přestávky, protože IM nemoc nevyléčí, pouze zabraňuje její manifestaci. U části pacientů přestane po určité době léčba IM zabírat. Možných příčin rezistence je několik. Nejčastěji dochází k bodovým mutacím v ATP-vazebném místě BCR-ABL tyrozinkinázy, které zabraňují vazbě IM a/nebo dojde k amplifikaci genu bcr-abl a k zmnožení jeho produktu. Kromě toho IM není schopen zasáhnout leukemické kmenové buňky, které neproliferují a jsou k tyrozinkinázovým inhibitorům (TKI) rezistentní (Corbin *et al*, 2011;Hamilton *et al*, 2012). Ze zmíněných důvodů byly navrženy inhibitory tyrozinkináz druhé generace, mezi něž patří nilotinib a dasatinib. Nilotinib také blokuje vazebné místo pro ATP, ale vazba je mnohem silnější než u IM, což zvyšuje jeho účinnost i při mutacích v tomto místě. Dasatinib interaguje jak s aktivní, tak i s neaktivní formou tyrozinkinázy a je schopen se vázat na různé konformace BCR-ABL. Dokáže potlačit i leukemické buňky rezistentní k IM. Není ale již tak specifický a léčba může mít více vedlejších příznaků (Quintas-Cardama *et al*, 2007). Kromě TKI se stále ještě uplatňuje léčba pomocí IFN-α, který má antiproliferační a imunomodulační účinek. Obrázek 5 přehledně znázorňuje možné cíle imunoterapie CML.

Obrázek 5. Signální dráhy ovlivněné BCR-ABL a možná terapeutika CML



Převzato a upraveno (Frazer *et al*, 2007)

2.10 Imunoterapie CML

Vzhledem k tomu, že kromě transplantace kostní dřeně se dodnes nenašla léčba CML, která by chorobu úplně vyléčila, hledají se další možné přístupy k léčbě této choroby. Díky velkým pokrokům v molekulární biologii, genové terapii a imunologii nádorů se otevírají nové možnosti léčby CML pomocí imunoterapie.

Sama skutečnost, že alogenní transplantace dokáže CML vyléčit, je důkazem, že dostatečně silná protinádorová T-buněčná odpověď dokáže zlikvidovat leukemické buňky. Ze stejného důvodu zabírá i transfúze dárcovských lymfocytů při relapsech po transplantaci. Chronická fáze CML nabízí dostatečný časový prostor pro imunoterapeutickou léčbu a léčba IM přitom zajišťuje redukci nádorové masy buněk. To znamená, že ubude buněk, které je třeba zničit, a které mohou „bojovat“ s imunitním systémem prostřednictvím svých imunosupresivních mechanizmů. Výhodou je, že krev i lymfa, ve které leukemické buňky cirkulují, je dobře přístupná buňkám imunitního systému (Vonka, 2010).

Zpočátku byl vývoj vakcín zaměřen na fúzní protein BCR-ABL, který se v normálních buňkách nevyskytuje, zejména na spoj mezi BCR a ABL s unikátní aminokyselinovou sekvencí. Před několika lety se ale ukázalo, že fúzní spoj nenese imunodominantní epitop a imunita proti němu nestačí k vyvolání dostatečné protinádorové odpovědi (Grunebach *et al*, 2006).

Nyní se pozornost upíná na antigeny asociované s leukémií (LAA) jako jsou WT1, hTERT, PRAME, proteináza 3 (PR3), RHAMM/CD168, MPP11, survivin, G250 a další. (Schmitt *et al*, 2006;Smahel, 2011) Imunogenní potenciál těchto LAA byl potvrzen u pacientů identifikací specifických aktivovaných T-lymfocytů. Žádný z těchto LAA nebyl ale nalezen u všech CML pacientů a pacienti se navzájem liší v jejich expresi. Z toho vyplývá, že před zahájením imunoterapie by bylo vysoce účelné znát antigenní strukturu leukemických buněk pacienta a podle toho vybrat odpovídající typ vakcíny, ve které by byly zastoupeny pacientovy leukemické antigeny. To je i dlouhodobým záměrem naší pracovní skupiny.

Zajímavé je zjištění získané v průběhu léčby CML pomocí IM. Při ní dochází ke vzniku velmi silné imunitní reakce proti autologním nádorovým buňkám. Imunita má však krátkou dobu trvání a po dosažení remise úplně mizí (Chen *et al*, 2008b). Tento jev vedl k vypracování matematického modelu průběhu a léčby nemoci, který predikuje, že pokud

by se vakcína podala v době, kdy pacientovi koluje v těle určité množství nádorových buněk, je velká šance k jeho vyléčení (Kim *et al*, 2008).

Většina dosud navržených a zkoušených vakcín proti CML byla založena na peptidech odvozených z fúzní zóny BCR-ABL. Pro lepší imunogennost byly některé peptidy optimalizovány tak, aby se zlepšila jejich vazba na HLA molekuly. Dále byly zkoušeny dendritické buňky a peptidy odvozené od WT-1 a PR3 a HSP 70. První buněčná vakcína proti CML, která byla testována v klinické studii, byla připravena z geneticky modifikované nádorové linie buněk K562 produkující GM-CSF (Smith *et al*, 2010). Přehled dosud provedených klinických studií s vakcínami proti CML je uveden v tabulce 3. Všechny vakcíny byly dobře snášené, po aplikaci většiny z nich došlo ke specifické imunologické odpovědi a ke snížení množství transkriptu bcr-abl a u některých pacientů i ke klinickému zlepšení. Kromě odpovědi CD8+ lymfocytů byla pozorována i odpověď lymfocytů CD4+. Další preklinické studie se zaměřují na kombinaci vakcíny s blokací VEGF-dráhy nebo s podáním hypometylačních agens (Kihslinger & Godley, 2007;Kessler *et al*, 2007).

Slabiny dosud provedených klinických studií jsou v tom, že byly založeny na malém počtu pacientů, jednotlivé studie se lišily jak použitou vakcínou, tak i protokolem vakcinace, a nebyly dvojitě slepé. Zároveň se zdá, že kritéria, podle kterých se klinické studie imunoterapií hodnotí, by potřebovala upravit a sjednotit. Do budoucna je třeba pokračovat v dalších dobře navržených preklinických a klinických studiích, shromažďovat výsledky, které dovolí (a) zmapovat podrobněji expresi LAA antigenů u jednotlivých pacientů a (b) určit povahu imunitní odpovědi proti nim a (c) definovat protinádorové mechanizmy. Výsledky umožní podrobnější charakterizaci imunologického profilu jednotlivých pacientů, podle kterého se pak navrhne vhodná vakcína. Důležité bude i načasování vakcinace tak, aby její účinnost byla co nejvyšší.

Tabulka 3. Přehled dosavadních imunoterapeutických vakcín proti CML podle (Vonka, 2010) a (Pinilla-Ibarz & Quintas-Cardama, 2009)

Vakcína	Adjuvans	Počet pacientů, předchozí léčba	Výsledky	Reference
Směs peptidů fúzní zóny BCR-ABL, nativní i optimalizované peptidy	QS-21	12 (IFN-α,HU)	potvrzena dobrá snášenlivost a imunologická odpověď 3 pacienti kompletní cytogenetická odpověď	(Pinilla-Ibarz *et al*, 2000)
Směs peptidů z fúzní zóny BCR-ABL	QS-21	14 (IM,IFN-α)	5 pacientů kompletní cytogenetická remise	(Cathcart *et al*, 2004)
Směs peptidů z fúzní zóny BCR-ABL	QS-21 GM-CSF	16 i AML a MDS (IM,IFN-α)	10 pacientů cytogenetická odpověď, z toho 5pacientů kompletní cytogenetická remise, z toho 3 pacienti RT-PCR negativní	(Bocchia *et al*, 2005)
Směs peptidů z fúzní zóny BCR-ABL, optimalizované	GM-CSF montanide	13 (IM)	imunologická odpověď, 3 pacienti dosáhli RT-PCR negativity	(Maslak *et al*, 2008)
Směs peptidů z fúzní zóny BCR-ABL (nativní a optimalizované)	GM-CSF, montanide	10 (IM)	6 pacientů molekulární odpověď	(Jain *et al*, 2009)
Směs peptidů z fúzní zóny BCR-ABL	PADRE GM-CSF	19 (IM)	14 pacientů imunitní odpověď, molekulární odpověď	(Rojas *et al*, 2007)
Autologní peptidový komplex HSP-70 izolovaný z leukocytů	-	20 (IM)	13 pacientů cytogenetická nebo molekulární odpověď pacientů	(Li *et al*, 2005)
Autologní dendritické buňky	-	3 (IFN-α,HU)	všichni imunologická odpověď	(Takahashi *et al*, 2003)
Autologní dendritické buňky	BCG	3 (IFN-α, AraC, HU)	všichni imunologická odpověď	(Ossenkoppele *et al*, 2003)
Autologní dendritické buňky	-	10 (IFN-α,HU,IM)	4 pacienti cytogenetická odpověď, imunologická odpověď	(Westermann *et al*, 2007)
Alogenní buněčná vakcína K562/GM-CSF	-	34 (IM)	došlo k redukci masy maligních buněk, molekulární odpověď 5 pacientů trvalá RT-PCR negativita	(Smith *et al*, 2010)
Peptidy WT-1 a PR3	GM-CSF montanide	9 (jen 1 CML)	imunologická odpověď, molekulární odpověď	(Rezvani *et al*, 2008)

QS-21 Quillaja saponaria, HU hydroxyurea, AraC cytarabine, AML acute myeloid leukemia, MDS myelodysplastic syndrom

3. VÝSLEDKY

3.1 Isolation and properties of gene-modified mouse bcr-abl-transformed cells expressing various immunostimulatory factors

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Cíl práce: Získat geneticky modifikované buňky B210 se stabilní expresí cytokinů IL-2, IL-12 nebo GM-CSF

Výsledky práce:

1) Byla vybrána a optimalizována transfekční metoda vhodná pro lymfoblastoidní typ buněk.
2) Opakovanými pasážemi v přítomnosti BrdU byly získány buňky B210cTK$^-$, z nichž byl vybrán nejvíce patogenní klon B210cTK$^-$/cl-2 pro genovou modifikaci.
3) Buňky B210cTK$^-$/cl-2 byly transfekovány plazmidy nesoucí gen pro cytokin IL-2, IL-12 nebo GM-CSF a pro HSV TK.
4) Transdukované buňky byly izolovány v médiu s HAT. Izolované sublinie produkovaly příslušný cytokin.
5) Modifikované buňky byly citlivé ke GCV, měly nezměněnou expresi proteinu BCR-ABL a molekul MHC I a MHC II a stabilně exprimovaly příslušný cytokin.
6) Pokusy na myších ukázaly, že všechny buněčné sublinie exprimující cytokiny ztratily onkogenní potenciál.

Podíl na práci: zavedení a ověření metody transfekce lymfoblastoidních buněk, selekce transdukovaných buněk a jejich klonování, vyšetření testy ELISA, western blot, průtoková cytometrie, sepisování práce

Isolation and properties of gene-modified mouse *bcr-abl*-transformed cells expressing various immunostimulatory factors

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B210 cells are murine (BALB/c) cells transformed by bcr-abl fusion gene. After intravenous administration they are capable of inducing leukaemia–like disease in syngeneic mice. From these cells a thymidine–kinase less subline was derived. It was significantly less pathogenic than the parental cells. However, a highly pathogenic clone denoted B210cTK$^-$/cl-2 was isolated from its population. As determined by Western blotting, these cells produced more p210$^{bcr\text{-}abl}$ protein than the parental B210 cells. To successfully transfect these cells a modified electroporation method was introduced. Bicistronic plasmids carrying gene for herpes simplex thymidine kinase (HSV TK) and the gene for either granulocyte-monocyte colony stimulation factor (GM-CSF), interleukin-2 (IL-2) or interleukin 12 (IL-12) were used for the transfection experiments. Gradually, cell lines producing these cytokines were isolated in media supplemented with hypoxantin, aminopterin and thymidine (HAT). All of them were highly sensitive to ganciclovir *in vitro* confirming that the cells produced HSV TK. The genetic modification of B210cTK-/cl-2 was associated neither with the alteration of p210$^{bcr\text{-}abl}$ production nor with any changes in expression of MHC class I molecules. From populations of each of the three lines several cell clones were isolated and tested for the production of the respective cytokines. The original uncloned population and several clones differing in the cytokine production were administered intravenously into mice. All animals survived without symptoms of the disease suggesting that the gene-modification was associated with the loss of pathogenicity.

Key words: CML, Bcr-Abl, HSV TK, cytokines, gene-modified tumour cells, pathogenicity

Chronic myeloid leukaemia (CML) is a malignant disease of the hematopoietic stem cells. The neoplastic cells are characterized by the Philadelphia (Ph+) chromosome, which results from the reciprocal translocation between the chromosomes 9 and 22. As a consequence, the *bcr-abl* fusion gene develops. Its product, most frequently the p210$^{bcr\text{-}abl}$ protein, has a high tyrosine-kinase activity surpassing markedly that exhibited by the wild ABL protein [1, 2]. It is generally accepted that the fusion protein produced plays a key role in the pathogenesis of CML. The recently introduced imatinib-mesylate, a potent inhibitor of the p210$^{bcr\text{-}abl}$-associated tyrosine–kinase activity, has made a considerable progress in the therapy of CML [3, 4]. Still, in the past few years there has nevertheless been an increasing interest in developing immunotherapeutic means for treatment of CML. It has been demonstrated that peptides derived from the fusion zone and covering the fusion point are capable of inducing specific immune responses in CML patients [5, 6] and studies in mouse experimental systems indicated that it is possible to induce immunity against the challenge with the syngeneic, highly oncogenic *bcr-abl*-transformed cells [7–9]. In our laboratory a project aiming to develop therapeutic vaccines against the leukaemia-like disease induced in mice by *bcr-abl*-transformed cells is under way. One of the strategies which we are trying to employ for this purpose is the development of cell-based vaccines expressing a variety of cytokines known to act as enhancers of anti-tumor immunity [10, 11].

Materials and methods

Cell lines and media. Ba-p210 (B210) cells are *bcr-abl*-transformed mouse (Balb/c) cells derived by Daley and Baltimore [12] and have been kindly provided to us by G.Q.Daley (Whitehead Institute, Cambridge Center, MA). Their *in vitro* and *in vivo* properties were described in more detail elsewhere [13,

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14]. In brief, they induce leukaemia-like disease in mice after intravenous administration. One TID_{50} corresponds to approximately 10^4 cells. Thymidine-kinase (TK)-less cells B210TK$^-$ were derived in our laboratory after repeated passages of B210 cells at gradually increasing concentrations of 5-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich corp., St. Louis, MO). Cells were cultivated in RPMI 1640 medium (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria), 4 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO_2 atmosphere. In the case of B210/TK$^-$ the medium was supplemented with BrdU (100 μg/ml). For the selection of cells successfully transfected with herpes simplex virus thymidine kinase (HSV TK) carried by bicistronic plasmids (see below), the RPMI was supplemented with hypoxantin, aminopterin and thymidine (HAT) (HAT Supplement, Gibco, Invitrogen, Carlsbad, CA). 293T cells (kindly provided by J. Kleinschmidt, DKFZ, Heidelberg, Germany) were used as a negative control in Western blotting. Their propagation was the same as in previous experiments [15].

Plasmids. The construction of plasmid carrying HSV TK (denoted pTR-IRES TK) [16] and bicistronic plasmids carrying the genes for HSV TK and either granulocyte-macrophage colony stimulation factor (GM-CSF) (denoted pTR-GM-CSF-IRES-TK) or interleukin 2 (IL-2), (denoted pTR-IL-2-IRES-TK) has been described [15]. The bicistronic pTR-IL-12-IRES-TK plasmid which carries the HSV TK gene and also the gene for mouse interleukin 12 (IL-12) was constructed (Fig. 1). The cDNAs of p40 (1008 bp) and p35 (648 bp) subunits of IL-12 were amplified using PCR, subcloned and sequenced. In the second step genes coding for the p35 and p40 subunits were linked up with the 24 base pair long sequence encoding a cleavage site for the cell endoprotease furin (Gly-Gly-Arg-Gly-Arg-Arg-Gly-Gly), [17]. With the use of XbaI and HindIII restriction enzymes the IL-12 fused gene was inserted into pTR-IRES-TK. Plasmid expressing green fluorescent protein (GFP), denoted pTR-UF2 [18] was used for monitoring transfection efficiency. Plasmids were propagated in E.coli DH5-α (Gibco, Invitrogen, Carlsbad, CA) and purified using Maxi Prep DNA isolation Qiagen kit (Qiagen, Hilden, Germany).

Electroporation. Electroporation was performed using Gene Pulser Electroporation system (Bio-Rad, Hercules, CA). Originally, counts of $5x10^6$ cells suspended in 500 μl of electroporation medium composed of HeBS (0.75 mM Na_2HPO_4, 5 mM KCl, 140 mM NaCl, 6mM glucose, 25mM Hepes, pH 7.05), were placed in 0.4 cm gap cuvette (Bio-Rad, Hercules, CA) and 15 μg of plasmid DNA was added. Electroporation was performed at room temperature (RT) exposing the cells to 250V, 975 μF. The cells were allowed to remain in the electroporation buffer for 10 min, and were then transferred onto tissue culture dishes with 4 ml of media. After 48 hrs of cultivation the cells were spun down and resuspended in the selection media. To optimise the procedure the experimental conditions were gradually modified (see the Results section) with the use of pTR-UF2 plasmid carrying the gene for GFP. The cells transfected with pTR-UF2 plasmid were monitored under fluorescent microscope 24 hrs after electroporation. Subsequently, the transfection efficiency was determined by flow cytometric analysis of GFP expression using Flow Cytometer EPICS XL (Beckman Coulter, Inc., Fullerton, CA). Counts of 5 x 10^5 cells were washed with PBS, resuspended in 0.5 ml PBS, and 10 μg/ml propidiumiodide (PI) was added immediately before the flow cytometric analysis. Living cells were gated and evaluated for GFP expression using WinMDI (version 2.8) software.

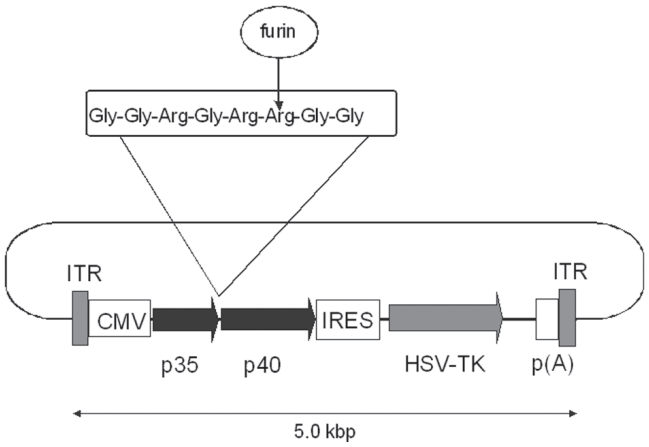

Figure 1. Recombinant plasmid carrying HSV TK and mouse IL-12 genes. It contains the adenovirus-associated virus type 2 (AAV-2) inverted terminal repeats (ITR), the cytomegalovirus immediate early promoter (CMV), mIL-12 gene consisting of p35 and p40 subunits linked with 24 bp sequence containing furin cleavage site, poliovirus type 2 internal ribosomal entry site sequence (IRES), herpes simplex virus thymidine kinase gene (HSV TK) and the bovine growth hormone polyadenylation signal (pA)

GCV sensitivity assay. Counts of 5 x 10^4 cells/well were seeded in 2 ml cultivation media with and without 40 μM GCV (Cymevene, Roche, Basel, Switzerland) and either HAT in 24-well plates. After 5-day cultivation cells were counted using the trypan blue exclusion.

Measurement of cytokine production. Counts of 5 x 10^5 cells were seeded in 3 ml medium in 6-cm culture dishes. The concentration of cytokine, viz mouse GM-CSF, IL2 or IL12, in culture supernatants was measured after 24 hours with the BD OptEIA™ Set Mouse GM-CSF or Mouse IL-2 or Mouse IL-12 (p70) (BD Biosciences, San Diego, CA) following the manufacturer's instructions. Cells were counted using the trypan blue exclusion test. The production level was calculated according to the formula: C/N (where C is the total amount of cytokine in culture medium and N is the final viable cells count) and was expressed in ng/10^6 cells/24 hrs.

Western blotting. Cells were lysed in a lysis buffer (4%SDS, 20% glycerol, 10% mercaptoethanol, 2 mM/l EDTA, 100 mM/l Tris-HCl (pH 8.0)) and after adding bromphenol blue they were boiled for 3 minutes. Lysates of 5 x 10^4 cells were separated by 7% SDS-PAGE electrophoresis. The protein's pattern

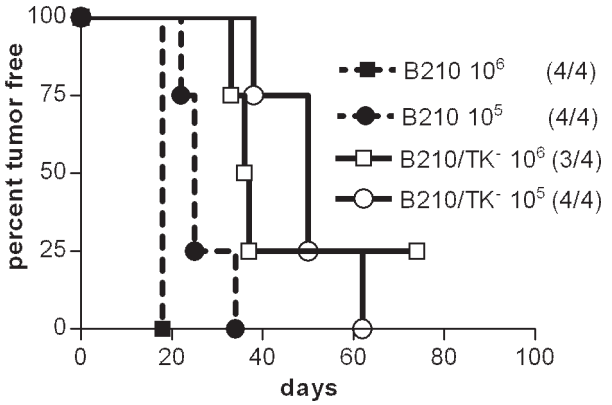

Figure 2. Pathogenicity for mice of B210 cells and its B210/TK- subline

was electroblotted onto nitrocellulose membrane. Any remaining binding sites were blocked in 10% skimmed milk at room temperature for 1 hour. The membrane was then incubated with mouse monoclonal anti-c-ABL antibody (Ab-3, Oncogene Research Products, Boston, MA). The incubation was provided under constant agitation at room temperature for 1 hour and then at 4°C overnight. The membrane was then washed with 0.1% Tween in PBS for 3 x 10 minutes and treated for 1 hour with peroxidase-labelled secondary anti-mouse antibody (Amersham Biosciences, Little Chalfont, UK). The blot was again washed for 3 x 10 minutes. Immunocomplexes were visualized using the ECL plus system (Amersham Biosciences, Little Chalfont, UK).

Flow cytometry for determination of MHC class I and II expression. Counts of 0.5 x 10^6 cells were washed twice with PBS. Detection of MHC class I molecules was performed after 30 min incubation at 4°C (i) with FITC-conjugated anti-mouse either H-2$K^d$$D^d$ monoclonal antibody (Cedarlane, Hornby, Ontario, Canada) or isotype control antibody (Sigma, St. Louis, MO), (ii) with phycoerythrin (PE)-conjugated either anti-mouse H-2L^d monoclonal antibody (Cedarlane, Hornby, Ontario, Canada) or with isotype control antibody (Cedarlane, Hornby, Ontario, Canada), and (iii) with FITC-conjugated anti-mouse either I-A^d monoclonal antibody (Cedarlane, Hornby, Ontario, Canada) or isotype control antibody (Sigma, St. Louis, MO).

Cell cloning. To isolate cell clones from the transduced cells, fresh cultivation medium was mixed with the spent medium, at a ratio 2:1. Spent medium was obtained from the culture of the particular transfected cell type at its growing phase. Before being mixed with the fresh medium, the spent medium was filtered through 0.22μm-Syringe-Filter (TPP, Trasadingen, Switzerland). To isolate cell clones, the transfected cells were diluted to obtain a final concentration of 0.3-cell/0.2 ml. The suspension in 0.2 volumes was distributed into 96-well plate (TPP, Trasadingen, Switzerland). Four hours after seeding all wells were carefully checked for the presence of cells and those containing only one cell were labelled. After approximately 14-day incubation the suspensions from these wells were sucked away and transferred into bigger plates. The cell lines derived were kept frozen in liquid nitrogen until being used.

Animals and oncogenicity assay . Six to 8 week-old female BALB/c mice were obtained from Charles Rivers, Germany. All experiments were carried out in accordance with the Guidelines for Animal Experimentation valid in the Czech Republic. For oncogenicity tests, cells were washed three times with PBS. If not indicated otherwise, counts of 10^6 in 0.2ml volumes of PBS were injected intravenously. Starting one week later, mice were monitored for the symptoms of the disease at least twice a week for up to 100 days.

Statistical analysis. For analysis of the growth curves of the tumours, the two-way analysis of variance was used. Calculations were done using Prism Software Version 3.0 (Graph-Pad Software, San Diego, CA).

Results

Derivation and pathogenicity of cTK- cells. B210 cells were passaged for a prolonged period of time at a gradually increasing concentration of BrdU. Finally, a cell line growing well in the presence of 100 μg of BrdU but incapable of replicating in the HAT media was isolated. The cell line was labelled B210cTK-. To determine its pathogenicity, two different doses (10^5 and 10^6) of these cells were tested in parallel with the parental cells. The results are shown in Figure 2. It can be seen that nearly all mice inoculated with the cTK- cells developed the disease; however, their survival was considerably prolonged ($p<0.01$). This indicated that the loss of cTK, but possibly other mutations which might have been induced by BrdU, resulted in decreasing the virulence of the cells. To obtain more information on the composition of the B210cTK- cell population, we isolated 11 clones and tested 8 of them for leukemogenic potency. The counts of 10^6 cells were administered i.v. The results are shown in Table 1. A marked variation in pathogenicity was apparent. While clone 1-derived cells did not induce disease in any of the mice inoculated, four

Table 1. Pathogenicity of cell clones derived from B210cTK- cells

Clone number	No. of mice with leukaemia/ No. of mice inoculated[1)]
1	0/4
2	3/3
3	2/4
4	3/3
7	3/3
9	3/3
10	2/3
11	2/3

[1)]ninety days after inoculation

other cell lines induced lethal leukaemia in all animals. The earliest onset of the deadly disease was observed in those inoculated with clone 2-cells.All animals died before day 49, while in the case of clones 4, 7 and 9 the last animal died on day 87, 72 and 87, respectively. Since one of the main aims in this study was to find out the impact of cytokine production on the pathogenicity of the gene-modified cells (see below), we selected clone-2 derived cells denoted B210cTK$^-$/cl-2 for further experiments. A large frozen stock of these cells was prepared and in subsequent experiments always the third passage of the thawed cells was used.

Transfection of B210 cells. Our initial attempts to transduce B210 cells using calcium-phosphate precipitation, metafectene (Biontex, Planegg, Germany) or transferin-polyethylenimine (Bender MedSystems, Burlingame, CA) failed. However, the subsequent tests indicated that using electroporation might solve the problem. Our efforts were aimed at optimising the condition of electroporation, i.e. to achieve sufficient percentage of transfected cells and to preserve their survival within acceptable limits. Multiple experiments were performed using pTR-UF2 plasmid carrying the gene for GFP. The efficacy of transfection was monitored by both fluorescent microscopy and flow cytometry. The highest expression of GFP was demonstrated 24 hours after electroporation. Later on, by changing the conditions of electroporation by substituting RPMI 1640 medium for the original elctroporation buffer and exposing the cells to 280-300V and 1050 μF we increased the transfection efficiency up to 16-18% of the surviving cells. The lower capacitance than 1050 μF decreased the transfection efficiency. Higher concentration of plasmid DNA (up to 30 μg) increased the number of GFP-positive cells without producing any dramatic impact on cell survival. The main results are summarized in Tables 2A and 2B. Thus, the optimum condition for electroporation in the present system appeared to be to use RPMI medium and to expose the cells to 280V and 1050 μF at room temperature.

Generation of gene modified B210 cells and their sensitivity to GCV. Based on these results we tried to prepare the cytokine producing B210 sublines. The B210cTK$^-$/cl-2 cells were separately transfected with pTR-GM-CSF-IRES-TK, pTR-IL-2-IRES-TK, pTR-IL-12-IRES-TK or pTR-IRES-TK plasmid, or mock transfected. The transfected cultures were kept in regular cultivation media for 48 hrs and then they were transferred into media containing HAT. Within three weeks marked cell proliferation was detected in all transfected cell cultures, whereas no cells survived in the mock-transfected cultures. To make certain that the cell lines isolated were not revertants to the cTK$^+$ phenotype but real transductants we tested their sensitivity to GCV. At a variance with the parental cells all the transfected cell lines were highly sensitive to GCV, which confirmed the production of HSV TK (results not shown). The transduced cells were labelled B210/2/GM-CSF, B210/2/IL-2, B210/2/IL-12 and B210/2/HSVTK, respectively.

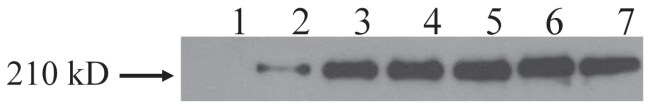

Figure 3. Production of p210$^{bcr-abl}$ in B210 cells and their gene-modified sublines as determined by Western blotting. Anti-c-ABL monoclonal antibody was used. Lane 1: 293T cells (negative control); lane 2: B210 (positive control); lane 3: B210cTK$^-$/cl-2; lane 4: B210/2/GM-CSF; lane 5: B210/2/IL-2; lane 6: B210/2/IL-12 and lane 7: B210/2/HSVTK. Each sample was a lysate from 5 x 10^4 cells.

Cytokine production by gene modified B210 cells. The cell lines were then tested for the cytokine production in ELISA. The B210cTK$^-$/cl-2 and B210/2/HSVTK served as negative controls. The cell lines B210/2/GM-CSF, B210/2/IL-2, and B210/2/IL-12 were confirmed as the producers of the respective cytokines forming 30 ng/10^{6}cells/24 hrs of GM-CSF, 8 ng/10^{6}cells/24 hrs of IL-2 and 160 ng/10^{6}cells/24 hrs of IL-12, respectively.

Immunoblotting detection of the p210$^{bcr-abl}$ protein. The Western blotting test with lysates of parental B210 cells and all the cell lines derived was made to check the expression of p210$^{bcr-abl}$ protein using mouse monoclonal anti-c-abl antibody. The results are shown at Fig. 3. As evident, both the B210cTK$^-$/cl-2 cells and all four transduced cells derived from them produced approximately the same amount of the p210$^{bcr-abl}$ protein, this indicating that the production of the cytokines or HSV TK was not associated with an alteration of p210$^{bcr-abl}$ protein production. However, it is noteworthy that the production of this pro-

Table 2. Influence of varying conditions of electroporation on the expression of green fluorescent protein (GFP) and cell survival

A

El. medium	U (V)	c (μF)	Temperature	% Cell survival	% GFP+
HeBS	250	1050	RT	35	6
PBS	250	1050	RT	30	3
RPMI	250	1050	RT	57	9
HeBS	250	1050	0°C	40	1,2
PBS	250	1050	0°C	28	1
RPMI	250	1050	0°C	45	3,1

B

El. medium	U (V)	c (μF)	Temperature	% Cell survival	% GFP+
RPMI	220	1050	RT	76	3
RPMI	240	1050	RT	62	8
RPMI	250	1050	RT	57	9,6
RPMI	260	1050	RT	60	11
RPMI	280	1050	RT	58	16
RPMI	300	1050	RT	42	18

El. Medium: electroporation medium used, U: voltage, c: capacitance, % Cell survival: percentage of living cells after 24 hrs, %GFP+: percentage of GFP positive cells among living cells after 24 hrs

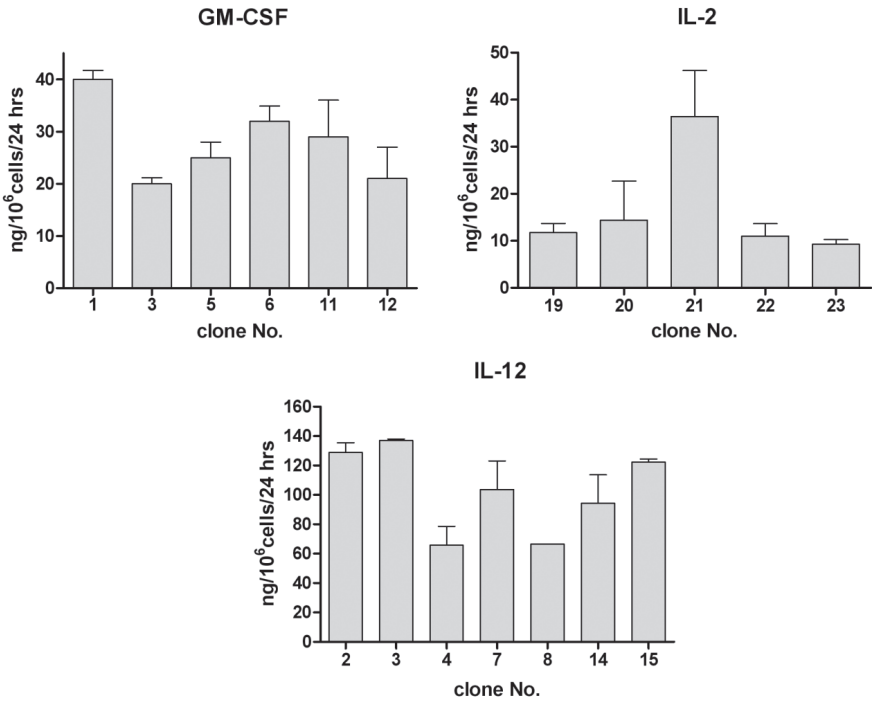

Figure 4. Cytokine production by clones derived from the respective B210 gene-modified cells. All clones were tested simultaneously in three repeated tests.

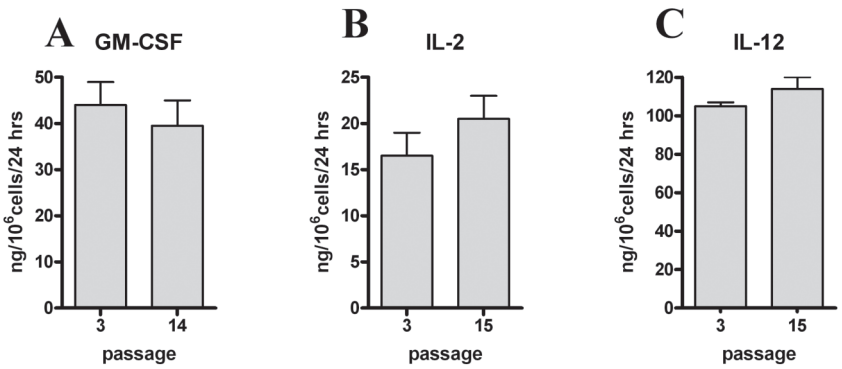

Figure 5. Production of the respective cytokines by the selected cloned cell lines in the course of passages *in vitro*. The following clones were selected: (A) B210/2/GM-CSF/cl-1; (B) B210/2/IL-2/cl-21; (C) B210/IL-12/cl-3

tein by all these cells was considerably higher than in the case of the parental B210 cells used as positive control. Similar results were obtained in all three repeated tests using two different sest of cell lysates.

Isolation of clones of the gene-modified cells and their efficacy as cytokine producers. From the cultures transfected with plasmids carrying the genes for GM-CSF, IL-2 and IL-12 cell clones were isolated and tested for the respective transduced gene products. The results are summarized in Fig. 4. It can bee seen that the production of the cytokines by individual clones markedly differed. The best producers were cultivated in the HAT medium up to fifteen passages, three times a week, and the production of the respective cytokines was measured by ELISA test. It may be seen in Fig. 5 that in the course of passaging the production of the cytokines did not dramatically change.

Determination of MHC class I and II expression of B210 derived cell clones. We also examined parental B210cTK$^-$/cl-2 and the derived cell lines for MHC class I and MHC class II expression. As shown elsewhere [13], MHC class I molecules

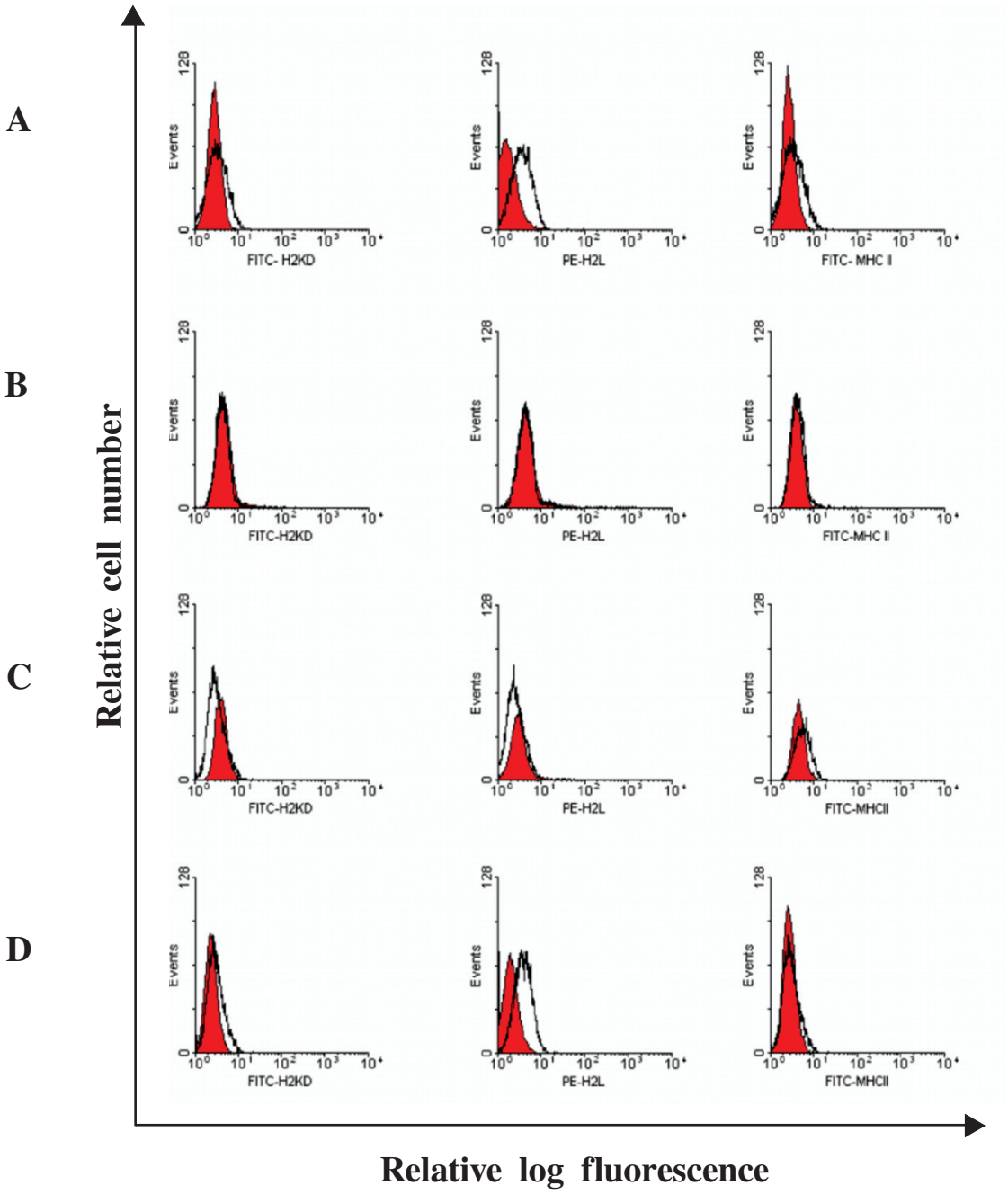

Figure 6. Flow cytometric analysis of MHC class I (H-2K^d and H-2L^d) and MHC class II (I-A^d) expression on gene-modified B210 cells:(A) B210cTK$^-$/cl-2; (B) B210/2/GM-CSF/cl-1; (C) B210/2/IL-2/cl-21; (D) B210/2/IL-12/cl-3. Empty histograms represent cells incubated with isotype control antibodies; filled histograms represent cells incubated with specific antibodies.

in B210 cells tend to be strongly downregulated. Data shown in Fig. 6 provide evidence that this property remained unchanged in all the transduced sublines tested.

Pathogenicity of the gene-modified cells for mice. To examine the influence of the cytokine production on the virulence of the respective cell lines, mice were inoculated with both the uncloned cell populations and with selected cloned sublines expressing either IL-2 or IL-12. In all instances 10^6 cells were administered i.v. The summary of the experiments is in Table 3. It can be seen that none of the cells expressing either GM-CSF or IL-2 or IL-12 was pathogenic for mice. On the other hand, the control cell line expressing

Table 3. Pathogenicity of the gene-modified cells for mice

Cell line	No.of mice with leukemia/ No.of mice inoculated[1)]
B210	4/4
B210cTK$^-$	3/4
B210cTK$^-$ /cl-2	3/3
B210/2/HSV TK	3/4
B210/2/GM-CSF	0/4
B210/2/IL-12	0/4
B210/2/IL-2	0/4
B210/2/IL-12/cl-2	0/3
B210/2/IL-12/cl-3	0/3
B210/2/IL-12/cl-4	0/3
B210/2/IL-12/cl-5	0/3
B210/2/IL-2/cl-19	0/3
B210/2/IL-2/cl-20	0/3
B210/2/IL-2/cl-21	0/3

1)In al instances 10^6 cells were administered intravenously

only HSV-TK induced deadly disease in 3 out of the 4 animals inoculated.

Discussion

In the present experiments the preparation of cytokine-producing *bcr-abl*-transformed cells was undertaken with the use of a system that had proved efficient in our previous experiments. This approach is based on the isolation of cTK-less cells and on making the use of bicistronic plasmids that carried together with the gene of interest also the HSV TK gene [15, 16] for transfection. To derive B210cTK$^-$ was not an easy task. For some reason it took about half a year of continuous propagating these cells at gradually increasing concentrations of BrdU, before cells growing at the 100 μg of this drug were isolated. These cells were less pathogenic than the parental cells. Clonal analysis revealed that the B210cTK$^-$ population was composed of cells mutually markedly differing in their pathogenicity for mice. Since we wanted to determine the impact of the production of selected cytokines on the pathogenicity of the transduced cells, we selected for the transfection experiments a clone of B210cTK$^-$ cells with the highest leukemogenic activity. Our attempts to use for transfection of the B210 cell progenies the techniques, which in our hands had been highly efficient for transfection of epithelial or fibroblastic cells [15, 19, 20], failed completely. However, we were quite successful when using electroporation technique. The optimal conditions for the transduction were defined using a plasmid that carried the gene for GFP. The highest transfection efficiency as well as the best cell survival was achieved with serum-free RPMI medium being used as the electroporation buffer. The transduced cells were producing reasonable amounts of the respective cytokines. The $p210^{bcr\text{-}abl}$ production by all transduced cells was approximately the same as detected in the B210cTK$^-$/cl-2 cells from which they were derived. Surprisingly, it was more efficient than by the parental B210 cells. At this writing, no reasonable explanation can be offered for this observation. We can only speculate that this phenomenon seen in repeated tests might be associated with mutations induced in the course of prolonged cultivation of the B210 cells in the presence of BrdU. On the other hand, the transduced cells did not differ from the parental cells in the expression of MHC class I and II molecules. Still, clonal analysis of the population of the transduced cells demonstrated a quite extensive inhomogeneity of the respective cell populations: the cell clones isolated differed widely in the production of all three cytokines. The cytokine production was apparently a stable property of the clones in the course of repeated passages *in vitro*. Since it was difficult to examine all the derived cell lines for their pathogenicity in mice, in addition to the uncloned populations we tested only several clones derived from B210/2/IL-2 and B210/2/IL-12 cells, which differed in cytokine production. All three cell lines were free of leukemogenic activity and also none of the cell clones tested was pathogenic, this suggesting that in none of them the production of the respective cytokine was below the critical level. Experiments are under way to further clarify this point. On the other hand, cells expressing HSV TK were capable of inducing leukaemia in mice, this indicating that the expression of this enzyme did not play any major role in the loss of pathogenicity observed in the case of cytokine-producing cell lines. The successful "attenuation" of the B210cTK$^-$/cl-2 cells provided us with a means useable as live vaccines in the therapy of experimental disease induced in mice by *bcr-abl*-transformed cells. Experiments are under way in which the potency of these vaccines for both prophylactic and therapeutic purposes is tested.

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3.2 Combined chemo- and immunotherapy of tumors induced in mice by bcr-abl-transformed cells

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Cíl práce: Otestovat na myších účinek kombinace chemoterapeutik a vakcinace genově modifikovanými buňkami B210/2/IL-2, B210/2/IL-12 a B210/2/GM-CSF

Výsledky práce:

1) Kombinace cyklofosfamidu (Cy) + IFN-α a Cy + IM výrazně zpomalila růst nádorů vyvolaných buňkami 12B1 jak oproti negativní kontrole, tak oproti podání samotného cyklofosfamidu.
2) Geneticky modifikované vakcíny z buněk B210 neochránily myši před vznikem nádorů, vyvolaných buňkami 12B1, ale významně oddálily jejich objevení. Výsledek byl podobný u všech tří buněčných linií nezávisle na tom, který cytokin exprimovaly.
3) Pokud se kombinovala chemoterapeutika s buněčnými vakcínami, nejlepšího výsledku bylo dosaženo po podání vakcíny B210/2/IL-2 společně s Cy + IFN-α nebo Cy +IM. Většina myší zůstala ochráněna a u ostatních se nádory objevily později a rostly pomaleji.

Podíl na práci: izolace genově modifikovaných buněk

Combined chemo- and immunotherapy of tumors induced in mice by *bcr-abl*-transformed cells

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Abstract. For our experiments we selected two oncogenic, *bcr-abl*-transformed mouse cell lines, viz. B210 and 12B1. Both cell types are capable of inducing leukemia-like disease in syngeneic BALB/c mice after intravenous inoculation. 12B1 cells can moreover form solid tumors after subcutaneous injection. Since immunotherapy would expectedly be most effective in animals in which the tumor mass had been reduced by other therapeutic means, we attempted to develop a combined therapeutic system for suppressing tumor growth. In the present study, mice inoculated with the aggressive 12B1 cells were treated with imatinib mesylate (IM), mouse interferon α (IFNα) and cyclophosphamide (Cy) in combination with genetically modified tumor cells engineered to produce various cytokines. These cell vaccines had been derived from B210 cells. Therapy with IM or IFNα alone or cell immunotherapy alone resulted in partial suppression of tumor growth. Of the different therapeutic regimens tested, a combination of repeated doses of IM, IFNα and cell vaccines with one relatively high dose of Cy (200 mg/kg) was the most effective, resulting in tumor-free survival of a large portion of mice. The spleens, livers and bone marrows of the successfully treated animals were tested for the presence of *bcr-abl*-positive cells by means of RT-PCR technique. Results were negative, this suggesting that the animals had been cleared of residual disease.

Introduction

Chronic myeloid leukemia (CML) is a lethal disease of blood stem cells. In the pathogenesis of this disease, the key role is played by the *bcr-abl* fusion gene, which originates from a translocation between chromosomes 9 and 22. The product of the fusion gene exists in three forms, viz. $p210^{bcr\text{-}abl}$, $p190^{bcr\text{-}abl}$ or $p230^{bcr\text{-}abl}$. Of these, $p210^{bcr\text{-}abl}$ is the most common. The *bcr-abl* fusion protein has a markedly increased activity of tyrosine-kinase (TyKi), which is coded for by the SH1 domain of the *abl* gene. It is generally accepted that this activity is responsible for both the cell transformation and the maintenance of the transformation state (1,2).

Targeting the TyKi activity of *bcr-abl* appears to be a highly attractive therapeutic strategy (3). Imatinib mesylate (IM), one of the 2-phenylaminopyrimidine derivatives, is a direct inhibitor of the TyKi activity of the BCR-ABL fusion protein (4-6). IM competitively inhibits the interaction of these proteins with adenosine triphosphate (ATP) (7) which is necessary for TyKi activity. IM inhibits the growth of *bcr-abl*-positive cells both *in vivo* and *in vitro* (8-10) and is capable of inducing long-term remissions and prolonging the life of CML patients considerably (11-13). In the treatment of chronic phase CML, IM has provided much better hematological and cytogenetic responses than INFα (14), which until recently was widely used for CML treatment. Already the early studies in mouse model systems also proved a high efficacy of IM, viz. retardation of the growth of tumors induced by *bcr-abl*-transformed cells (4,9,15). In the past few years several mechanisms of resistance to IM have been recognized (13,15-17). Furthermore, in spite of its high specificity and low toxicity, some dose-dependent side effects of IM have been reported (12,18). Quite recently impairment of proliferation and function of $CD4^+CD25^+$ cells (19) and a strong, though transient anti-leukemia immune reaction (20) have been observed in IM-treated patients. On the basis of the experience with this drug, combination of IM with other antileukemic agents has been proposed and examined. Burchert *et al* (21) have reported that the concurrent or sequential combinatory therapy with IFNα and IM, taking advantage of their different effector mechanisms, has been more effective in the treatment of CML than any current monotherapy. Also our results (Sobotkova *et al*, unpublished data) indicated that a combination of IM with INFα was more effective in suppressing leukemia-like disease induced by the *bcr-abl*-transformed Ba-P210 (B210) cells developed by Daley and Baltimore (22) than its treatment with either of these substances alone (23). A combination of IM with INFα and other substances *in vitro* has resulted in additive or synergistic antiproliferative effects, with *bcr-abl*-positive cell lines having been used (24-26). For example, Kano *et al* (25) have shown some synergistic *in vitro* cytotoxic effects of IM

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Key words: chronic myeloid leukemia, mouse *bcr-abl*-transformed cells, cell vaccines, imatinib mesylate, cyclophosphamide, interferon α

and recombinant IFNα and an additive effect of 4-hydroperoxy-cyclophosphamide [the active form of cyclophosphamide (Cy)]. Because of the immunomodulatory activity of Cy (27-30) one could expect additional beneficial *in vivo* effects of this drug if used either alone or in combination with the above drugs and/or with experimental vaccines.

In the study reported below, the therapy of disease induced in mice by *bcr-abl*-transformed cells was attempted with IM, INFα and Cy, applied either alone or in various combinations and in combination with vaccines based on another syngeneic *bcr-abl*-transformed cell line that had been engineered to produce either interleukin-2 (IL-2) or interleukin-12 (IL-12) or the granulocyte-monocyte colony-stimulating factor (GM-CSF). To be able to easily reveal possible synergistic effects, we employed suboptimal treatment regimens with IM and INFα.

Materials and methods

Cell lines and media. Two BALB/c mouse cell lines transformed by the *bcr-abl* gene (b3a2) and expressing $p210^{bcr-abl}$ protein were used. 12B1 cells were obtained through the courtesy of E. Katsanis (University of Arizona, Tuscon, AZ, USA). They had been derived by transformation of primary bone marrow cells with a retrovirus-derived vector carrying the *bcr-abl* fusion gene (31). Ba-P210 (B210) cells were kindly provided by G.Q. Daley (Whitehead Institute of Biochemical Research, Cambridge, MA, USA). They had been derived from interleukin 3 (IL-3)-dependent Ba/F3 cells (22). Their transduction by the *bcr-abl* gene carried by a retroviral vector had made them IL-3-independent. We described the basic *in vitro* and *in vivo* characteristics of these two cell lines in more detail elsewhere (10,32). In brief, both express approximately the same amounts of the $p210^{bcr-abl}$ protein but differ in MHC class I expression (it is down-regulated in B210 cells) and in oncogenic potential. While B210 induce leukemia-like disease at doses exceeding $5x10^4$ cells only after intravenous (i.v.) inoculation, 12B1 induce leukemia at doses $<10^2$ cells after i.v. inoculation and, in addition, induce solid tumors after subcutaneous (s.c.) inoculation of doses equal to or $>10^3$ cells. These tumors exhibit a high metastatic activity to spleen, liver and bone marrow. Both cell lines are highly susceptible to IM *in vitro*. After i.v. inoculation of $5x10^5$ B210 cells, the animals develop leukemia in the course of the third week. After s.c. inoculation of 5.10^3 12B1 cells, animals form rapidly growing solid tumors that appear after 12-14-day incubation. Bcr-abl-negative HL-60 cells were used as a negative control in PCR (see below). All cell lines were passaged in RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% FCS (PAA Labs., Linz, Austria), 2 mmol/l glutamine and antibiotics. In the case of 12B1 cells, the medium was furthermore enriched with 1 mmol/l sodium pyruvate and 50 μmol/l 2-mercaptoethanol.

Cell vaccines. After repeated passages of B210 cells in the presence of increasing concentrations of 5-bromo-2'-deoxyuridine (BUDR), a thymidine-kinase-less (TK^-) subline was isolated. These cells, designated $B210TK^-/cl-2$, grew well in the presence of 100 μg BUDR, but did not grow in medium supplemented with hypoxanthine, aminopterin and thymidine (HAT media Supplement, Invitrogen, Carlsbad, CA, USA) and were oncogenic for mice after i.v. inoculation. After transfection with bicistronic plasmids carrying genes for herpes simplex virus TK (HSV TK) and for various cytokines, genetically modified cell lines were isolated in HAT media. The construction of these plasmids was described previously (33). In addition to HSV TK, the gene-modified cells expressed IL-2 (B210/IL-2, IL-2 production 46.5 ng/10^6/24 h), IL-12 (B210/IL-12, IL-12 production 130 ng/10^6/24 h) or GM-CSF (B210/GM-CSF, GM-CSF production 40 ng/10^6/24 h). *In vitro*, all three cell lines were highly sensitive to ganciclovir, this confirming HSV TK production, and were non-oncogenic after i.v. inoculation (Petrackova *et al*, unpublished data).

Animals used and tumor induction. Seven- to 8-week old BALB/c female mice were used in all experiments. They had been obtained from Charles Rivers, Germany. The mice were inoculated s.c. with $5x10^3$ 12B1 cells in 0.2 ml PBS. In therapeutic experiments, 10^6 B210/IL-2, B210/IL-12 or B210/GM-CSF cells in 0.2 ml PBS were repeatedly injected intraperitoneally (i.p.) (see the Results section). The animals were inspected for tumor development at least twice a week and tumor size was measured with a caliper. Animals carrying tumors exceeding 20 mm in their longest diameter were humanely sacrificed. All animal studies were done in accordance with the Guidelines for Animal Experimentation valid in the Czech Republic.

Reagents. Imatinib mesylate (IM, STI571, Glivec) was a generous gift from Novartis (Basel, Switzerland). It was dissolved in distilled water (1 mg/ml), sterilized by filtration through a Millipore filter, distributed into vials and kept at -20˚C until use. It was inoculated i.p., one dose (50 mg/kg) per day, five times a week for one or two weeks, starting on day 3 or 10 after the s.c. inoculation of 12B1 cells. Cyclophosphamide (Cy) (Farmos, Finland) was given in a single i.p. dose, 200 mg/kg, on day 3 after inoculation of 12B1 cells. Recombinant mouse interferon α (INFα) (Calbiochem, Merk Biosciences, Darmstadt, Germany) was administered starting on day 3 or day 10, one dose (1000 IU) per day, five times a week, for one or two weeks.

PCR used for detection of bcr-abl-positive cells in animal tissues.

Sampling. Livers and spleens were taken from a portion of the mice surviving the treatment without tumor development and from some tumor-bearing animals. The tissues were immediately frozen in liquid nitrogen and stored at -70˚C until examination. Bone marrow was also sampled from some animals. It was obtained by irrigating tight bone with 1 ml of sterile PBS. Cells were counted with a haemocytometer. RNA extraction (see below) was done immediately after collection of cells. Similarly treated organs from diseased animals served as positive controls.

RNA extraction. Prior to RNA extraction, the samples (250 mg of thawed liver or spleen tissue, or $4\text{-}10x10^6$ bone marrow cells) were homogenized with a TH 220 hand-held homogenizer with disposable tips (Cole Parmer, IL, USA) in

1 ml RTL (a guanidium thiocyanate containing buffer provided by the producer) and supplemented with ß-merkapto-ethanol provided in the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA was isolated by means of the Qiagen RNeasy mini kit with On-Column DNase digestion using an RNase-free DNase set, in accordance with the manufacturer's protocol (Qiagen). The concentration of RNA was measured with a UV spectrometer (BioMate 3, Thermo Fisher Scientific, Inc., USA) and the quality of RNA was checked on agarose gel. Prior to reverse transcription, any possibly remaining DNA was removed by treating 2 μg amounts of the RNA extracts with 1 U/μg of DNase I (Roche Diagnostics GmbH, Mannheim, Germany) at 37˚C for 30 min in a total volume of 20 μl. The enzyme was subsequently inactivated by incubation at 65˚C for 10 min. RNA samples were stored at -70˚C until their examination.

Reverse transcription. DNase-treated RNA (2 μg) was reverse transcribed using an oligo $(dT)_{18}$ primer, 50 U MMLV reverse transcriptase (Promega, Madison, WI, USA) and 20 U Rnasin (Promega) in a total volume of 20 μl. After an initial denaturation of RNA at 70˚C for 10 min, the master mix was placed on ice and the sample was incubated at 37˚C for 60 min.

PCR. The quality of cDNA was assessed by PCR with primers specific for the house-keeping ß-actin gene (forward 5'CCACTGGGACGACATGGAGAAGAT3'; reverse 5'CAT GGCTGGGGTGTTGAAGGTC3'), which amplify the 166-bp-long fragment. The expression of the *bcr-abl* gene was monitored by means of nested PCR with a set of external primers that amplify the 327-bp-long fragment (forward 5'TTCAGAAGCTTCTCCCTG3'; reverse 5'CTCCACTGGC CACAAAAT3') and a set of internal primers that amplify the 245-bp-long fragment from the b3a2 spliced gene (forward 5'GTGAAACTCCAGACTGTC3'; reverse 5'CAACGAAAA GGTTGGGGT3'). Fifty microlitres of the first reaction mixture contained 5 pM/μl of each primer, 1.5 mM $MgCl_2$, 2 mM dNTPs, 0.5 U of Taq polymerase (Fermentas, Vilnius, Lithuania) and 1X PCR buffer with $(NH_4)_2SO_4$ (Fermentas). For the second PCR, 1 μl of 10-fold diluted PCR product of the first reaction was used. Initial denaturation at 94˚C for 5 min was followed by 35 cycles, each consisting of 1 min at 94˚C, 90 sec at 57˚C, 90 sec at 72˚C and a final extension at 72˚C for 7 min. The reaction mixture and cycling conditions for the second PCR were the same as for the first one. As a positive control, RNA extracted from B210 cells, and as a negative control, RNA extracted from HL60 cells, was used.

Statistical analysis. Tumor development was analyzed in 2x2 contingency tables by the two-tailed Fisher's exact test. For analysis of the growth curves of the tumors, the two-way analysis of variance was used. Calculations were done using the Prism Software Version 3.0 (Graph-Pad Software, San Diego, CA). A difference between groups was considered significant at $p<0.05$.

Results

Effects of Cy, IM and IFNα on the growth of 12B1-induced tumors. Animals inoculated s.c. in the right back with 5x10³ 12B1 cells were treated with Cy (200 mg/kg) alone or in combination with either IM (50 mg/kg/day) or INFα (1000 IU/day). Cy was administered in a single dose on day 3, while the other substances were given once a day for 5 or 10 days starting on day 3 or day 10, after the cell administration. The results of a representative experiment are shown in Fig. 1. It is evident from Fig. 1a that although nearly all mice developed tumors before the end of the observation period, Cy given alone or in combination with either IM or INFα induced a considerable delay in their appearance. This delay was statistically significant ($p<0.02$) in all the combinations tested except that in which INFα was given on days 3-7. Fig. 1b shows that tumors grew at a much slower rate in mice treated with the drug combinations, more so in those treated with Cy in combination with IM ($p<0.001$) than with INFα (non-significant) for the early or late treatment and $p<0.01$ for the early+late treatment. Furthermore, treatment with the Cy plus IM combination was significantly more efficient than treatment with Cy alone ($p<0.01$). Similar results were obtained in repeated experiments.

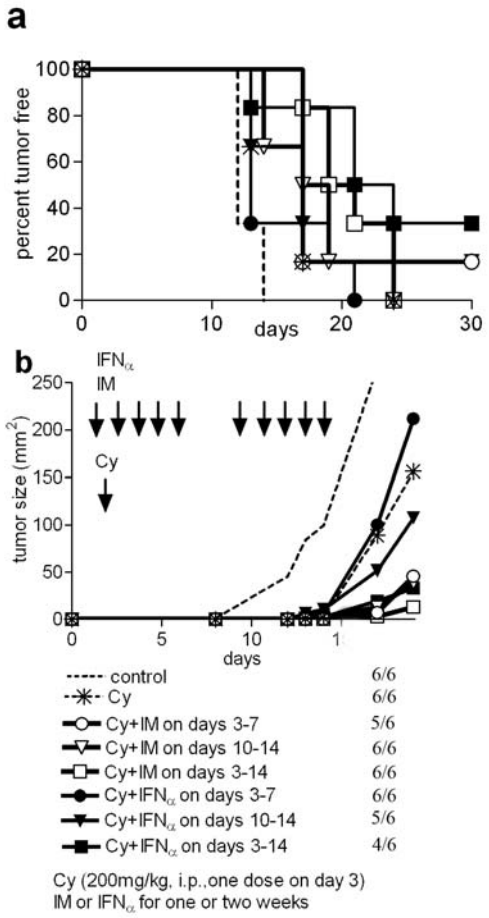

Figure 1. Effect of Cy, IM and IFNα on the growth of tumors induced by 12B1 cells. (a) tumor development, (b) tumor growth. The measurement of tumor size was terminated when it reached or just exceeded 20 mm in its longest diameter. Arrows indicate the days after 12B1 cell inoculation when the drugs were administered.

Effect of cell vaccines producing IL-2, IL-12 or GM-CSF on the growth of 12B1-induced tumors. Animals inoculated s.c. with 5x10³ 12B1 cells on day 0 were repeatedly i.p. injected

Table I. Presence of *bcr-abl*-positive cells in treated and untreated mice inoculated with 12B1 cells.

12B1 cells	Therapy	Tumor present	Day of sampling[a]	Bone marrow	Spleen	Liver
i.v.	-	+	12	2/2[b]	2/2	2/2
	-	+	18	NT[c]	2/2	2/2
s.c.	-	+	15	NT	3/3	3/3
s.c.	Cy+IFNα+B210/IL-2	-	124	0/4	0/4	0/4
s.c.	Cy+IM+B210/IL-2	-	123	0/2	0/2	0/2
s.c.	Cy+B210/IL-12	-	123	0/1	0/1	0/1
s.c.	Cy+IFNα+B210/GM-CSF	-	123	0/1	0/1	0/1

[a]Number of positive animals/number tested in RT-PCR. [b]After 12B1 cell inoculation. [c]Not tested.

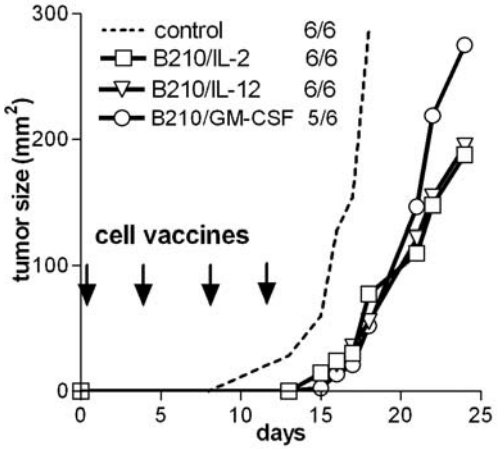

Figure 2. Effect of live B210 cell-based vaccines expressing different cytokines on the growth of tumors induced by 12B1 cells. Arrows indicate the days after 12B1 inoculation when the vaccines were administered.

with B210-cell vaccines producing IL-2, IL-12 or GM-CSF (10^6 cells per dose). The vaccines were given on days 0, 3, 7 and 10. It is evident from Fig. 2 that the therapy did not prevent tumor development, but it significantly postponed the appearance of tumors as compared with control animals ($p<0.01$ in the case of all three vaccines). The tumors grew at a somewhat slower rate in animals injected with either the IL-2 or IL-12-producing cells than in those injected with GM-CSF-producing cells; however, 1 of the 6 animals in the latter group survived without tumor formation.

Effect of cell-vaccine therapy combined with Cy, IM and IFNα administration on the growth of tumors induced by 12B1 cells. Having obtained some basic information as to the effectiveness of the drugs tested and evidence that the cell-based vaccines can produce some, though a limited effect on 12B1 tumor formation, we decided to combine the two approaches. In the ensuing experiment the cell vaccines were administered in the same way as previously. A single dose of Cy was i.p. injected on day 3, either alone or in combination with the repeated administration of IM and/or IFNα. The latter substances were injected on days 3 to 7 and 10 to 14. The amount of each drug per dose was the same as in the preceding experiments. Results are shown in Fig. 3.

It can be seen that the administration of Cy alone resulted in a significant postponement of tumor appearance ($p<0.05$), but did not prevent tumor formation in any of the animals. A similar effect was observed in animals treated with mixtures of Cy and either IM or INFα or both. Animals which received Cy and any one of the three vaccines also responded similarly. However, when the combined Cy+cell-vaccine treatment was supplemented with either IM or INFα in the case of the B210/IL-2 vaccine (Fig. 3a and b), or with INFα in the case of the B210/IL-12 vaccine (Fig. 3c and d), tumors appeared later and grew slower ($p<0.001$) than in the non-vaccine-treated animals. Most importantly, some animals did not develop tumors at all. This was most frequent in those treated with the B210/IL-2 vaccine. Two of the 6 mice which, in addition to the Cy+cell vaccine, also received IM, and 4 of the 6 animals which received INFα in addition to the Cy+vaccine, remained tumor-free until the end of the observation period. The protective effects of the B210/IL-2 and B210/IL-12 vaccines administered in the above combinations were significantly higher than in the groups treated with Cy alone ($p<0.01$, <0.001 and <0.02, respectively). On the other hand, no beneficial effects of the B210/GM-CSF vaccine were apparent (Fig. 3e and f).

The vaccine treated mice surviving tumor-free for four months were tested for the presence of *bcr-abl* transcripts in cells obtained at autopsy from their livers, spleens and bone marrows. The results presented in Table I testify to the absence of leukemic cells in all of the surviving animals. On the other hand, both control animals with 12B1-induced tumors possessed *bcr-abl*-positive cells in the organs tested, this indicating a high propensity for metastizing of these tumors.

Discussion

The recently enhanced interest in the combination of chemo- and immunotherapy of CML is paralleled by an increased interest in suitable animal models, in which specific immune responses to *bcr-abl*-positive cells can be studied much more easily than in human patients. In the study reported herein, we used 12B1 mouse cells, which carry the *bcr-abl* gene and express the $p210^{bcr-abl}$ protein and the drugs most widely used for the treatment of human CML, i.e. IM (now) and INFα (until recently). The treatment regimen with these substances was intentionally suboptimal, because we expected that under these

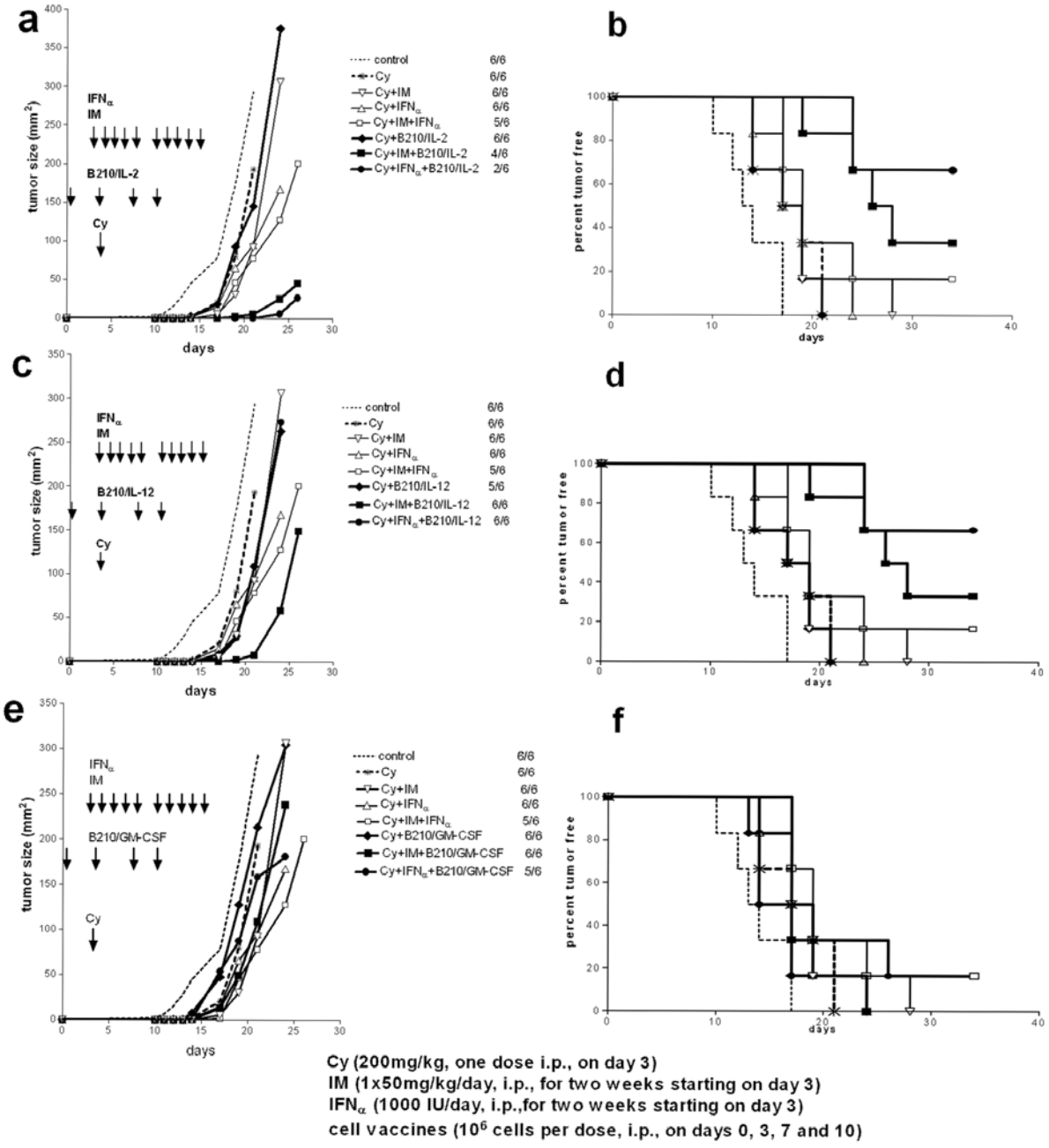

Figure 3. Effect of therapy with Cy, IM and/or IFNα combined with live B210 cell-based vaccine administration on the growth of tumors induced by 12B1 cells. (a and b) Treatment with B210/IL-2 vaccine, (c and d) treatment with B210/IL-12 vaccine and (e and f) treatment with B210/GM-CSF vaccine. Arrows indicate time after 12B1 cell inoculation when the drugs and the vaccines were administered.

conditions any possibly synergistic effects would be more evident. These drugs were supplemented with Cy and with specific vaccines based on heterologous but syngeneic B210 cells, which also express the $p210^{bcr\text{-}abl}$ protein and, in addition, several immunostimulatory factors known from many previous studies to enhance anti-tumor reactions.

In agreement with our previous results obtained with B210 cells (Sobotkova *et al*, unpublished data) and with results obtained in patients with CML (21,34-36), combinations of two or more of the above-mentioned drugs increased their effectiveness in the treatment of tumors induced by 12B1 cells. The mutual interactions of these substances are not quite clear at this writing. While the mechanism of action of IM is understood well, this is not so with the other two agents used. It is believed that the beneficial effects of INFα in the treatment of CML are mediated by both its immunomodulatory and antiproliferative effects (37-39). Similar effects can be expected in the case of Cy. Although this substance has been extensively used as a cytostatic drug in cancer treatment, recent evidence has made it clear that it suppresses regularory T cells (28,30,40,41) and may have some other beneficial immunomodulatory effects (42-44). In those experiments lower doses of Cy (28) than in our present undertaking have been used. Thus it is likely that the antiproliferative effect of the substance played a major role in our system. This conjecture of ours is further supported by our earlier observation that a reduction of the Cy dose markedly decreased its effects (unpublished data).

The vaccines used in our present study were prepared with B210 cells transformed by the bcr-abl fusion gene and expressing the $p210^{bcr\text{-}abl}$ protein. Gene-modified cells expressing IL-2, IL-12 or GM-CSF were employed. Since they were free of oncogenic potential (Petrackova *et al*, unpublished data), they were used for therapeutic vaccination as live cells. In the therapeutic regimen chosen, none of the vaccines was capable of completely suppressing tumor development. However, all three delayed tumor formation significantly. Hoping that the concurrent administration of the chemo- and immunotherapy would result in a synergistic effect, we combined the two therapeutic modes. The combination was indeed more effective than the drug therapy alone. The effects were most conspicuous with the B210/IL-2 vaccine and weakest with the B210/GM-CSF vaccine. Some of the animals treated with the vaccine-drug combinations survived without developing tumors. The livers, spleens and bone marrows of these animals were checked by RT-PCR for the presence of *bcr-abl*-transcripts. The results were negative, which indicated that these animals were free of *bcr-abl*-positive cells and could thus be considered free of residual disease.

Presumably, the different 'therapeutic' activities of the vaccines were a consequence of the biological activities inherent in the cytokines tested and they seem to be in line with the experience of other investigators as well as with our earlier observations in another system (45). Still, it is possible that also the amount of the cytokine produced played a role. Since live cells were used for the vaccination in our experiments, they continued to replicate *in vivo* for some time, with the replication rate, though limited, most likely varying between the cell lines tested and thus producing varying amounts of the immunizing antigens as well as of the cytokines. Experiments are under way in which cell vaccines derived from clones differing markedly in the production of GM-CSF are being tested.

At this writing, it is not understood which tumor-cell antigens are involved in establishing immunity to the highly aggressive 12B1 cells. Although we originally speculated that the new epitope carried by the fusion zone of the $p210^{bcr\text{-}abl}$ protein might mainly be involved, and in fact quite convincing evidence obtained in another laboratory (46-49) strongly indicated that this was the case, the results we obtained with a variety of genetic vaccines based on the fusion zone have not yet confirmed this (unpublished data). On the other hand, we have obtained evidence that other regions of the $p210^{bcr\text{-}abl}$ protein are able to induce protection against 12B1 cells (unpublished data). Furthermore, other antigens present in leukemic cells might also be involved. It has been reported that WT1 and proteinase 3 (Pr-3) are overexpressed in human leukemic cells and that it is possible to induce a beneficial immune reactivity in patients or experimental animals by peptide-based or other types of vaccines (50-53). Our attempts to demonstrate the presence of either WT1 or Pr-3 by using monoclonal antibodies against their mouse analogues have failed (unpublished data). However, it is conceivable that other proteins involved in the $p210^{bcr\text{-}abl}$ activities or in the transformation procedure itself are overexpressed in either cell line and act as immunogens. Experiments aiming to identify at least some of the proteins involved are underway.

To summarize, combination of chemotherapy and immunotherapy with vaccines based on gene-modified cells expressing IL-2 or IL-12 prevented development of tumors in a significant portion of mice inoculated with the highly aggressive $p210^{bcr\text{-}abl}$ -positive 12B1 cells.

Acknowledgements

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3.3 Properties of bcr-abl-transformed mouse 12B1 cells secreting interleukin-2 and granulocyte-macrophage colony-stimulating factor: I. Derivation, genetic stability, oncogenicity and immunogenicity

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IF 2,571

Cíle práce: Získat a otestovat geneticky modifikované buňky 12B1 se stabilní expresí cytokinu IL-2 nebo GM-CSF

Výsledky práce:

1) Model s buňkami 12B1 cTK- a selekčním médiem HAT selhal, takže byl zvolen systém selekce transdukovaných buněk na základě rezistence k blasticidinu.
2) Byly připraveny plazmidy pBSC/IL-2-Bsr a pBSC/GM-Bsr, které obsahují gen pro daný cytokin a gen pro rezistenci k blasticidinu.
3) Buňky 12B1 byly elektroporovány s výše uvedenými plazmidy a transdukované buňky byly selektovány v médiu s blasticidinem. Podařilo se získat několik klonů buněk 12B1 stabilně exprimujících IL-2 a GM-CSF. Klony se vzájemně lišily intenzitou produkce cytokinů. U všech zkoušených klonů byla zjištěna podobná exprese proteinu BCR-ABL.
4) Při testování patogenity vybraných klonů se ukázalo, že klon exprimující IL-2 má oproti mateřským buňkám nižší onkogenní potenciál. Kultury odvozené z nádorů nebo z orgánů leukemických myší rostly v přítomnosti blasticidinu hůře a měly velmi nízkou produkci IL-2, která se zvedla až po několika pasážích v médiu s blasticidinem. Výsledky tak ukázaly malou genetickou stabilitu těchto buněk *in vivo.*
5) Klon 12B1 exprimující velké množství GM-CSF neztratil svůj onkogenní potenciál a u inokulovaných myší došlo k orgánovému poškození. Kultury izolované z nádorů vykazovaly stejně vysokou expresi GM-CSF jako původní inokulované buňky a byly rezistentní k blasticidinu. Tyto buňky byly *in vivo* geneticky stabilní.

6) Pokud se porovnávala patogenita klonů buněk 12B1 exprimujících různé množství GM-CSF, nejméně patogenní byly buňky s nejnižší produkcí.
7) Myši byly imunizovány ozářenými buňkami 12B1 produkujícími GM-CSF nebo IL-2 a čelenžovány buňkami 12B1. Myši, imunizované buňkami s produkcí GM-CSF zůstaly zdravé, zatímco myši imunizované buňkami sekretujícími IL-2 byly chráněny jen zčásti.
8) Pokud byly podle stejného schématu imunizovány myši živými buněčnými vakcínami odvozenými od buněk B210, byl nejlepší výsledek opět dosažen s buňkami exprimujícími GM-CSF. Účinnost vakcinace byla ale ve srovnání s modifikovanými buňkami 12B1 nižší. Buňky B210 exprimující IL-2 nevyvolaly žádnou protekci proti čelenži buňkami 12B1.

Podíl na práci: příprava a konstrukce plazmidů, elektroporace buněk, selekce transdukovaných buněk a jejich klonování, ELISA testy, western blot, práce se zvířaty, vyhodnocení myších pokusů, sepisování práce

Properties of bcr-abl-transformed mouse 12B1 cells secreting interleukin-2 and granulocyte-macrophage colony-stimulating factor: I. Derivation, genetic stability, oncogenicity and immunogenicity

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Abstract. The highly oncogenic bcr-abl-transformed mouse (Balb/c) 12B1 cells were transfected with plasmids carrying genes for either mouse interleukin-2 (IL-2) or the mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and the gene for blasticidine resistance. From the transduced cells several clones widely differing in the production of either cytokine were isolated. For further experiments, clones with the highest secretion of the cytokines were selected. When administered subcutaneously to mice, the IL-2-secreting cell line was approximately hundred times less pathogenic than the parental cells. A portion of animals developed small, spontaneously regressing tumours and most of them became resistant to challenge with the parental cells. Cell populations from either solid tumours or from organs infiltrated by the tumour cells predominantly consisted of cells which did not produce IL-2 and had lost resistance to blasticidine. This indicated that the IL-2 secreting cells were genetically unstable in the course of their propagation *in vivo*. On the other hand, the GM-CSF-secreting cells were more pathogenic than the parental cells, induced extensive organ damage and remained genetically stable in the course of their growth *in vivo*. The pathogenicity of different GM-CSF secreting clones directly depended on the magnitude of production of this cytokine. When used in the form of inactivated vaccines, the GM-CSF-secreting cells were more immunogenic than the IL-2-secreting cells. In comparative experiments, similar results were obtained with GM-CSF- and IL-2-secreting cells derived from B210 cells, another bcr-abl transformed cell line.

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Key words: chronic myeloid leukaemia, bcr-abl, gene-modified cells, pathogenicity, immunogenicity

Introduction

Numerous studies have shown that cellular vaccines engineered to secrete various immunostimulatory cytokines are capable of surpassing the poor immunogenicity of most tumour-associated antigens and can generate a potent, specific and long-lasting anti-cancer immunity. Thus, the use of gene-engineered whole-cell vaccines is gradually developing into a promising approach in immunotherapy. Their augmented immunogenicity is apparently due to local coupling of cytokine production and antigen presentation. The lack of serious untoward reactions, as well as the fact that the host is exposed to the whole spectrum of antigens, this securing the involvement of the immunologically most important ones, provides further support for this concept.

Of the immunostimulatory cytokines, attention has especially been paid to interleukin-2 (IL-2) and the granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-2 secreting cellular vaccines have repeatedly been used with partial success in both preclinical and clinical studies (1-14). GM-CSF secreted by gene-engineered tumour cells has been found to be the most potent stimulatory factor among the ten cytokines tested (15) and it has since become the most frequently used cytokine in the construction of cell-based cancer vaccines. GM-CSF secreting vaccines have been used in numerous preclinical and clinical studies, for reviews see (16-18).

Although most clinical studies have dealt with solid tumours, there seems to be a growing interest in utilizing gene-modified cells for the treatment of chronic myeloid leukaemia (CML) and other haematological malignancies. Both autologous and allogenic cell vaccines are under consideration (17,19). Favourable results have been obtained in postremission acute myeloid leukaemia patients with a vaccine composed of a mixture of autologous cells and K562 cells, i.e. a cell line derived from a CML patient, which were engineered to secrete GM-CSF (20). Encouraging results have been shown in lethally irradiated GM-CSF-secreting autologous cells for the treatment of acute myeloid leukaemia and myelodysplasia patients who had received allogenic stem cell transplantations (21). Quite recently, it was further shown in a preclinical

model that the administration of a GM-CSF-secreting myeloid leukaemia cellular vaccine prior to autologous bone marrow transplantation significantly prolonged the survival of mice (22). Recently, also a therapeutic vaccine based on GM-CSF-secreting K562 cells was successfully used for the treatment of CML patients (23).

In the past few years we tried to obtain some more information on immune reactions to bcr-abl-transformed cells in the murine system. The key element of these studies was the development of a variety of vaccines aimed at inducing protection against challenge with these cells (24,25) and at testing their immunotherapeutic potential (13). In the previous paper of ours (26), we showed that bcr-abl-transformed mouse (Balb/c) B210 cells, which had been gene-modified by transfection of mouse genes for IL-2, IL-12 or GM-CSF immunostimulatory factors, had lost their capability of inducing leukaemia in syngeneic animals. It was of interest to determine how a similar genetic modification would influence the pathogenicity and immunogenicity of another mouse (Balb/c) bcr-abl-transformed cell line, namely 12B1 cells, which are more oncogenic than the B210 cells after intravenous administration and, in addition, form solid lymphoma-like tumours after subcutaneous (s.c.) administration.

In this study we explored the properties of 12B1 cells expressing murine IL-2 and GM-CSF proteins. We also tested their immunogenic potency and compared it with that induced by similarly modified B210 cells.

Materials and methods

Cell lines and media. 12B1 is a murine leukaemia cell line derived by the transformation of Balb/c bone marrow cells with a retrovirus-derived vector carrying the human bcr-abl (b3a2) fusion gene (27). The 12B1 cells were kindly provided by Dr E. Katsanis (University of Arizona, Tuscon, AZ). Their *in vitro* and *in vivo* properties have been described in more detail elsewhere (28,29). They have the phenotype of early pre-B cells, are CD19 positive and induce leukaemia-like disease in mice after intravenous administration. In our hands, 1 TID_{50} corresponds to approximately 10^2 cells. After s.c. inoculation 12B1 cells induce solid tumours, with 1 TID_{50} corresponding to approximately $10^{2.5}$ cell. The cells were cultivated in RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories, Linz, Austria), 4 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37˚C in 5% CO_2 atmosphere. For the selection of cells successfully transduced with plasmids (see below), the medium was supplemented with blasticidine (25 μg/ml) (InvivoGen, San Diego, CA). To obtain cTK-less cells, we cultivated 12B1 cells in RPMI-1640 medium with a gradually increasing concentration of 5-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich). The 12B1 TK-less cells grew well at a concentration of 100 μg BrdU/ml. The derivation and properties of IL-2- and GM-CSF-secreting B210 cells, designated B210/2/IL-2/cl-21 (producing 30 ng of the cytokine per 10^6 cells/24 h) and B210/2/GM-CSF/cl-1 (producing 40 ng of the cytokine per 10^6 cells/24 h) have been described elsewhere (26). These cells were derived from clone no. 2 isolated from the population of B210 TK-less cells, which was the most pathogenic of the TK-less clones tested. B210/2 will be referred to as parental cells. All of the B210 derived cells were passaged in RPMI supplemented with 10% FCS and enriched with hypoxanthin-aminopterin-thymidine (HAT) (HAT Supplement, Gibco, Invitrogen, Carlsbad, CA). The cells were kept in liquid nitrogen. In all experiments the third passage of rethawed cells was used. 293T cells (kindly provided by Dr J. Kleinschmidt, DKFZ, Heidelberg, Germany) were used as a negative control in Western blotting. Their propagation was the same as in previous experiments (8).

Plasmids. Plasmids pBSC/IL-2 (30) and pBSC/GM-CSF were used. Both plasmids were constructed and kindly provided by Dr M. Šmahel (Institute of Hematology and Blood Transfusion, Prague). The latter plasmid was constructed using pBKGM (31). From this plasmid, cDNA of mouse GM-CSF was cut out and ligated into the pBSC plasmid (Dr M. Šmahel, unpublished data). Plasmids pBSC/IL-2 and pBSC/GM-CSF were linearized with *Sac*II restriction enzyme (New England Biolabs, Beverley, MA). The resulting ends were treated with T4-DNA polymerase (Promega, Madison, WI) and *Not*I restriction enzyme (New England Biolabs) creating one blunt and one *Not*I end. To supplement the plasmids with a gene for a selection marker, we isolated a fragment from pBLAST42mAngio (InvivoGen, San Diego, CA) containing the SV40 promoter, the gene for blasticidine resistance (BsrS2) and the poly A end. First we linearized the plasmid with *Bsr*GI restriction enzyme (New England Biolabs). The ends were treated with Klenow fragment (New England Biolabs) and then with *Not*I restriction enzyme (New England Biolabs) to produce one blunt and one *Not*I overlapping end. This fragment was then ligated into linearized pBSC/IL-2 and pBSC/GM-CSF plasmids. The new constructs were denoted pBSC/IL-2-Bsr and pBSC/GM-Bsr, respectively. The plasmid pTR-UF2 (32) carrying gene for green fluorescent protein (GFP) was used for monitoring the transfection efficiency. Plasmids were propagated in *Escherichia coli* DH5-α strain (Gibco) and purified using the Maxi Prep DNA Isolation Qiagen kit (Qiagen, Hilden, Germany). For the experiment with 12B1 TK-less cells, we used bicistronic plasmids pTR-GM-CSF-IRES-TK and pTR-IL2-IRES-TK (8).

Electroporation. Before electroporation, plasmids pBSC/GM-Bsr and pBSC/IL-2-Bsr were linearized with *Kpn*I enzyme (New England Biolabs). Plasmids pTR-UF2, pTR-GM-CSF-IRES-TK and pTR-IL-2-IRES-TK were used in non-linearized form. Electroporation was performed using the Gene Pulser Electroporation system (Bio-Rad, Hercules, CA) as described previously (26). Electroporation was carried out at room temperature exposing the cells to 280 V, 1050 μF. The cells were then transferred into tissue culture dishes containing 4 ml of regular cultivation media. After 48 h cultivation the cells were spun down and resuspended in the respective RPMI-1640 medium. Cells transfected with pBSC/GM-Bsr and pBSC/IL-2-Bsr were cultivated in media supplemented with 25 μg/ml of blasticidine, those transfected with the HSV TK gene-carrying plasmids in media with HAT and those transfected with pTR-UF2 in regular media. The transfection efficiency was determined by flow cytometric analysis of GFP expression in 12B1 cells transfected with plasmid pTR-UF2 24 h after electroporation.

Cell cloning. To isolate cell clones from transduced cells, fresh cultivation medium was mixed with conditioned medium, at a 4:1 ratio. The conditioned medium was obtained from the culture of the particular transfected cells at its logarithmic growth phase. Before mixing it with the fresh medium, the conditioned medium was filtered through a 0.22 mm-Syringe-Filter (TPP, Trasadingen, Switzerland). To isolate cell clones, transfected cells were diluted to the final concentration of 0.3 cell/0.2 ml and distributed in 0.2 ml aliquots into a 96-well plate (TPP). Four h after seeding, all wells were carefully checked for the presence of cells and those containing only one cell were marked. After about 14 day incubation the cell suspensions from these wells were transferred into bigger plates. Again, the cell lines derived were kept frozen in liquid nitrogen until being used and the third passage of rethawed cells was used.

Animal experiments. Six to 8 weeks old female Balb/c mice were obtained from Charles Rivers, Germany. All experiments were carried out in accordance with the Guidelines for Animal Experimentation valid in the Czech Republic. For oncogenicity tests and for challenge, cells were washed 3 times with PBS and the appropriate counts of cells in 0.2 ml of PBS were injected s.c. When applied as vaccines in immunization/challenge experiments, the cells were inactivated using γ-radiation (100 Gy) (IBL 437C irradiator-^{137}Cs-irradiation source, CIS Bio International, Gif-Sur-Yvette Cedex, France), washed 3 times in PBS, and injected in 0.2 ml of PBS intraperitoneally. Gene-modified B210 cells, which had lost their pathogenicity for mice, were used as live vaccines. In both cases two doses of $3x10^6$ cells were administered at a two-week interval, and 2 weeks later the animals were challenged with $5x10^3$ parental 12B1 cells. Mice were monitored 3 times a week for up to 90 days. When tumours reached the size of 400 mm^2, the mice were humanely sacrificed. Mice which did not develop a tumour and manifested symptoms of leukaemia-like disease were sacrificed when impaired vitality, limited mobility, bristled hair and/or hind-leg paresis were observed.

Generation of cell cultures from tumours or organs infiltrated by tumour cells. Tumours or selected organs were excised from the mice under sterile conditions. They were mechanically disrupted in small volumes of complete RPMI medium to produce cell suspensions, filtered through a cell strainer and centrifuged. The cell pellets were resuspended in regular media and cultivated. Third passage cultures were split into media with or without blasticidine. Their growth activity and cytokine production (see below) were monitored.

RT-PCR. To detect bcr-abl-expressing cells RT-PCR was performed as described previously (12), except that the concentration of RNA was determined with Nanodrop (Nanodrop Technologies, Wilmington, DE). Spleens, livers and bone marrows were tested.

Measurement of cytokine production. Concentrations of cytokines in cell culture media were measured by ELISA using the BD OptEIA™ set mouse GM-CSF or mouse IL-2 kits (BD Biosciences, San Diego, CA), following the manufacturer's instructions. Counts of $1x10^6$ cells were seeded in 3 ml medium in 6-cm culture dishes. After 24 h cells were counted and spun down and the supernates were tested for the content of the cytokines. Cell viability was determined using the trypan blue exclusion test. The production level was calculated according to the formula: C/N (where C is the total amount of cytokine in culture medium and N is the final viable cell count) and was expressed in ng/10^6 cells/24 h.

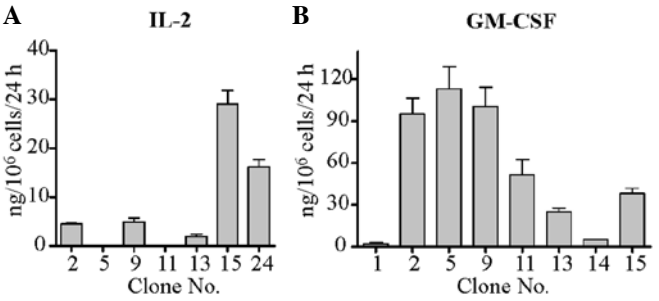

Figure 1. Cytokine production by cell clones derived from 12B1 cells transduced by plasmids carrying genes for (A) mouse IL-2 and (B) mouse GM-CSF. The data are from 3 independent experiments.

Western blotting. Cell lysates were prepared and Western blotting with anti-c-ABL monoclonal antibody (Ab-3, Oncogene Research Products, Boston, MA) was performed as described previously (26). Then the membrane was washed 2 times in PBS with 0.1% Tween and reprobed with anti-β-tubulin monoclonal antibody (Sigma-Aldrich, Steinheim, Germany).

Flow cytometry. Flow cytometry for monitoring GFP-expressing cells was performed on the Beckman Coulter EPICS XL. For analysis of the results WinMDI 2.8 software was used.

Statistical analysis. For analysis of survival the log-rank test and for the analysis of tumour growth two-way ANOVA test were used. Calculations were done using Prism software version 5.0 (Graph-Pad Software, San Diego, CA).

Results

Isolation of genetically modified 12B1 cells. Originally, we tried to isolate gene-modified 12B1 cells similarly to that performed in B210 cells (26). Thus, our plan was to isolate TK-less cells by repeated passages in the presence of increasing concentrations of BrdU, transfect them with plasmids carrying genes for the respective cytokines and HSV TK and select the transduced cells in HAT media. These early experiments confronted us with two unexpected outcomes. First, when compared with the isolation of B210 TK-less cells, which involved many passages requiring more than 6 months, the isolation of 12B1 TK-less cells, i.e. cells growing well in the presence of 100 μg BrdU, was easily achieved in a few weeks. Second, when these cells were transfected with plasmids carrying the HSV TK gene, cells growing in HAT media were selected readily; however, cells growing well in the same media also appeared in cultures of mock-transfected cells. Apparently, revertants to the TK$^+$ phenotype were present in the populations of the putative TK-less 12B1 cells. Both observations, i.e. easy isolation of TK-less cells and their rapid reversion, demonstrated a

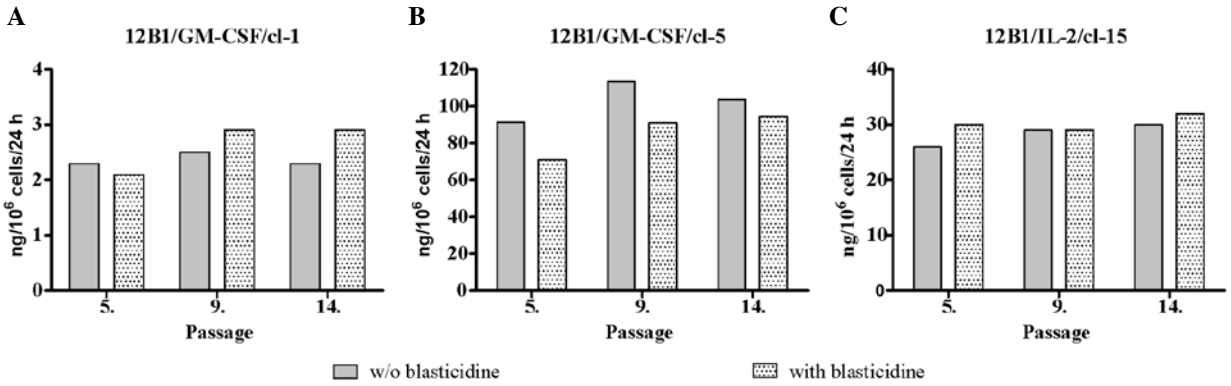

Figure 2. Stability of expression of the respective cytokines in the course of passages *in vitro* either in the presence or absence of blasticidine. Clones (A) 12B1/GM-CSF/cl-1, (B) 12B1/GM-CSF/cl-5 and (C) 12B1/IL-2/cl-15 were tested.

genetic instability of the 12B1 cells and suggested that another approach had to be used for the isolation of the gene-modified cells.

For this purpose we constructed plasmids carrying either the IL-2 or the GM-CSF gene and the blasticidine resistance gene, as described in Materials and methods. Cells were transfected by electroporation; the efficiency was determined by flow cytometry measuring GFP-positive 12B1 cells after transfection with the pTR-UF2 plasmid. In repeated tests, 12 to 18% of live cells were GFP+. A total of 24 clones and a total of 12 clones were isolated from the populations of the IL-2-transfected and GM-CSF-transfected cells, respectively, growing in blasticidine containing selective media. Seven clones of pBSC/IL-2-Bsr-transfected cells and 8 clones of pBSC/GM-Bsr-transfected cells were tested for the production of the respective cytokines. The production was expressed in ng/10^6 cells/24 h. As indicated in Fig. 1, all of the GM-CSF-transfected clones produced the cytokine but only 5 out of 7 IL-2-transfected clones were positive. Furthermore, individual clones markedly differed in the magnitude of production.

For subsequent experiments we selected clone no. 15 for IL-2-secreting cells, designated 12B1/IL-2/cl-15, and clones no. 5 and no. 1 for the GM-CSF-secreting cells, designated 12B1/GM-CSF/cl-5 and 12B1/GM-CSF/cl-1. Before their inoculation into animals, we determined the stability of the cytokine production *in vitro* by passaging these cells in the presence or absence of blasticidine. The results are summarized in Fig. 2. It is evident that the extent of production remained unaltered in both the selective and non-selective media, this proving the *in vitro* genetic stability of the transduced cell lines and also the lack of influence of the product of the blasticidine resistance gene on the growth of the transduced cells in the non-selective medium.

Western blotting. The results of Western blotting are shown in Fig. 3. They indicate that there were no marked differences in the expression of bcr-abl protein among the cell lines tested.

Pathogenicity of transduced cells. Groups of mice were inoculated s.c. with 10^3-10^6 of the gene-modified cells. Mice inoculated with 10^3 and 10^4 of the parental 12B1 cells served as controls. The results are summarized in Fig. 4. All mice inoculated with 10^6 12B1/IL-2/cl-15 cells died before day 45.

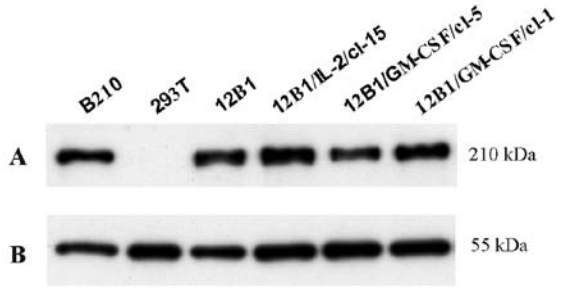

Figure 3. Production of bcr-abl protein by 12B1 cells and their gene-modified cell lines, as determined by Western blot analysis. Protein lysates were separated in 7% gel, transferred onto PVDF membrane and treated with anti-c-ABL monoclonal antibody to detect $p210^{bcr\text{-}abl}$ protein (A). The same membrane was reprobed with anti-β-tubulin monoclonal antibody (B). B210 cells served as a positive and 293T cells as a negative control.

It may be of interest that all animals in this group developed small subcutaneous tumours, which regressed by day 25 and later on all of them died of leukaemia. Their death was markedly delayed when compared with mice, which had been inoculated with parental 12B1 cells in doses 100 and 1000 times lower. Without exception animals inoculated with the parental cells developed rapidly growing subcutaneous tumours. Mice inoculated with 10^5 12B1/IL-2/cl-15 cells also developed small subcutaneous tumours, which regressed by day 21. One mouse developed a big tumour later on and the other two mice survived. All animals inoculated with the 10^4 and 10^3 IL-2-secreting cells remained healthy throughout the observation period, this indicating that the secretion of IL-2 was associated with attenuation of the cells. The surviving animals were challenged on day 79 with 10^5 12B1 cells, i.e. with approximately 300 TID_{50}, given s.c. Four of 7 mice inoculated survived this indicating that the inoculation of a rather small amount of 12B1/IL-2/cl-15 cells was capable of eliciting protection against challenge with a high dose of the parental cells in the majority of animals. After additional 75 days, the survivors were rechallenged with the same dose of 12B1 cells. Three of 4 mice remained healthy and were free of bcr-abl-positive cells in their bone marrow, liver and spleen, as demonstrated by RT-PCR (results not shown). Similar results were obtained in the repeated experiment, in which 9 out of 10 surviving animals remained healthy after challenge given

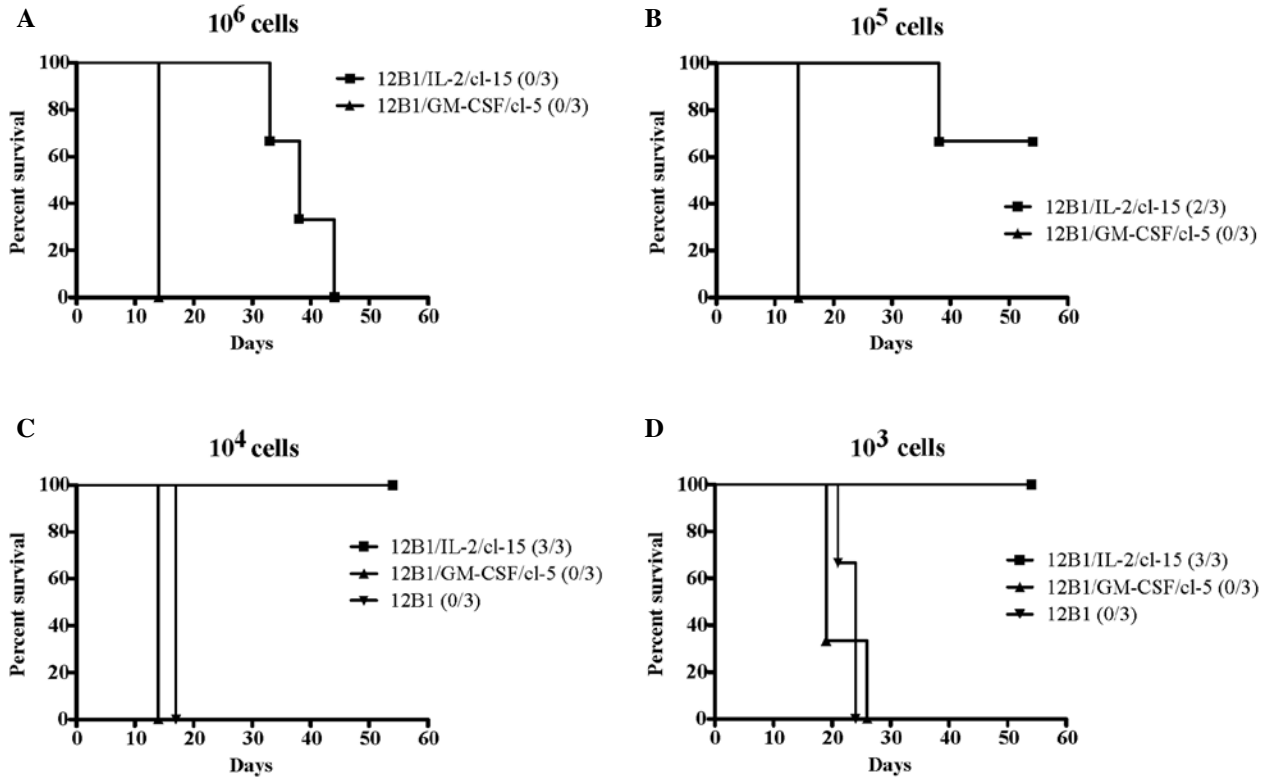

Figure 4. Pathogenicity of 12B1-derived cell lines designated 12B1/IL-2/cl-15 and 12B1/GM-CSF/cl-5 and parental 12B1 cells. Mice were inoculated s.c. with: (A) 10^6, (B) 10^5, (C) 10^4 and (D) 10^3 cells.

on day 75. On the other hand, all animals inoculated with 12B1/GM-CSF/cl-5 cells developed tumours similar to those induced by the control parental 12B1 cells. In addition, these animals had bristled hair and showed a loss of weight, and their autopsy revealed extensive organ damage never seen in mice inoculated with the parental 12B1 cells (33). Similar results were obtained in repeated experiments.

The induction of tumours by the gene-modified cells provided us with a possibility to test the genetic stability of the cells under investigation *in vivo*. Several tumours that

Table I. Cytokine production of the cell suspensions derived from mice inoculated with 12B1/IL-2/cl-15 and with 12B1/GM-CSF/cl-5 cells.

Cells inoculated	Mouse no.	Day of autopsy	Source of cells	Passage	Production of cytokine[a]
12B1/IL-2/cl-15	1	38	Tumour	3	0.6
				2+3 Bl[b]	40
	2	44	Spleen	3	0.7
				2+4 Bl	50
			Liver	2	0.5
				2+4 Bl	20
			Blood	2	2.7
				2+4 Bl	18
	3	49	Tumour	4	0
				2+6 Bl	25
12B1/GM-CSF/cl-5	4	19	Tumour	3	127
				2+4 Bl	115
	5	23	Tumour	3	120
				2+4 Bl	111

[a]Production of cytokine is expressed in ng/10^6 cells/24 h; [b]'Bl' indicates passages carried out in the presence of blasticidine.

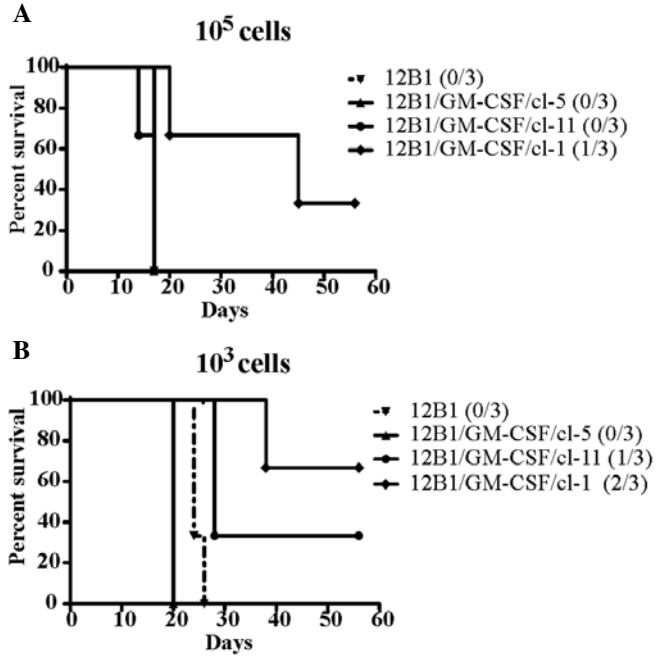

Figure 5. Pathogenicity of 12B1-derived cell lines differing in the production of GM-CSF; 12B1/GM-CSF/cl-5: 110 ng/10^6 cells/24 h; 12B1/GM-CSF/cl-11: 50 ng /10^6 cels/24 h; 12B1/GM-CSF/cl-1: 3 ng/10^6 cells/24 h. Mice were inoculated s.c. with (A) 10^5 and (B) 10^3 cells.

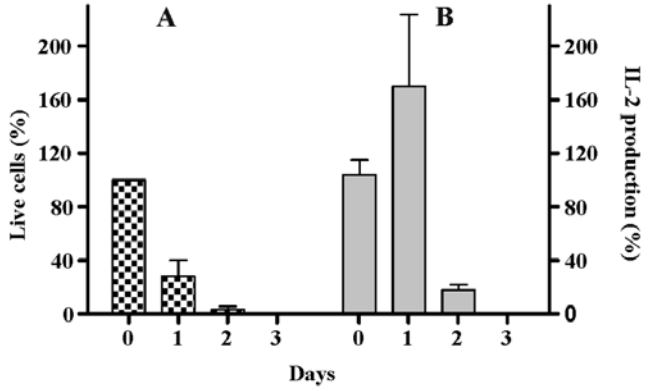

Figure 6. Survival of and cytokine production by irradiated (100 Gy) 12B1/IL-2/cl-15 cells. The total of $3x10^6$ of irradiated cells was placed in cultivation media. At 24 h intervals the cell suspension was spun down, (A) the viability of the cells and (B) the cytokine contents in the supernates were determined and the cells were resuspended in fresh media. The data originate from 3 independent experiments.

had developed in mice after the administration of the gene-modified cells were surgically removed, and cell suspensions were mechanically prepared. In one animal inoculated with 12B1/IL-2/cl-15 cells, which did not develop a solid tumour but leukaemia, cell cultures were prepared from its liver, spleen and blood. Cells were cultivated in parallel in media either containing or not-containing blasticidine. The data shown in Table I demonstrate marked differences between the cells derived from the 12B1/IL-2/cl-15 and the 12B1/GM-CSF/cl-5 inoculated mice. Initially, 12B1/IL-2/cl-15 derived cells grew poorly in the presence of blasticidine and produced low amounts of IL-2 both in the presence and absence of the antibiotic. However, after a few passages in its presence, blasticidine-resistant cells were readily selected and they proved to be efficient producers of the cytokine. These findings demonstrate the instability of 12B1/IL-2/cl-15 cells *in vivo*. Clearly, the cell populations of the tumour cells and the populations of cells infiltrating various organs were not homogeneous, being composed of a great majority of cells in which the two transgenes were not expressed, either owing to their loss or silencing, and a minority of transduced cells. The results obtained with cells derived from 12B1/GM-CSF/cl-5 induced tumours were dramatically different. From the very beginning, the cells grew very well in the presence of blasticidine and already the first passage cells secreted high amounts of GM-CSF, comparable with its production by the cells inoculated.

To examine the relationship between GM-CSF production and cell pathogenicity, we tested, in parallel, three GM-CSF-secreting cell clones markedly differing in the production of the cytokine. In addition to 12B1/GM-CSF/cl-5, producing 110 ng GM-CSF/10^6 cells/24 h, clones denoted 12B1/GM-CSF/cl-11 producing 50 ng/10^6 cells/24 h and 12B1/GM-CSF/cl-1 producing only 3 ng/10^6 cells/24 h of the cytokine, were tested. The results are shown in Fig. 5. While at a dose of 10^5 cells all animals except those inoculated with the lowest cytokine producer, i.e. 12B1/GM-CSF/cl-1 cells, died of leukaemia before their tumour reached the critical size justifying euthanasia, a more pronounced difference became apparent after the administration of 10^3 cells. The most efficient producer of the cytokine, 12B1/GM-CSF/cl-5 cells, was the most pathogenic, causing death of the animals more rapidly than the parental 12B1 cells or clones producing lesser amounts of the cytokine. Clearly 12B1/GM-CSF/cl-1 cells, with the lowest production of GM-CSF, were the least pathogenic. These observations suggested a relationship between GM-CSF production and pathogenicity. It may be of interest that mice, which survived both the high and the low cell dose administered, were free of bcr-abl-expressing cells, as determined by RT-PCR, at the end of the observation period (data not shown).

Efficacy of irradiated gene-modified cells in immunization/challenge experiments. Next, the efficacy of irradiated gene-modified 12B1 cells as tumour vaccines was investigated. Since we realized that the vaccine efficacy would depend on the ability of the irradiated cells to secrete the cytokine, before using them as vaccines we tested the viability of the irradiated cells by the trypan blue exclusion test and their capability of secreting the respective cytokine. As shown in Fig. 6, the irradiated 12B1/IL-2/cl-15 cells were dead within 48 h. After an initial increase, the cytokine production rapidly dropped in parallel with their dying and virtually stopped after 48 h. The same phenomenon was observed in all cell lines tested irrespective of whether they secreted IL-2 or GM-CSF. Irradiated 12B1/GM-CSF/cl-5 cells, 12B1/IL-2/cl-15 cells and the parental 12B1 cells ($3x10^6$/mouse) were injected intraperitoneally twice at a 2-week interval and challenged as described in Materials and methods. As shown in Fig. 7A, all animals vaccinated with irradiated 12B1/GM-CSF/cl-5 cells remained tumour-free throughout the observation period, whereas 2 out of 6 mice vaccinated with irradiated 12B1/IL-2/cl-15 developed tumours before day 24 after challenge. Vaccination with irradiated

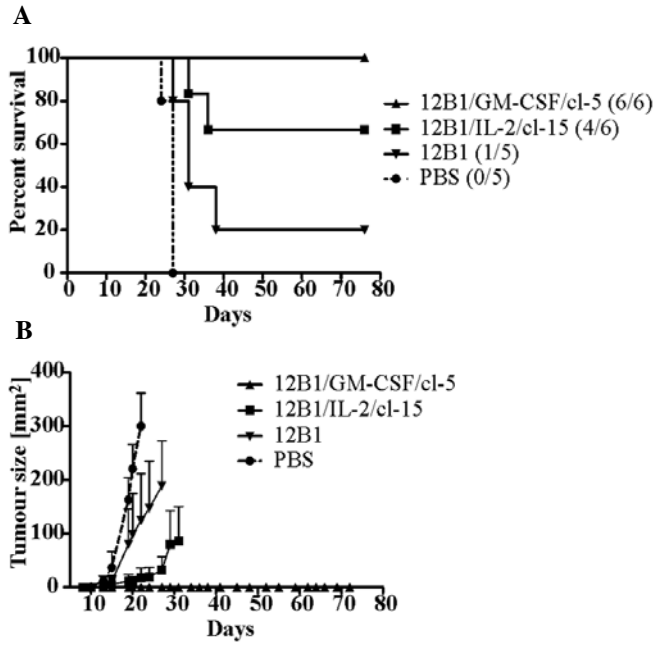

Figure 7. Immunogenicity of irradiated 12B1/GM-CSF/cl-5, 12B1/IL-2/cl-15 and parental 12B1 cells. Mice were immunized i.p. with two doses of $3x10^6$ cells in 2-week interval. Two weeks after the second dose the animals were challenged s.c. with $5x10^3$ parental 12B1 cells and monitored for (A) survival and (B) tumour growth. Graph (B) represents mean tumour size ± SEM. Statistics: Survival: 12B1/GM-CSF/cl-5 vs. 12B1 or PBS $p< 0.01$; 12B1/IL-2/cl-15 vs. PBS $p < 0.01$; 12B1 vs. PBS $p< 0.02$, the other differences are NS. Tumour growth: The growth trends of all the groups are mutually different with high significance ($p<0.001$). The experiment was repeated with similar results.

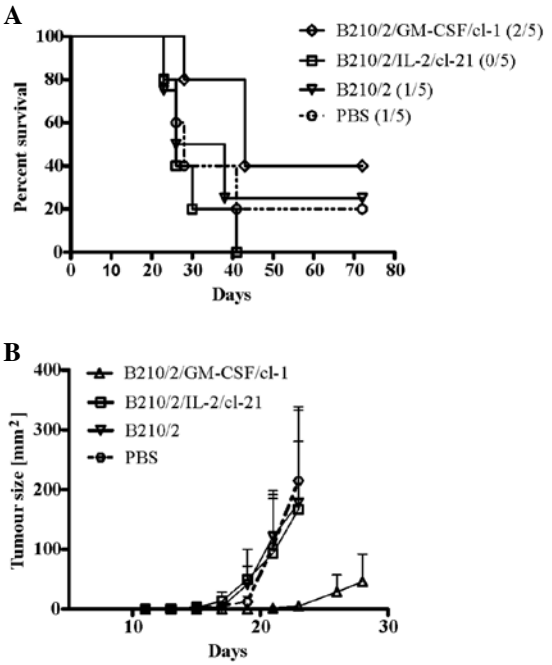

Figure 8. Immunogenicity of live B210/2/GM-CSF/cl-1, B210/2/IL-2/cl-21 and parental B210/2 cells. Mice were immunized in 2-week interval with $3x10^6$ cells i.p.. Two weeks after the second immunization animals were challenged s.c. with $5x10^3$ parental 12B1 cells and monitored for (A) survival and (B) tumour growth. Graph (B) represents mean tumour size ± SEM. Statistics: Survival: B210/2/GM-CSF/cl-1 vs. B210/2/IL-2/cl-21 $p<0.05$; the other differences were NS. Tumour growth: B210/2/GM-CSF/cl-1 vs. B210/2 or B210/2/IL-2/cl-21 $p<0.001$, B210/2/GM-CSF/cl-1 vs. PBS $p<0.01$, the other differences are NS. The experiment was repeated with similar results.

parental cells was even less effective: 4 out of 5 mice developed tumours before day 40. As indicated in Fig. 7B, the tumours in immunized mice grew more slowly than in the non-immunized animals. Surviving animals were rechallenged on day 78 with $5x10^3$ of parental 12B1 cells. All 6 mice vaccinated with 12B1/GM-CSF/cl-5 cells remained healthy for the subsequent 60 days. Bone marrows, livers and spleens from these animals were checked for the presence of bcr-abl-expressing cells by RT-PCR, with negative results (data not shown). Similar effects of vaccination were observed in a repeated test. In this experiment 12B1/GM-CSF/cl-1-based vaccine was also used. This vaccine was less potent in inducing protective immunity than the vaccine prepared from the high-producer cells but more potent than those prepared from the parental cells or 12B1/IL-2/cl-15 cells (data not shown).

Since also B210 cells secreting IL-2 and GM-CSF were available, it was of interest to compare the immunogenicity of these cells with their 12B1-derived counterparts. As these cells had completely lost their pathogenicity for mice (26), they were administered in the form of live vaccines, using the same immunization schedule as in the case of gene-engineered 12B1 cells. As challenge, parental 12B1 cells were used. The results are shown in Fig. 8. In general, the immunization effect of B210-based vaccines was less potent than in the case of vaccines based on the homologous, gene-engineered 12B1 cells. However, it was clear again that the GM-CSF-secreting cells were a more potent immunogen that the IL-2-secreting cells. The latter cells failed to induce any protection. Similar results were obtained in a repeated test.

Discussion

One of the main present trends in the immunotherapy of cancer is based on the assumption that gene-engineered tumour cells secreting immunostimulatory cytokines might be effective in the therapy of cancer. In the recent past, employing the bcr-abl-transformed B210 TK-less cells and HSV TK as the selection gene, we derived several gene-modified cell lines producing IL-2, IL-12 or GM-CSF. Invariably, these cells had lost their pathogenicity for mice (26). Possibly even more important were some additional observations which indicated that (i) the administration of these cells resulted in a postponement of tumour formation in mice which had been inoculated with the highly aggressive sister mouse cell line, 12B1, and (ii) the administration of these cells prevented tumour development in a substantial proportion of 12B1-inoculated animals, if combined with cyclophosphamide plus imatinib mesylate and/or interferon α given in doses which *per se* were incapable of suppressing tumour development (13). In this respect, the IL-2-secreting cells were the most effective. Although both B210 and 12B1 produce comparable amounts of the $p210^{bcr-abl}$ protein, they differ in a number of other characteristics (28,29; Krmenčiková *et al*, unpublished). Therefore, it was of interest to examine the properties of similarly gene-engineered 12B1 cells. In the present study we report on the properties of 12B1 cells transduced to secrete IL-2 and GM-CSF. Clearly, these cell lines differed considerably from similarly transduced B210 cells. First, another system had to be employed for their derivation. Since the 12B1 TK-less cells proved to be

genetically unstable, and thus not suitable for the isolation of the transduced cells, plasmids with the HSV TK gene were replaced with plasmids carrying the blasticidine resistance gene for the selection of transduced cells. Second, the transduced cells had not lost their pathogenicity for mice. Still, the 12B1-IL-2-secreting cells were clearly less oncogenic than the parental cells. In a significant proportion of animals the small subcutaneous tumours which had developed, regressed spontaneously and these animals became resistant to challenge with the parental 12B1 cells. Third, the analysis of the tumour cell populations demonstrated a genetic instability of the 12B1-IL-2 cells *in vivo*, this contrasting with the previously detected genetic stability of the transduced cells in the course of multiple tissue culture passages. In an *in vitro* analysis of the tumour cell populations, the *in vivo* instability manifested itself both by a decreased production of the cytokine and increased sensitivity to blasticidine. However, cultivation of these cells in the presence of blasticidine resulted in a rapid selection of the transduced IL-2 producing cells from the tumour cell population. These observations suggested that the cells in which the transgenes had either been lost or silenced had a selective advantage for *in vivo* growth over those preserving the transduced phenotype. Fourth, the behaviour of the GM-CSF-expressing sublines was very different. These results suggested that, at variance with IL-2-secreting cells, the GM-CSF-secreting cells had a selective advantage *in vivo*. Their capability of forming subcutaneous tumours was not diminished, and, in addition, the disease induced by these cells was clearly more severe than that induced by the parental cells. Data were obtained suggesting that this extra-pathogenicity depended on the amount of the GM-CSF produced. Subsequent experiments indicated that the growth of the 12B1/GM-CSF/cl-5 cells *in vivo* was associated with extensive damage to several organs. These pathological findings are described and discussed separately (33).

Finally, in the present series of experiments, we tested the ability of these cells, used in the form of irradiated vaccines, to induce immunity against challenge with the parental cells. While in some other systems the secretion of the cytokines monitored was not compromised by lethal irradiation (34,35) and was reported to continue for up to 5 (31) or even 12 days (36), we observed a quick shut-off of their production in our system. In spite of the apparently short-time secretion *in vivo*, vaccines based on our gene-engineered cells were clearly more immunogenic than the parental cells. The GM-CSF secreting vaccine proved to be a more potent immunogen than the IL-2 secreting vaccine, which was in agreement with the evidence obtained in other systems in the past (15). Again, there was a relationship between the amount of GM-CSF produced and the immunization effect: the vaccine based on the low-producer 12B1/GM-CSF/cl-1 cells was somewhat less effective than that based on the high producer 12B1/GM-CSF/cl-5 cells, this underscoring the key role of the cytokine in eliciting the anti-tumour immunity. These data clearly indicated that the high-producer cells, absolutely unsuitable for use as live vaccines, are preferable for the development of inactivated vaccines. In this respect it was of interest to test the efficacy of the similarly engineered B210 cells which, because of complete loss of pathogenicity, could be employed as live attenuated vaccines. In the present study we used them for immunization against the heterologous 12B1 cells. Although one could conjecture that the live vaccines would be more immunogenic than the inactivated ones, the opposite was true, this most likely expressing the difference in the antigenic make-up of B210 and 12B1 cells, but also the lower production of the cytokines, though it was in the case of GM-CSF still above the critical level (37). The protection induced by the B210-GM-CSF-secreting cells was incomplete and none was detected when IL-2-secreting cells were used for immunization. This finding was in contrast with the results obtained when using the same cell lines as therapeutic vaccines. In those experiments, in which the same lines of transduced cells were employed, IL-2-secreting cells acted as a more potent immunogen than the GM-CSF secreting tumour cells (13). Those data may indicate that the quite common trend to gene-engineered therapeutic cancer vaccines to secrete GM-CSF does not need to have general validity. They suggest that the preferences for optimal cytokine treatment may differ owing to a variety factors related to the magnitude of the cytokine secretion, specificities of the various tumour systems but also whether live or non-viable cell vaccines are employed.

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3.4 Properties of bcr-abl-transformed mouse 12B1 cells secreting interleukin-2 and granulocyte-macrophage colony-stimulating factor: II. Adverse effects of GM-CSF

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Cíle práce: Objasnit patogenitu buněk 12B1 exprimujících vysokou dávku GM-CSF a popsat vývoj orgánových změn

Výsledky práce:

1) Přestože se u myší, kterým byly inokulovány buňky produkující GM-CSF, a u myší, které dostaly rodičovské buňky 12B1, objevily nádory současně, byla zvířata první skupiny v daleko horším zdravotním stavu.
2) V sérech myší, kterým byly podány buňky produkující GM-CSF, byla již 8. den detekována zvýšená hladina GM-CSF, a to ještě před tím, než se začaly objevovat nádory. Se vzrůstající hladinou GM-CSF koreloval nárůst populace MDSC a dalších nezralých myeloidních buněk ve slezině.
3) U obou skupin myší byla patrná splenomegalie, ale u zvířat inokulovaných buňkami produkujícími GM-CSF byla daleko výraznější. Zvětšení slezin nebylo způsobeno infiltrací nádorovými buňkami, ale nárůstem nezralých myeloidních buněk.
4) Orgánové poškození s četnými krevními výrony bylo pozorováno ve slezině, plicích, játrech a ledvinách. Poškození byla detailně popsána.
5) Neutralizační protilátky proti GM-CSF dokázaly podstatně zmírnit orgánové poškození způsobené buňkami produkujícími GM-CSF, což dokazuje, že poškození bylo způsobené nadprodukcí GM-CSF.

Podíl na práci: příprava buněk, inokulace buněk myším, kultivace buněk z nádoru, ELISA testy, průtoková cytometrie, sepisování práce

Properties of bcr-abl-transformed mouse 12B1 cells secreting interleukin-2 and granulocyte-macrophage colony stimulating factor (GM-CSF): II. Adverse effects of GM-CSF

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Abstract. Granulocyte-macrophage colony stimulating factor (GM-CSF) is considered to be the most effective immunostimulating factor for the construction of gene-engineered anti-cancer vaccines. In some tumour cells, this type of genetic modification has resulted in the loss of the oncogenic potential. This was not the case with bcr-abl-transformed mouse 12B1 cells. A cell line, designated 12B1/GM-CSF/cl-5 producing more than 100 ng/10^6 cells/24 h, displayed higher pathogenicity than the parental, non-transduced cells. Although the tumours induced by the parental 12B1 cells and 12B1/GM-CSF/cl-5 cells appeared nearly at the same time and then grew at an approximately equal rate, the latter mice were in a much poorer clinical condition. In these animals the growth of the tumours was associated with gradually increasing blood levels of GM-CSF. In both groups of animals splenomegaly was observed; it was much more pronounced in the case of 12B1/GM-CSF/cl-5-inoculated animals. While in the case of animals inoculated with the parental cells the splenomegaly was probably mainly due to infiltration with tumour cells, in the animals inoculated with the GM-CSF-secreting cells splenomegaly and derangement of parenchymal organs, such as lungs, liver and kidneys, were more complex, including congestion and infiltration with hemopoietic cells, predominantly immature cells of myeloid lineage. The most conspicuous of these changes was the hyperaemia of the lungs. No such alterations were seen in animals inoculated with the parental cells. On the other hand, the contents of T regulatory cells were comparable in both groups and they increased in parallel at the end of the observation period. When GM-CSF neutralizing antibody was administered to animals inoculated with the 12B1/GM-CSF/cl-5 cells, the pathological changes observed within the organs were suppressed, this proving that the overproduced GM-CSF and not any other substance, played the key role in their induction.

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Key words: bcr-abl, gene-modified cells, granulocyte-macrophage colony stimulating factor, organ damage

Introduction

The granulocyte-macrophage colony stimulating factor (GM-CSF) is a cytokine with pleiotropic effects on the immune system, capable of eliciting well co-ordinated cellular and humoral immune responses. It plays roles both in the priming and effector phases of the immune response. Since the demonstration that among tumour cells genetically engineered to express different cytokines those secreting GM-CSF are the most immunogenic (1,2), tumour cell vaccines secreting this cytokine have run into the focus of interest. During the latest decade, inactivated GM-CSF secreting cancer vaccines have been used in numerous preclinical and clinical studies (reviewed in refs. 2-5).

However, several reports have suggested that GM-CSF is a dual-role player: it may either augment the anti-cancer response or suppress it. This apparently depends on the magnitude of its production. Convincing evidence has been obtained that the levels of GM-CSF exceeding a certain upper limit recruit myeloid-derived suppressor cells (MDSC) that facilitate tumour progression by suppressing both innate and adaptive immune reactions (reviewed in refs. 6-8) and by displaying proangiogenic activity (9). Furthermore, GM-CSF induces the expression of milk fat globule EGF-8 (MFG-E8), which suppresses anti-tumour immune reactions through a variety of mechanisms including the expansion of T regulatory cells (Tregs) (10,11), and which has recently also been shown to promote the oncogenic potential of at least some cancer cells by stimulating their proliferation (12). Also some other studies have demonstrated that high GM-CSF production may have harmful consequences. It has been reported that an overexpression of GM-CSF in the lungs of rats resulted in pulmonary fibrosis (13). Transgenic mice with constitutive overproduction of GM-CSF exhibited damage to several organs, including eye, skeletal muscles and lungs, in which alveolar proteinosis and accumulation of lymphocytes in the peribronchial area were observed (14). In a more recent study with transgenic animals, the GM-CSF gene equipped with the promoter and

flanking sequences of the human CRP gene was used (15). Hepatosplenomegaly with increased extramedullar haematopoiesis and high numbers of activated monocytes in the blood of the animals was observed; however, no changes in the lungs or kidneys were reported. The subsequent administration of LPS resulted in an extreme increase of circulating GM-CSF levels and rapid death of the animals owing to an endotoxic shock. Similar was the outcome of an experiment in which animals were injected intravenously with a high dose of recombinant adenovirus encoding murine GM-CSF (16). Adenoviruses are known to infect hepatocytes after intravenous inoculation. The inoculation of the recombinant adenovirus resulted in a transitory appearance of GM-CSF blood levels, hepatosplenomegaly and a massive accumulation of mononuclear cells in the liver. The administration of LPS on day 7 was followed by severe liver damage characterized by massive haemorrhagic injury and extensive apoptosis of hepatocytes associated with a marked increase of alanine aminotransferase (ALT). The authors concluded that the augmented susceptibility to LPS was a consequence of the accumulation of mononuclear cells in the liver. All animals died within 24 h after its administration. Damage to other organs was not reported.

Recently, we reported on some properties of the mouse bcr-abl-transformed, GM-CSF-secreting 12B1 cells (17). These cells not only retained their oncogenic potential, but also were apparently more pathogenic than the parental cells. Autopsy revealed alterations of a variety of organs. In the present report we describe the pathological changes induced by the 12B1/GM-CSF-secreting cells in individual organs and their development.

Materials and methods

Cell lines and media. 12B1 is a murine leukaemia cell line derived by the transformation of Balb/c bone marrow cells with a retrovirus-derived vector carrying the human bcr-abl (b3a2) fusion gene; the 12B1 cells are of early B cell lineage (18). They were kindly provided to us by E. Katsanis (University of Arizona, Tuscon, AZ). *In vitro* and *in vivo* properties of the 12B1 cells were described in more detail elsewhere (19,20). More recent data indicate that they differ from the other bcr-abl-transformed mouse cell lines in a number of other properties (Krmenčíková *et al*, unpublished). They express CD19 but are considerably bigger than the mature B-lymphocytes and have large segmented nuclei. These properties were utilized for identification of the tumour cells in the splenocyte population (see below). When administered intravenously, they induce acute leukaemia-like disease, with 1 TID_{50} corresponding to approximately 10^2 cells. After subcutaneous inoculation, 12B1 cells induce solid tumours, with 1 TID_{50} corresponding to approximately $10^{2.5}$ cells. Cells were cultivated in RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria), 4 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in 5% CO_2 atmosphere. 12B1/GM-CSF/cl-5 cells producing about 110 ng of GM-CSF/10^6 cells /24 h, were isolated after electroporation with a plasmid carrying the genes for mouse GM-CSF and blasticidine resistance, as described (17). They were grown in medium supplemented with blasticidine (25 μg/ml) (InvivoGen, San Diego, CA). In all experiments the third passage derived from a large frozen stock was used. For animal experiments, the cells were washed three times with PBS and the appropriate counts of cells in 0.2 ml of PBS were injected subcutaneously.

Animal experiments. Six to eight-weeks old female Balb/c mice were obtained from Charles Rivers, Germany. All experiments were carried out in accordance with the Guidelines for Animal Experimentation valid in the Czech Republic. The 12B1 and 12B1/GM-CSF/cl-5 cells were washed three times with PBS and, if not indicated otherwise, counts of $5x10^3$ in 0.2 ml volumes of PBS were injected subcutaneously. The mice were monitored for symptoms of the disease and were sacrificed humanely. Blood samples were collected for determining the GM-CSF levels in the sera. At autopsy, several organs were taken for histopathological and immunohistochemical investigation. Mice were followed for up to 18 days.

To neutralize GM-CSF in inoculated mice, a monoclonal anti-mouse GM-CSF antibody (MP122E9, 0.5 mg/ml, R&D Systems, Minneapolis, MN, USA) was used. Mice inoculated subcutaneously with $5x10^3$ 12B1/GM-CSF/cl-5 cells were divided into two groups, each group containing 3 animals. One group was treated with anti-GM-CSF antibody, while the other one remained untreated. Each animal in the first group received 100 μg of anti-GM-CSF antibody on day 0 (i.e. simultaneously with cell administration) and 200 μg on day 5. A third group of animal received only the antibody and no cells. The antibody was administered intraperitoneally. This experiment was terminated on day 14.

Histology. Organs for histopathological investigation were fixed in 10% buffered formalin (in PBS). Paraffin-embedded samples were sliced using a microtome (Leova), to 3 to 5 μm thick slices. After paraffin wash out, they were stained with hematoxylin and eosin (H&E) (Dako, Denmark). The preparations derived from the kidneys were also stained with trichrome (Mallory Trichrome kit, Bamed, Czech Republic).

Immunohistochemistry. Sections of deparaffinised samples were incubated with rabbit anti-human ABL1 polyclonal antibody (pTyr272) (LifeSpan, Biosciences, Spain) diluted (1:50) for 1 h. This antibody reacts with human but not with murine abl protein. Samples were washed with PBS, incubated with histidine (EXBIO, Czech Republic) for 30 min and subsequently treated with the Universal Immuno-Enzyme Polymer reagent Histofine (Nichrei Biosciences, Tokyo, Japan) following the manufacturer's instructions. DAB+ substrate chromogen (Dako) was used for visualization. Finally, cells were stained with hematoxylin-eosin (Dako).

Generation of cell cultures from tumours and organs infiltrated by tumour cells. Tumours and selected organs were removed from the mice under sterile conditions. They were mechanically disrupted in small volumes of complete RPMI medium to produce cell suspensions, which were filtered through a cell strainer and centrifuged. The cell pellets were resuspended and cultivated in regular media. Third passage of cell cultures was split into media with and without blasticidine. Growth activity and cytokine production were monitored.

Measurement of cytokine production in sera and cell cultures. Sera from the inoculated animals were stored at -20˚C until investigation. Counts of $1x10^6$ cells from cell cultures were seeded in 3 ml medium. After 24 h they were spun down. The supernates were stored at- 20˚C until investigation. The concentration of GM-CSF in sera and media was determined by ELISA kit (BD OptEIA™ Set Mouse GM-CSF, BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Flow cytometry. Single-cell suspensions of splenocytes were prepared by mechanical disruption in a small volume of RPMI and filtration through a cell strainer. Red blood cells were removed by 5-min treatment with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) at room temperature. The cells were spun down and washed with RPMI + 2% FCS. Then $1x10^6$ cells were incubated with 0.125 μg of rat anti-mouse-CD16/32 (BD Pharmingen, San Diego, CA) to block the Fc receptor and washed with FACS buffer (PBS, 2% FCS, 0.09% sodium azid). For cell phenotype characterization, the following antibodies were used: FITC-Gr-1 (eBioscience, San Diego, CA), PE-CD11b (BD Pharmingen), FITC-CD4 (BD Pharmingen), PE-Cy5-CD25, PE-CD19 (US Biologicals, Swampscott, MA) and PE-FoxP3 (eBioscience). The cells were incubated with cell-surface reacting antibodies for 30 min on ice in the dark. Then the cells were washed, fixed and permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) according to the manufacturer's instructions. For the determination of Treg cells, the permeabilized cells, already labelled with CD4 and CD25 antibodies, were incubated 30 min with the PE-FoxP3 antibody. Flow cytometry was performed using Beckman Coulter EPICS XL. For analysis of the results FlowJo 7.6 software was used.

Results

Pathological changes in mice inoculated with 12B1/GM-CSF/cl-5 cells. Mice inoculated subcutaneously with the 12B1/GM-CSF/cl-5 cells and the parental 12B1 cells were followed for the development of tumours and pathological changes in various organs. Two mice from the 12B1/GM-CSF/cl-5-inoculated group and two mice from the 12B1-inoculated group were sacrificed at 2-day and 4-day intervals, respectively. In general, starting with day 6, mice which had received the 12B1/GM-CSF/cl-5 cells were in much poorer state than the 12B1-inoculated mice. They had bristled hair and exhibited weight loss. In these animals, macroscopic pathological changes were apparent at autopsy already on day 8. i.e. 4 days prior to the appearance of tumours. Among these, splenomegaly (Fig. 1A-c) and extensive foci of congestion of lungs (Fig. 1B-c) dominated. Such changes in lungs were not seen in animals inoculated with the parental 12B1 cells (Fig. 1B-b).

Tumour formation. Tumours were palpable from day 10 to 12 after cell inoculation in both groups and tumour growth did not significantly differ between mice inoculated with the parental 12B1 and the 12B1/GM-CSF/cl-5 cells (Fig. 2). Histologically, tumours in both groups were characterized by poorly differentiated cells with frequent mitoses. At the end of the observation period, a difference was encountered. While tumours induced by the 12B1/GM-CSF/cl-5 cells remained vital, extensive foci of necrosis were seen in tumours induced by the parental cells (results not shown).

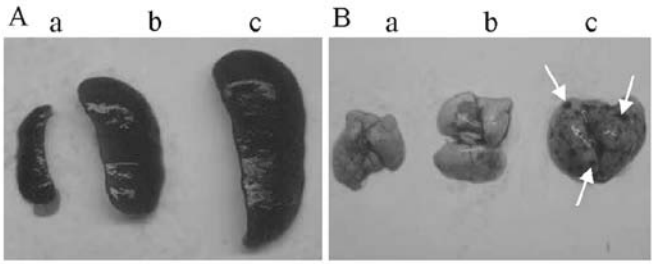

Figure 1. Spleens and lungs of mice inoculated with 12B1 and 12B1/GM-CSF/cl-5 cells: differences in size and appearance. (A) Spleen (a) healthy mouse, (b) mouse inoculated with parental 12B1 cells, (c) mouse inoculated with 12B1/GM-CSF/cl-5 cells. (B) Lung (a) healthy control mouse, (b) mouse inoculated with parental 12B1 cells, (c) mouse inoculated with 12B1/GM-CSF/cl-5 cells. Focuses of congestion are apparent (arrows). Day 18.

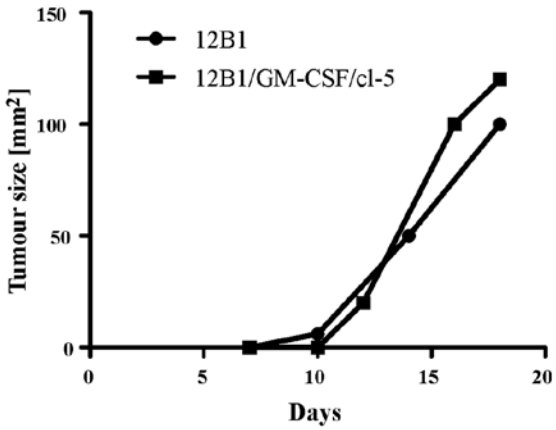

Figure 2. Tumour growth in mice inoculated with 12B1 and 12B1/GM/cl-5 cells. Mice were inoculated subcutaneously with $5x10^3$ cells. Tumour growth was monitored at two-day intervals. All animals eventually developed tumours.

Serum levels of GM-CSF and its production by tumour-derived cell cultures. The production of GM-CSF in sera of the inoculated animals was measured by ELISA. Throughout the observation period, GM-CSF was undetectable in sera of mice inoculated with parental 12B1 cells and in control healthy mice. In mice inoculated with 12B1/GM-CSF/cl-5 cells, the level of GM-CSF in serum started to increase on day 8 and reached its peak, i.e. 280 ng/ml, on day 16 (Fig. 3). Spleens and tumours of mice from this group of animals were taken on days 16 and 18. Their portions were mechanically homogenized and the cells were cultivated *in vitro* in complete RPMI medium either with or without blasticidine. The cells grew very well in both media. After three passages, we collected the media and tested them in ELISA for the contents of GM-CSF. Cells derived from the spleens produced 83±12 ng/10^6 cells/24 h and cells derived from tumours produced 92±16 ng/10^6 cells/24 h, indicating that the ability of the cells to secrete the cytokine remained essentially unchanged in the course of their growth *in vivo*.

Changes in spleens and composition of splenocyte populations. In both groups of inoculated animals marked splenomegaly was

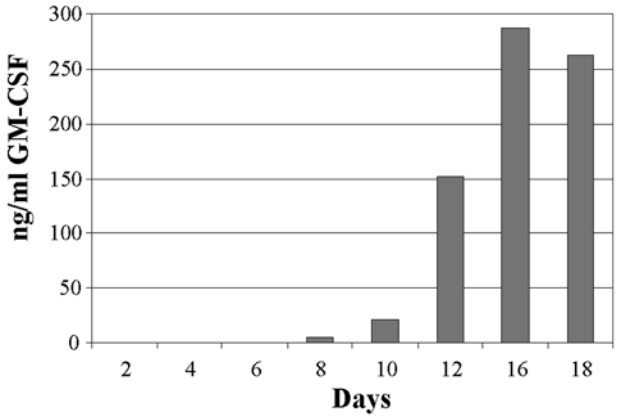

Figure 3. GM-CSF levels in sera of mice inoculated with 12B1/GM-CSF/cl-5 cells as determined by ELISA. Sera from two animals sacrificed on the days indicated were pooled. GM-CSF levels were undetectable in sera from healthy mice or mice inoculated with parental 12B1 cells.

observed from day 8. The presence of GM-CSF in the blood of mice that had received the 12B1/GM-CSF/cl-5 cells correlated with their spleen enlargement. By the end of the observation period, the size of the spleens showed a nearly 20-fold increase over the spleens of healthy animals and it also considerably surpassed the size of spleens of animals inoculated with the parental 12B1 cells. Spleens from the latter animals showed approximately only a 6-fold increase over spleen size of healthy animals.

Results of the analysis of the splenocyte populations are shown in Figs. 4 and 5. As indicated in Fig. 4A and B in mice inoculated with GM-CSF producing cells there was a gradual increase of $CD11b^+$ cells. From day 6, the population of Grl^+CD11b^+ started to expand. Between day 2 and 18 their content increased from 2 to 21% and of Grl^-CD11b^+ cells from 2 to 34% of spleen cells. In the case of spleen cells from mice inoculated with parental 12B1, only a mild increase of cells with these markers was observed on day 16 and 18, respectively. By the end of the observation period the Grl^+CD11b^+ cells represented only 3.9% of spleen cells and the population of Grl^-CD11b^+ cells increased to 5.4%. On the other hand, there was little difference between the two groups of animals in the percentage of $CD4^+CD25^+FoxP3^+$ regulatory cells (Treg) within the $CD4^+$ population. Until day 16 similarly low percentages of Tregs were detected in both groups and their relative count did not differ from Treg counts in healthy animals. Only on day 18, i.e. at the end of the observation period, there was a marked but similar increase in Tregs in both parental 12B1-inoculated and 12B1/GM-CSF/cl-5-inoculated animals (Fig. 4C). To get basic information on the rate of infiltration of the spleens by the tumour cells, at the end of the observation period the splenocytes were also tested for their presence. The results are shown in Fig. 5. They suggest that the spleens of the two groups of animals were infiltrated with tumour cells to a different degree. On day 18 tumour cells represented 40% of the cells isolated from the spleens of mice inoculated with the parental cells but only 5% of cells isolated from the spleens of mice inoculated with 12B1/GM-CSF/cl-5 cells. To further confirm these data the spleens were investigated immunohistochemically making

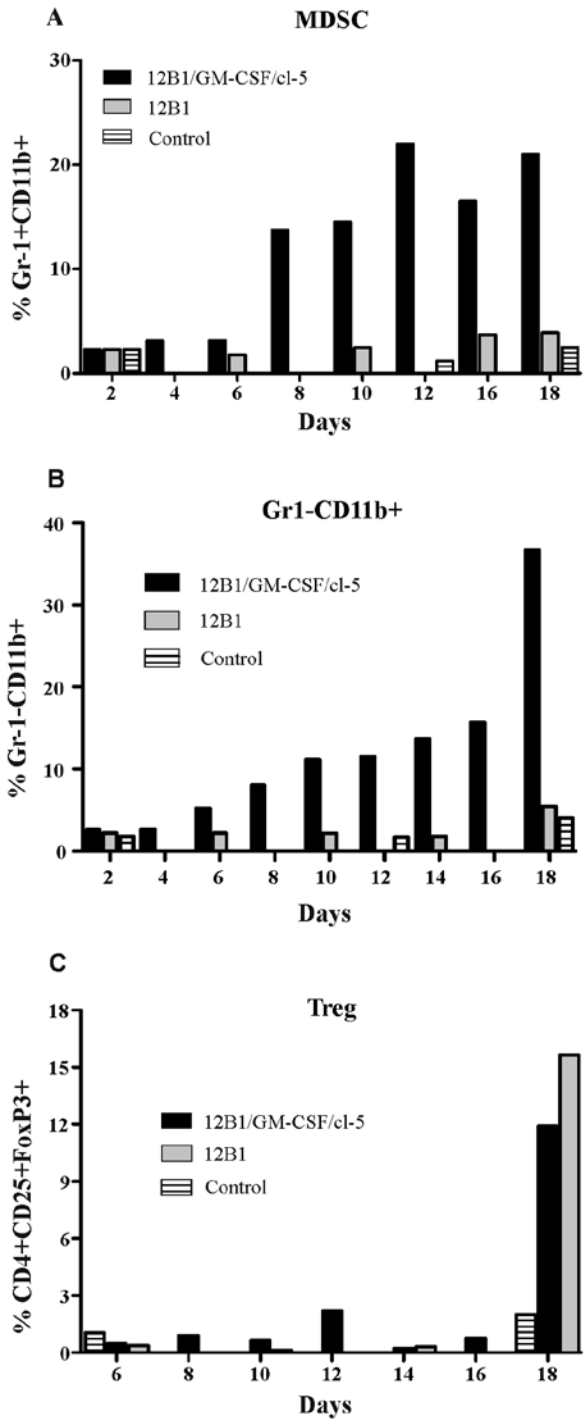

Figure 4. Presence of $CD11b^+$ cells and Treg cells in spleens of mice inoculated with 12B1 and 12B1/GM-CSF/cl-5 cells as determined by FACS. (A) Grl^+CD11b^+ cells, (B) Grl^-CD11b^+ cells, (C) $CD4^+CD25^+FoxP3^+$ cells within $CD4^+$Tcell subset. Day 18.

use of an antibody reactive with human but not murine abl protein. The results are shown in Fig. 6. No reactive cells were detected in spleens of healthy animals. They were much more frequent in mice inoculated with parental cells than in those which received GM-CSF-producing cells.

Histological investigation of the spleens disclosed normal white and red pulp without marked congestion in animals

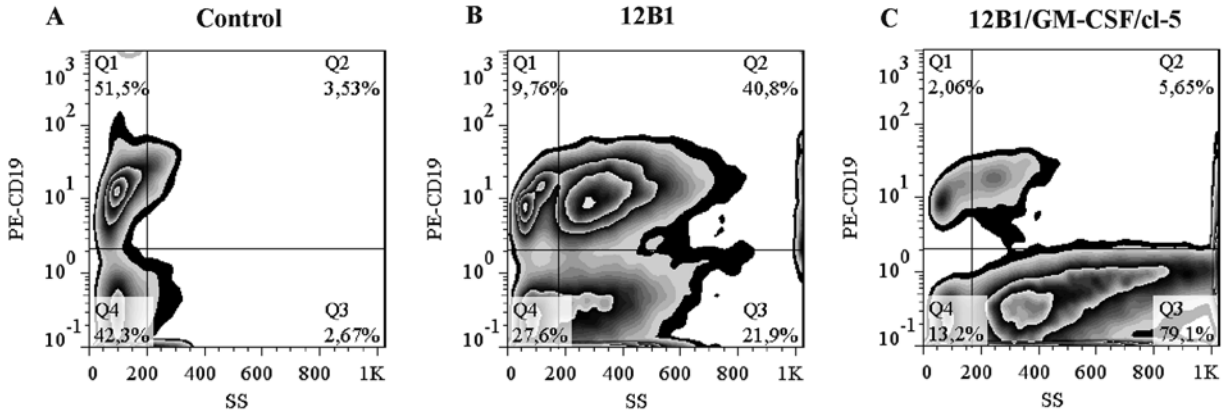

Figure 5. Presence of tumour cells in spleens from mice inoculated with 12B1 and 12B1/GM-CSF/cl-5 cells as determined by FACS at the end of the observation period. The results are presented as density plots. B-lymphocytes are gated in quadrant 1 (Q1) and tumour cells in quadrant 2 (Q2) on the basis of side scatter and CD19 positivity. (A) Healthy control mouse, (B) mouse inoculated with parental 12B1 cells, (C) mouse inoculated with 12B1/GM-CSF/cl-5 cells. Day 18.

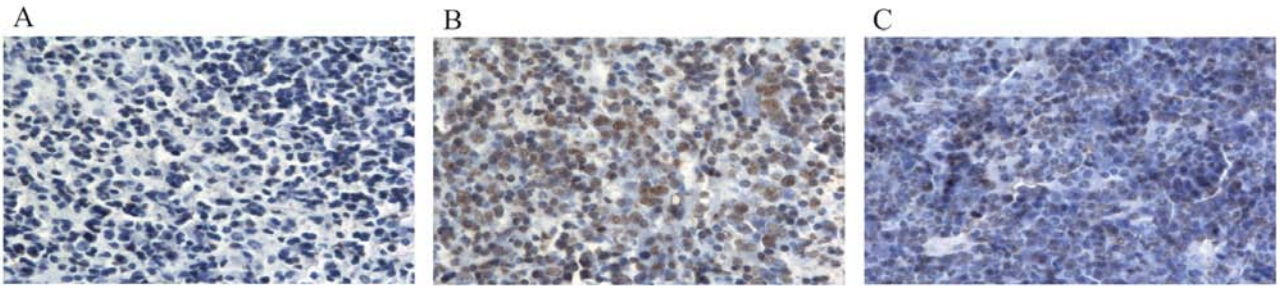

Figure 6. Immunohistochemical investigation of spleens of mice inoculated with 12B1 and 12B1/GM-CSF/cl-5 cells. For detection of tumour cells carrying human bcr-abl gene, rabbit antibody reactive with human but not with murine abl protein was employed. Histofine Universal Immuno-enzyme Polymer Reagent and DAB substrate chromogen were used for visualization. (A) Healthy control mouse, (B) mouse inoculated with 12B1 cells, (C) reduced number of positive tumour cells in mouse inoculated with 12B1/GM-CSF/cl-5 cells. Day 18.

inoculated with the parental 12B1 (Fig. 7A-a). On the contrary, the spleens of 12B1/GM-CSF/cl-5-inoculated animals exhibited marked changes in their structure. The red pulp was congested, expanded and largely replaced the white pulp, and extramedullary haematopoiesis became much more apparent. Multiple megakaryocytes were detected (Fig. 7A-b and c).

Changes in lungs. From day 10, marked alterations were observed in the lungs of mice inoculated with 12B1/GM-CSF/cl-5 cells. As already mentioned, macroscopical inspection revealed focuses of congestion already at the autopsy. Histological examination of lungs of mice inoculated with the parental 12B1 cells did not reveal any structural changes of the lung parenchyma (Fig. 7B-a). On the other hand, the lungs in 12B1/GM-CSF/cl-5-inoculated mice displayed morphological alterations. Interalveolar septa were extended due to congestion and infiltration with immature hematopoietic cells was apparent. Focal collapse and pneumorrhagia were also present (Fig. 7B-b and c).

Changes in livers. In mice inoculated with 12B1/GM-CSF/cl-5 cells, hepatomegaly accompanied with irregular hemorrhagic foci and other changes was observed. On the other hand, the structure of liver in animals inoculated with the parental 12B1 cells remained unaffected (Fig. 7C-a). Fig. 7C-b and c shows the liver of an animal inoculated with 12B1/GM-CSF/cl-5 cells. In the latter, prominent congestion and focal haemorrhage were found. Apart from focal infiltrates of tumour cells, massive infiltration of liver parenchyma with hematopoietic cells resembling a myeloproliferative syndrome was also present. Reactive changes of hepatocytes manifested themselves by a variability of the size of the nuclei and binuclear hepatocytes were observed.

Changes in kidneys. Kidneys of animals inoculated with the parental cells were free of any marked pathological changes (Fig. 7D-a). At the autopsy, the first changes of kidneys in animals inoculated with the 12B1/GM-CSF/cl-5 cells were observed on day 12. They were characterized by congestion and focal haemorrhages and progressed until the end of the observation period. Histological examination discovered congestion, higher cellularity of glomeruli and focal infiltrates with haematopoietic cells, predominantly of immature myeloid lineage (Fig. 7D-b). Proximal tubuli displayed massive deposition of hyaline droplets as revealed by trichrome staining (Fig. 7D-c).

Neutralization of GM-CSF by specific anti-GM-CSF antibody. To prove that the pathological changes had been induced by overexpressed GM-CSF, the GM-CSF-neutralization test was performed as described in the Materials and methods. Lungs

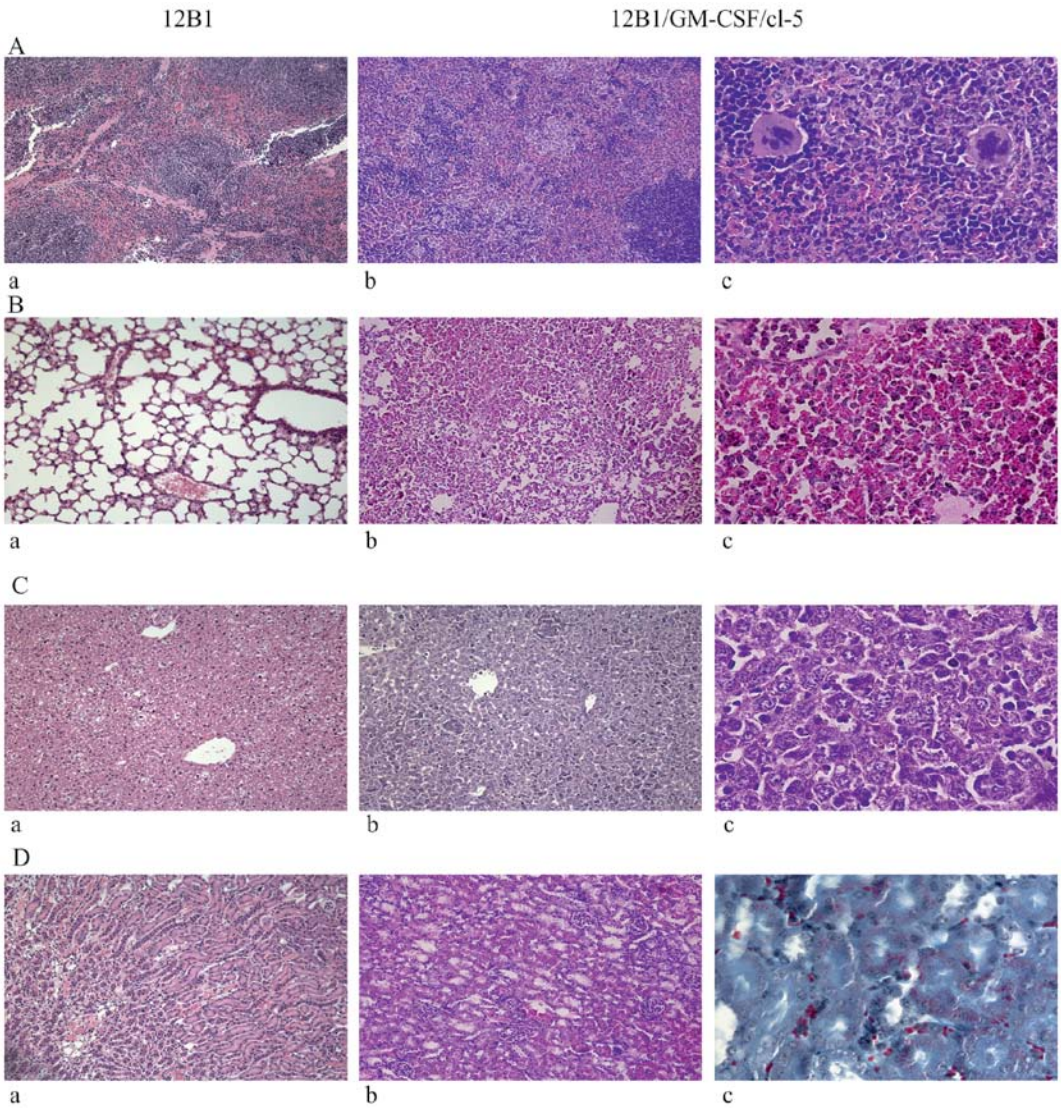

Figure 7. Histological investigation of various organs. (A) (a) Spleen of a mouse inoculated with parental 12B1 cells (H&E, 100x). (b) Spleen of a mouse inoculated with 12B1/GM-CSF/cl-5 cells (H&E, 100x). Congestion, extensive foci of extramedullary hematopoiesis, reduction of white pulp. (c) Spleen of a mouse inoculated with 12B1/GM-CSF/cl-5 cells (H&E, 400x). Detail of extramedullary hematopoiesis with megakaryocytes and immature myeloid cells. Day 16. (B) (a) Lung of a mouse inoculated with parental 12B1 cells (H&E,100x). (b) Lung of a mouse inoculated with 12B1/GM-CSF/cl-5 cells (H&E, 100x). Focal collapse of parenchyma with pneumorrhagia. (c) Lung of a mouse inoculated with 12B1/GM-CSF/cl-5 cells (H&E, 400x). Immature myeloid cells within the lung parenchyma, focal pneumorrhagia. Day 16. (C) (a) Liver of a mouse inoculated with parental 12B1 cells (H&E, 100x). (b) Liver of a mouse inoculated with 12B1/GM-CSF/cl-5 cells (H&E, 100x). Small focal infiltrates of tumour cells and extramedullary hematopoiesis. (c) Liver of a mouse inoculated with 12B1/GM-CSF/cl-5 cells (H&E, 400x). Extramedullary hematopoiesis within liver parenchyma, reactive changes of hepatocytes and immature myeloid cells in sinusoids. Day 16. (D) (a) Kidney of a mouse inoculated with parental 12B1 cells. (b) Kidney of a mouse inoculated with 12B1/GM-CSF/cl-5 cells (H&E, 100x). Congestion, higher cellularity of glomeruli. (c) Kidney of a mouse inoculated with 12B1/GM-CSF/cl-5 cells (Mallory trichrome, 400x). Numerous hyaline droplets within epithelia of proximal tubules, focal interstitial infiltrates of immature myeloid cells. Day 12.

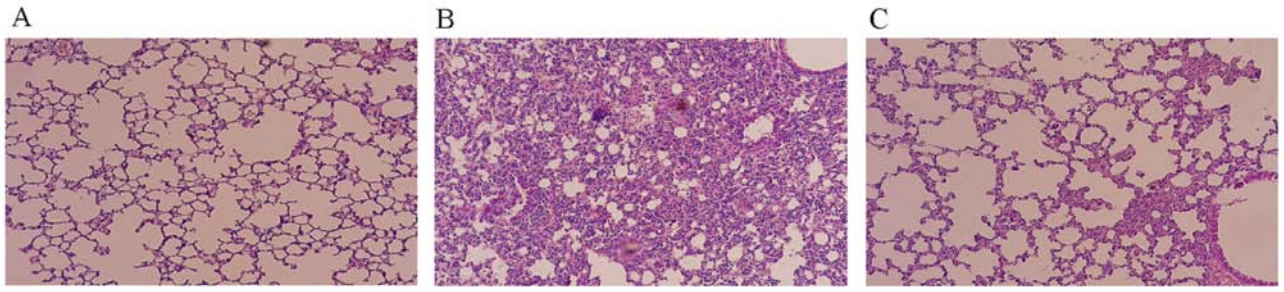

Figure 8. Lungs of mice inoculated with 12B1/GM-CSF/cl-5 cells: neutralization effect of antibody against mouse GM-CSF. (A) Lung of a mouse which did not receive the cells but was inoculated with GM-CSF-neutralizing antibody. (B) Lung of a mouse inoculated with 12B1/GM-CSF/cl-5 cells. (C) Lung of a mouse inoculated with 12B1/GM-CSF/cl-5 cells and treated with GM-CSF-neutralizing antibody. Day 16.

of mice treated and non-treated with the GM-CSF antibody are shown in Fig. 8. Both of these animals possessed tumours of approximately the same size (8 mm in their longest diameter) at the time of autopsy. It is evident that the haemorrhagic changes characteristic for the 12B1/GM-CSF/cl-5-inoculated animals were essentially suppressed by the administration of the antibody. The extent of organ injury was also markedly reduced in spleens, livers and kidneys (data not shown). The findings were similar in all three animals which had been antibody-treated and in all three animals which had not been treated with the antibody. No pathological changes were detected in animals which had received only the antibody.

Discussion

We recently observed that, contrary to our expectation, the mouse bcr-abl-transformed 12B1 cells gene-engineered to secrete high amounts of GM-CSF did not lose their oncogenic potential, but in fact were more pathogenic than the parental cells (17). The pathogenicity of the GM-CSF-secreting cells was dependent on the amount of the cytokine produced, this suggesting that the cytokine itself might be responsible for the additional detrimental effects.

Although harmful effects of high doses of GM-CSF have repeatedly been reported, to the best of our knowledge no study describing them in various organs in some detail has been published until now. Since GM-CSF is the most frequently used cytokine for the preparation of vaccines based on inactivated gene-engineered tumour cells, we considered it useful to investigate its putative adverse effects more closely. For this purpose we employed the high producer cell clone designated 12B1/GM-CSF/cl-5 and compared its pathogenicity with the parental 12B1 cells. The ability of these two cell lines to induce subcutaneous tumours did not differ substantially, but there were marked differences in the clinical picture. However, other differences were also evident. The sera of animals inoculated with the GM-CSF-secreting cells, but not of those inoculated with the parental cells, contained detectable amounts of GM-CSF and its levels increased in parallel with the growth of the tumours and gradual spleen enlargement. The cell cultures derived from the tumours or organs infiltrated with tumour cells produced approximately the same amount of cytokine as did the cells which had been inoculated. Since this suggested that the production of the cytokine was unaltered during the growth of the cells *in vivo*, one has to assume that a tumour sized 1 cm^3 produced up to 100 μg of the cytokine per day. In animals inoculated with the gene-modified cells, but not in those inoculated with the parental cells, marked adverse effects on a variety of organs were detected. Possibly the most striking was the damage to lungs which even macroscopically exhibited strong congestion and focal bleeding. Histological investigation revealed complex changes characterized by congestion and thickening of interalveolar septa, focal collapse and pneumorrhagia. Thus, our findings differ from those reported by Metcalf *et al* (14). The difference may be associated with the lower levels of GM-CSF achieved in these authors' transgenic animals and also with the fact that macrophages and not rapidly replicating tumour cells were the major producer of the cytokine in their system. We also found prominent alterations of the spleens. Splenomegaly was detected in animals inoculated with both the parental and the GM-CSF-secreting cells; however, it was much more pronounced in the latter animals. In animals inoculated with the gene-modified but not in those inoculated with the parental cells, a gradual replacement of white pulp by the red pulp and a very extensive congestion associated with a substantially increased extramedullary haematopoiesis were seen. Originally, we had thought that the splenomegaly was due to the infiltration with tumour cells. However, this did not appear to be the case. As indicated by both FACS analysis and immunohistochemistry, tumour cells represented only a small fraction of splenocytes in animals inoculated with the GM-CSF secreting cells. The splenomegaly in animals inoculated with the parental cells was possibly largely due to the infiltration of this organ by tumour cells. An additional observation dealt with the different presence of immature myeloid cells among the splenocytes of the two groups of animals. It is known that GM-CSF can elicit a heterogeneous population of immature myeloid cells, designated MDSC, characterized by the $Gr1^+CD11b^+$ phenotype, the products of which impair immune responses. MDSC were much more frequent in animals inoculated with the GM-CSF-secreting cells which is in agreement with the earlier observations (10,21). Also the $Gr1^-CD11b^+$ cells, representing a mixture of other immature myeloid cells were much more frequent in animals inoculated with the 12B1/GM-CSF/cl-5 cells than in animals inoculated with the parental cells. On the other hand, no marked difference was observed in the relative contents of Treg cells. This seems to be at variance with some earlier observations which have indicated that GM-CSF can support the expansion of Treg cells (22,23), though our present observation may be obscured to a certain degree by the different sizes of the spleens. Other organs displaying pathological changes in the 12B1/GM-CSF/cl-5-inoculated animals were the livers and the kidneys. In both of them congestion and focal haemorrhages were observed. Extramedullary haematopoiesis was detected in the liver. In the kidneys massive deposits of hyaline droplets within the proximal tubuli epithelia were seen. As far as we are aware, damage to kidneys in animals overexpressing GM-CSF has not previously been reported.

Thus, the direct adverse effect of the megadoses of GM-CSF, which were active in our experiments, apparently overshadowed the immunosuppressive activity of this cytokine, although one of the mechanisms responsible for the immunosuppression, i.e. the recruitment of MDSC, was also operative and most likely contributed to the pathological changes described. MDSC could also contribute to the effective vascularization of the tumours induced by the GM-CSF-secreting cells. At variance with the advanced tumours induced by the parental cells they were without necrotic foci.

Although the present results strongly suggested that overproduced GM-CSF was closely related to the pathological changes described in this report, there was a theoretical possibility that some other factor produced by the gene-engineered cells was involved. Therefore, we checked whether the development of the pathological changes could be suppressed by the administration of GM-CSF-neutralizing antibody to mice inoculated by the 12B1/GM-CSF/cl-5 cells. As the chief indicator of the antibody effect the extensive bleeding into lungs seen in these animals was chosen. The administration of the antibody nearly completely suppressed it and protective effects could also be

seen in other organs monitored. This proved beyond reasonable doubt that GM-CSF was responsible for at least a great majority of the organ damage observed. *Sublata causa, tollitur effectus*.

Definitely, the present findings should not discourage those involved in the development of vaccines secreting GM-CSF, because the use of live vaccines for the immunotherapy of cancer patients is not under serious consideration. Still, the present data may invite some caution when planning the immunization schemes and when using recombinant GM-CSF along with other vaccines.

To summarize, in our experiments we observed substantial alterations of lungs, spleens, kidneys and livers caused by a high amount of GM-CSF secreted by the tumour cells. We demonstrated that the pathological changes observed were induced by this cytokine and were not due to some unintentional changes in the biology of the cells that might have been caused by their transduction or subsequent selection and cultivation.

Acknowledgements

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4. DISKUZE

Tato dizertační práce je zaměřena na přípravu modifikovaných buněčných vakcín proti myším buňkám transformovaným genem bcr-abl, který hraje klíčovou roli v patogenezi lidské CML. Imunogenní potenciál vakcín jsem se snažila zvýšit vnesením genů pro cytokin IL-2, GM-CSF, eventuelně IL-12. Toto očekávání se potvrdilo v provedených experimentech. Součástí předložené práce je popis toxického efektu GM-CSF, který jsem pozorovala po podání živých buněk 12B1 produkujících tento cytokin.

4.1 Buňky B210 a 12B1 jako leukemický model

Pokud chceme studovat jakékoliv onemocnění a hledat nové léčebné přístupy, je nezbytné začít s pokusy na experimentálních zvířecích modelech. Většinou vyhovují k tomuto účelu laboratorní myši imbredních linií, které mají homozygotní genotyp. Jelikož v přírodě neexistuje u myší onemocnění CML, je myší model CML založen na myších buňkách, do kterých je arteficiálně vložen lidský fúzní gen bcr-abl. Buňky tím získají onkogenní potenciál a u myší vyvolají nemoc podobnou lidské leukémii.

Jak buňky B210 tak buňky 12B1, které jsem použila ve svých pokusech, byly transformovány pomocí retrovirového vektoru nesoucího lidský gen pro BCR-ABL. Retrovirový vektor byl v případě buněk 12B1 vnášen přímo do buněk z kostní dřeně myší BALB/c, zatímco buňky B210 vznikly vnesením retrovirového vektoru do buněčné linie BA/F3. Tato linie byla odvozena z buněk kostní dřeně myší BALB/c a je závislá na přítomnosti IL-3 v růstovém médiu (Palacios & Steinmetz, 1985). Transdukcí ztratily buňky tuto cytokinovou závislost. Obě buněčné linie vyvolávají u myší po intravenózním podání nemoc podobnou akutní leukémii. Klinicky se projevuje hepatosplenomegálií, zduřením uzlin, nahrbením, zježením srsti a u některých zvířat dojde k paréze zadních končetin. Při intravenózním podání jsou buňky 12B1 přibližně 100x onkogennější než buňky B210. Pokud jsou buňky B210 podány subkutánně, nemoc nevyvolají, zatímco po stejné aplikaci buněk 12B1 se vytvoří v místě vpichu nádor charakteru lymfomu.

Obě buněčné linie mají lymfoidní charakter, B210 jsou charakterizovány jako pro-B a 12B1 jako pre-B lymfocyty. Buňky B210 jsou *in vitro* MHC I negativní, zatímco 12B1 buňky jsou MHC I pozitivní.

Z obou buněčných linií jsme se rozhodli připravit buněčné linie, které by exprimovaly vybrané cytokiny, a sledovat jejich onkogenní a imunogenní potenciál. Začali jsme s modifikací buněk B210.

4.2 Transfekce buněk B210 a 12B1

Nejprve bylo třeba zvolit vhodnou transfekční metodu. Obvyklé transfekční postupy, jako je kalciumfosfátová precipitace nebo lipofekce, které se na našem oddělení úspěšně používaly pro genové modifikace epiteliálních či fibroblastových buněk, byly neúčinné. O lymfoidních buňkách je známo, že jsou obtížně transfekovatelné, a že z nevirových metod je relativně účinná elektroporace. Abych zjistila podmínky, při kterých je elektroporace v našem systému účinná, transfekovala jsem buňky B210 plazmidem pTR-UF2, který nese gen pro zelený fluoreskující protein (GFP). Transfekované buňky se pak dají rozpoznat pod mikroskopem, protože v procházejícím ultrafialovém světle svítí zeleně. Pomocí průtokové cytometrie se dá přesně změřit, jaké procento buněk bylo úspěšně transfekováno. GFP-pozitivní buňky B210 a 12B1 jsme měřili po 24 hodinách, protože po 48 hodinách bylo pomocí propidiumiodidu detekováno velké množství GFP+ mrtvých buněk. Vysvětlujeme si to tím, že silná exprese GFP je pro tento typ buněk toxická.

Nejúčinnější transfekce byla prováděna při pokojové teplotě, napětí 280V a kapacitanci 1050 μF, v bezsérovém RPMI médiu. Za těchto podmínek bylo po 24 hodinách GFP-pozitivních okolo 16-18% buněk. Způsob transfekce buněk B210 a výsledné podmínky elektroporace, ke kterým jsem došla sadou optimalizačních pokusů, se podobají podmínkám, které zvolila skupina G. Q. Daleyho k transfekci BA/F3 buněk, od kterých byly odvozeny buňky B210, a to elektroporaci při 250 V a 960 μF (Klucher *et al*, 1998). Pro transfekci buněk 12B1 jsem také zkusila několik různých podmínek elektroporace, při nichž jsem vycházela z předchozích experimentů. Podařilo se dosáhnout podobné účinnosti transfekce jako u buněk B210 při nastavení 250 V a 1050 μF a při užití RPMI bez FCS jako elektroporačního média. Je třeba zdůraznit, že úspěšnost transfekce je závislá na velikosti plazmidu, jeho čistotě a formě, ale i na kondici transfekovaných buněk a jejich „ochotě“ k transfekci. U buněk B210 jsem použila plazmid v cirkulární formě a podařilo se mi izolovat stabilně transfekované buňky. Buňky 12B1 byly nejprve také transfekovány cirkulárním plazmidem. Ale po zamražení a rozmražení buněk produkce testovaných

cytokinů podstatně klesla. Abychom měli větší šanci získat stabilně transfekované buňky 12B1, použitý plazmid jsem linearizovala. Podařilo se pak získat klony, u kterých jsem nezaznamenala propad produkce cytokinů ani po zamražení a rozmražení, ani po jejich delší kultivaci. Účinnost elektroporace jsem vždy kontrolovala paralelní transfekcí plazmidu pTR-UF2 a měřením GFP-pozitivních buněk průtokovou cytometrií.

4.3 Selekční model transfekovaných buněk

Už v minulosti se v naší laboratoři moji kolegové zabývali přípravou buněčných vakcín, hlavně na papilomavirovém modelu. Používali při tom systém sebevražedných genů, ve kterém kromě genu pro imunostimulační faktor vnesli do buněk s nefunkční tymidinkinázou (cTK-) i gen pro herpesvirovou tymidinkinázu (HSV TK). Díky produktu tohoto genu mohli jednak selektovat transfekované buňky, a jednak při pokusech *in vivo* cíleně zabíjet nádorové buňky podáním antivirotika gancikloviru, analogu tymidinu (Vonka *et al*, 1998;Janouskova *et al*, 2003;Jinoch *et al*, 2003).

Pro využití obdobného modelu v systému buněk transformovaných fúzním genem bcr-abl bylo nejdříve nutné vypěstovat buňky deficientní v produkci buněčné tymidinkinázy. Toho se docílilo opakovaným pasážováním buněk v postupně se zvyšující koncentraci bromdeoxyuridinu (BrdU). Takto byly po téměř půlročním snažení odvozeny buňky B210cTK$^-$, které se dobře množily v přítomnosti 100 μg BrdU/ml a nerostly v médiu s hypoxantinem-aminopterinem-tymidinem (HAT). Buňky B210cTK$^-$ vykazovaly nižší onkogenní potenciál než buňky mateřské. Proto byly buňky klonovány a vybrané klony byly testovány na onkogenitu. Klon B210cTK$^-$/cl-2, který měl nejvyšší onkogenní potenciál, byl vybrán pro následné genetické modifikace. Pro transfekci buněk B210cTK$^-$/cl-2 jsem použila bicistronické plazmidy pTR-IL-2-IRES-TK, pTR-IL-12-IRES-TK a pTR-GM-CSF-IRES-TK, které připravili v naší laboratoři O. Janoušková a P. Jinoch. Tyto plazmidy obsahují gen pro HSV TK, za nímž následuje IRES sekvence a gen pro cytokin IL-2, IL-12 nebo GM-CSF. Po transfekci buněk těmito plazmidy jsem buňky nasadila do RPMI média. Po 48 hodinách jsem jim médium vyměnila za RPMI obsahující HAT, který zajišťuje selekci transformantů. Jako kontrolu selekce jsem použila tzv. mock transfekované buňky, které byly elektroporovány v nepřítomnosti plazmidu. Během dvoutýdenní kultivace transfekovaných buněk v selekčním médiu se namnožily

modifikované buňky, zatímco kultura mock-transfekovaných buněk v selekčním médiu hynula.

Kultivace buněk 12B1 v médiu s BrdU byla neočekávaně snadná, v koncentraci 100 μg/ml se množily buňky už po několika týdnech pasážování. Tyto buňky jsem elektroporovala a nasadila do selekčního média s HAT. Posléze se ale ukázalo, že v něm rostou i buňky z mock-transfekce. Buňky 12B1cTK- byly zřejmě geneticky nestabilní a v buněčné populaci se objevovalo mnoho revertant cTK+. Jelikož účinnost transfekce byla relativně nízká a selekční systém selhal, bylo potřeba zvolit jiné řešení modifikace buněk 12B1.

Použila jsem plazmidy pBSC/IL-2 a pBSC/GM-CSF, které konstruoval v naší laboratoři M. Šmahel. Tyto plazmidy však nemají vhodný marker pro selekci v eukaryotických buňkách. K tomuto účelu se hodil gen pro rezistenci na blasticidin, který jsem izolovala z plazmidu pBLAST42mAngio (InvivoGen). Upravené plazmidy, ve kterých má gen pro blasticidinovou rezistenci i gen pro cytokin vlastní promotor, byly označeny jako pBSC/IL-2-Bsr a pBSC/GM-Bsr. Expresi obou genů jsem zkontrolovala ve snadno transfekovatelných buňkách 293T, a to kultivací transfekovaných buněk v médiu s blasticidinem a testem ELISA. Buňky 12B1 jsem před transfekcí otestovala na jejich citlivost k blasticidinu. K selekci jsem zvolila koncentraci 25 μg/ml, která netransfekované buňky spolehlivě zabíjí.

Transfekované buňky vytvořily po dvou až třech týdnech selekce dostatečně velkou populaci vhodnou k zamražení, k testování *in vitro* a ke klonování. Klonování probíhalo tak, že jsem buňky naředila do koncentrace 0,3 buňky/0,2 ml RPMI s HATem (B210) nebo v RPMI s blasticidinem (12B1) a rozpipetovala je do dvou 96-jamkových destiček. U obou linií se ukázalo, že takové podředění buňkám nesvědčí a klony se mi nedařilo získat. Abych buňkám imitovala prostředí s vyšší koncentrací buněk, obohatila jsem médium ke klonování o kondiciované médium (*spent* médium), odebrané v logaritmické fázi růstu daných transfekovaných buněk. V takto upraveném médiu se podařilo jak u buněk B210, tak u buněk 12B1 odvodit linie pocházející z jedné buňky.

4.4 Charakteristika modifikovaných buněk B210

Získané modifikované buňky B210/2/IL-2, B210/2/IL-12 a B210/2/GM-CSF byly citlivé na GCV, což dokazovalo expresi funkční HSV TK. Produkci cytokinů jsem

testovala jak po zamražení a rozmražení, tak i v průběhu dlouhodobého pasážování *in vitro*. Jevila se jako stabilní. Všechny tři linie exprimovaly podobné množství proteinu BCR-ABL. Zajímalo nás také, zda modifikací buněk nedošlo ke změně v expresi MHC I a MHC II. Ukázalo se, že všechny získané linie stejně jako mateřské buňky nevytváří *in vitro* prokazatelné množství těchto molekul.

Na myších se testovala patogenita modifikovaných buněk a několik jejich klonů s různou expresí cytokinů. Buňky se aplikovaly myším intravenózně v dávce 10^6, která v případě nemodifikovaných mateřských buněk B210, stejně jako v případě klonu B210cTK$^-$ /cl-2, vyvolala u všech myší fatální leukémii. Myši inokulované modifikovanými buňkami zůstaly ve všech případech zdravé. Z toho vyvozuji, že produkce cytokinů byla dostatečná k vyvolání imunitní odpovědi a zabránění vývoje leukémie. Z myší, které dostaly buňky exprimující jen HSV TK, onemocněly 3 ze 4. To napovídá, že exprese HSV TK nehrála žádnou zásadní roli při ztrátě onkogenního potenciálu modifikovaných buněk.

Jelikož byly modifikované buňky B210 nepatogenní, další série pokusů byla založena na použití těchto buněk jako živých vakcín. V pokusech byly k vyvolání nemoci použity buňky 12B1, protože buňky B210, pokud se podají jinak než intravenózně, nevyvolají nemoc a navodí velmi silnou protekci proti intravenózní čelenži homologními buňkami. Efekt modifikovaných buněk B210 by pak nemohl být na takovémto modelu sledován, kdežto po podání agresivnějších buněk 12B1 by byl patrný. Pro čelenž se buňky 12B1 podávaly subkutánně. V místě vpichu se posléze vytvořil nádor. Ten se dal snadno měřit, takže jsme mohli hodnotit dobu objevení nádoru, jeho růst a přežívání myší.

Modifikované buňky B210 byly nejprve testovány v profylaktickém uspořádání pokusu. Myši byly imunizovány dvakrát v dvoutýdenním intervalu dávkou 3 x 10^6 buněk intraperitoneálně a po dvou týdnech dostaly jako čelenž dávku 5 x 10^3 buněk 12B1. Většina myší nebyla ochráněna a vytvořila nádor. Nejlépe dopadla skupina myší, která byla vakcinována buňkami B210/2/GM-CSF. Dvě z pěti myší zůstaly zdravé a u ostatních myší byl ve srovnání s kontrolními zvířaty pozorován významně pomalejší růst nádorů.

Vakcíny byly dále testovány v terapeutickém uspořádání pokusu. Pokud byly podány v dávce 10^6 4x za sebou v den 0, 3, 7 a 10, došlo po subkutánním podání buněk 12B1 v den 0 k statisticky významnému oddálení vývoje nádorů. Nádory u zvířat vakcinovaných buňkami B210/2/IL-2 a B210/2/IL-12 se vyvíjely stejně. Nádory u myší vakcinovaných buňkami B210/2/GM-CSF rostly o něco rychleji, ale v této skupině zůstala jedna ze šesti myší zdravá.

Výsledky byly povzbudivé, ale bylo jasné, že k vyléčení myší je třeba kombinovat více léčebných postupů. V následujících experimentech byly myším podány buněčné vakcíny v kombinaci s cyklofosfamidem (Cy), IM a myším IFN-α , a to v suboptimálních dávkách. Jak jsme předpokládali, samotná chemoterapeutika či IFN-α, ať jednotlivě nebo ve vzájemné kombinaci pozdržela vývoj nádorů, ale myši nevyléčila. Povzbudivější byly výsledky kombinace antileukemických léčiv s vakcínami. Nejlepších výsledků bylo dosaženo kombinací buněk B210/2/IL-2 a Cy s IFN-α. V této skupině jen dvě ze šesti myší vytvořily nádory a ty se objevily opožděně a rostly pomaleji. Podobně se vyvíjel růst nádorů u myší, kterým byly aplikovány buňky B210/2/IL-2 a Cy s IM, i když se nádory vytvořily u čtyř ze šesti myší. Naproti tomu vakcíny B210/2/IL-12 a B210/2/GM-CSF byly podstatně méně účinné. Stojí za zmínku, že podobná chemo-imunoterapie byla v minulosti zkoumána na myším modelu s buňkami transformovanými HPV-16. Byla také účinnější než podání samotné vakcíny nebo samotných chemoterapeutik (Sobotkova *et al*, 2004).

Synergický efekt kombinace antileukemických léčiv, jako je IM, IFN-α a další, byl již opakovaně popsán, např. (Thiesing *et al*, 2000;Kano *et al*, 2001;Marley *et al*, 2002). Mechanizmus účinku IM je znám, některé práce ale upozorňují na jeho možné imunosupresivní účinky na T-buňky, makrofágy a DC (Wolf *et al*, 2007;Dietz *et al*, 2004;Seggewiss *et al*, 2005;Cwynarski *et al*, 2004). IFN-α má imunomodulační a antiproliferační účinek. Podporuje diferenciaci monocytů na dendritické buňky, aktivaci T-lymfocytů a makrofágů a cytotoxicitu NK buněk. Rovněž indukuje apoptózu nádorových buněk, inhibuje angiogenezi a nutí dormantní leukemické kmenové buňky k aktivaci buněčného cyklu (de Castro *et al*, 2003;Burchert & Neubauer, 2005). Přestože lékem číslo jedna jsou u CML tyrozinkinázové inhibitory, IFN-α se znovu dostává do centra pozornosti a uvažuje se o použití kombinace TKI + IFN-α (Kiladjian *et al*, 2011;Simonsson *et al*, 2011), O cyklofosfamidu se ví, že inhibuje supresivní aktivitu populace Treg buněk. V nízkých dávkách napomáhá odpovědi Th1-lymfocytů a aktivaci, maturaci a sekreci cytokinů dendritických buněk a zvyšuje expresi interferonů typu 1 důležitých pro imunologickou paměť (Wada *et al*, 2009;Emens, 2010). V našem případě byla dávka cyklofosfamidu vyšší než imunomodulační, tudíž se pravděpodobně uplatnil hlavně jeho cytostatický efekt. Každopádně můžeme konstatovat, že kombinace vakcinace s Cy, IM a IFN-α měla v našich pokusech synergický účinek.

Asi by bylo zajímavé provést obdobné pokusy se stejnými buněčnými vakcínami, ale inaktivovanými ozářením. Ačkoliv v některých leukemických myších modelech se

imunita podařila navodit jen po podání živých vakcín (Nakazaki *et al*, 1998), uvádí se, že ozářené buňky vysílají apoptické signály nebezpečí, takže jsou čitelnější pro APC buňky a tudíž i imunogennější (Simmons *et al*, 2007).

4.5 Charakteristika modifikovaných buněk 12B1

Podobně jako v případě buněk B210, také u buněk 12B1 jsem se pokusila určit vliv genové modifikace na onkogenní potenciál buněk. Z klonů buněk 12B1 exprimujících IL-2 jsem pro další pokusy zvolila klon s nejvyšší produkcí označený jako 12B1/IL-2/cl-15. Z klonů buněk exprimujících GM-CSF jsem vybrala tři klony lišící se významně produkcí cytokinu. U všech těchto klonů byla naměřena stabilní produkce příslušného cytokinu i během dlouhodobého pasážování *in vitro.* V porovnání s mateřskými buňkami se produkce proteinu BCR-ABL nijak výrazně nelišila.

Na rozdíl od modifikovaných buněk B210, obdobně modifikované buňky 12B1 neztratily svůj onkogenní potenciál. Nicméně u buněk 12B1/IL-2/cl-15 byl výrazně nižší než u mateřských buněk. To se projevilo zvláště po inokulaci relativně malých dávek buněk. Po dávkách 10^3 a 10^4 nemodifikovaných mateřských buněk všechny myši onemocněly, zatímco po inokulaci stejného množství modifikovaných buněk zůstaly všechny myši zdravé. Zajímavé bylo, že po aplikaci 10^5 a 10^6 buněk 12B1/IL-2/cl-15 docházelo k vytvoření malých nádorů, které ale do 25. dne vymizely a většina myší zemřela později na leukémii. To naznačuje, že vedle kvantitativních změn došlo i ke kvalitativní změně onkogenního potenciálu. Myši, které při testech onkogenního potenciálu buněk 12B1/IL-2/cl-15 neonemocněly, byly čelenžovány velmi vysokou dávkou buněk 12B1 a více jak polovina jich zůstala zdravá. To svědčí o imunogenním potenciálu buněk 12B1/IL-2/cl-15. Navíc se ukázalo, že populace buněk kultivovaných z nádorů nebo z orgánů infiltrovaných leukemickými buňkami jsou nehomogenní. V médiu s blasticidinem rostly zpočátku špatně a celková produkce IL-2 byla nízká ve srovnání s naočkovanými buňkami. Až po několika pasážích v přítomnosti blasticidinu se vyselektovaly buňky exprimující IL-2 a jeho produkce se stala srovnatelnou s inokulovanými buňkami. Pravděpodobně se objevily revertantní buňky, kterým ztráta či umlčení transgenu pro blasticidinovou rezistenci a IL-2 dala růstovou výhodu.

Ani buňky 12B1 exprimující GM-CSF neztratily onkogenní potenciál. Ten závisel na míře jejich produkce GM-CSF. Klon 12B1/GM-CSF/cl-1 s nejnižší produkcí 3

ng/10^6bb/24 hod byl jak v dávce 10^5, tak i v dávce 10^3 méně onkogenní než mateřské buňky. Klon 12B1/GM-CSF/cl-11 s produkcí 50 ng/10^6bb/24 hod byl v dávce 10^5 přibližně stejně onkogenní jako mateřské buňky 12B1, ale v dávce 10^3 už dvě ze tří myší zůstaly zdravé. Nejvíce patogenní se jevil klon 12B1/GM-CSF/cl-5 s produkcí GM-CSF 110 ng/10^6bb/24 hod. Ačkoliv se myším naočkovaným těmito buňkami vytvořily nádory ve stejnou dobu jako po inokulaci mateřských buněk, zvířata byla v daleko horší kondici. Byla naježená, méně pohyblivá, ztrácela na váze a měla řadu orgánových poškození (viz kapitola 4.6).

Jelikož jsme modifikované, ale onkogenní buňky 12B1 připravovali pro účely buněčných vakcín, bylo třeba je před aplikací zvířatům inaktivovat. Zvolili jsme gama-záření o síle 100 Gy. Ozářením ztratí buňky proliferační aktivitu, většina z nich zůstane v G2/M fázi buněčného cyklu, ale neztratí schopnost exprimovat vnesený transgen. Uvádí se, že ozářené nádorové buňky exprimují více transgenu než buňky neozářené (Simova *et al*, 1998;Vereecque *et al*, 2003;Berezhnoy *et al*, 2008). Chtěla jsem ověřit, zda v našich buňkách také došlo ke zvýšení produkce daného cytokinu. Vzhledem k tomu, že buňky po ozáření velmi rychle umíraly, těžko se produkce cytokinu vztahovala na množství buněk, které byly schopné jej produkovat. Zjistila jsem, že *in vitro* bylo po 24 hod živých jen 30% buněk a po 48 hod už jen 5%. Ozářené buňky v místě inokulace jsme nezkoumali. Pokud předpokládáme podobný průběh jejich životnosti *in vivo* jako *in vitro*, dochází v místě vakcinace k významnější produkci cytokinu jen prvních 48 hod, což je poměrně krátká doba. Pro srovnání, ozářené K562 buňky transdukované genem GM-CSF produkují po ozáření 100 Gy cytokin po dobu 10 dní, jejich životnost je také 10 dní (Borrello *et al*, 1999). Dranoff a spol. pozorovali produkci GM-CSF *in vitro* u ozářených melanomových buněk B16F10 7 dní (Dranoff *et al*, 1993). V místě vakcinace ozářenými buňkami B16F10 byla detekována zvýšená hladina GM-CSF i 21 dní po jejich aplikaci (Simmons *et al*, 2007). Uvádí se, že pro vyvolání nádorové imunity vakcinací buňkami exprimujícími GM-CSF je u myší potřeba uvolnit 35 – 300 ng GM-CSF/10^6bb/24 hod (Dranoff *et al*, 1993;Serafini *et al*, 2004). Vakcína připravená z 3 x 10^6ozářených buněk 12B1/GM-CSF/cl-5 produkovala *in vitro* v průběhu prvních 24 hod kolem 300 ng GM-CSF, což je v udávaném limitu.

V profylaktickém uspořádání pokusu jsme myším nejprve podali 2x v dvoutýdenním intervalu ozářené modifikované i nemodifikované buňky 12B1 v dávce $3x10^6$ a po dalších dvou týdnech jsme myši čelenžovali dávkou 5 x 10^3 mateřských buněk 12B1. Nejlépe myši ochránila vakcína z buněk 12B1/GM-CSF/cl-5, klonem s nejvyšší

produkcí cytokinu. Všechny myši zůstaly bez nádoru a zdravé, a to i po rečelenži provedené 78. den. Po aplikaci vakcíny z ozářených mateřských buněk 12B1 jedna z pěti myší přežila a nádory rostly oproti kontrole o něco pomaleji. Při opakování pokusu jsme použili pro vakcinaci i klon 12B1/GM-CSF/cl-1 s nízkou produkcí GM-CSF. Po vakcinaci těmito buňkami přežily čtyři ze šesti myší a dvěma myším rostly nádory pomaleji než u kontrolních neimunizovaných myší a myší imunizovaných ozářenými mateřskými buňkami. Vakcína tak neochránila všechna zvířata, ale byla účinnější než vakcína z mateřských buněk nebo buněk 12B1/IL-2/cl-15.

Modifikované buňky 12B1 jsme zkoušeli i v terapeutickém uspořádání. Výsledky nejsou uvedeny v žádném z přiložených článků, protože byly velmi heterogenní. Ozářené modifikované i nemodifikované buňky 12B1 jsme podali myším intraperitoneálně v dávce $3x10^6$ v den 0, 3, 7 a 10. V den 0 jsme zároveň aplikovali myším subkutánně $5x10^3$ buněk 12B1. V prvním pokusu zůstalo všech 6 myší vakcinovaných inaktivovanými buňkami 12B1/IL-2/cl-15 zdravých. Myši neonemocněly ani po rečelenži 65. den. To byl velmi povzbuzující výsledek, který se nám ale, bohužel, nepodařilo zopakovat. Důvody nízké reprodukovatelnosti se nám zatím nepodařilo objasnit.

Přípravu vakcín z modifikovaných buněk 12B1 jsme standardizovali, ale přesto docházelo po ozáření k určité variabilitě v přežívání buněk a v jejich cytokinové produkci. Ozářené buňky 12B1 měly krátkou dobu životnosti, nicméně exprese cytokinu stačila k tomu, aby se projevily rozdíly ve vakcinaci nemodifikovanými a modifikovanými buňkami.

4.6 Efekt nadprodukce GM-CSF po podání buněk 12B1/GM-CSF/cl-5 myším

Když jsme testovali patogenitu modifikovaných buněk 12B1, pozorovali jsme u myší inokulovaných buňkami 12B1/GM-CSF/cl-5 výrazně horší zdravotní stav a posléze jsme u nich detekovali četná a rozsáhlá orgánová poškození.

Jelikož GM-CSF se v dnešní medicíně využívá pro přípravu buněčných vakcín, rozhodli jsme se blíže charakterizovat patologické změny vyvolané buňkami produkujícími tento cytokin a jejich vývoj v závislosti na hladině cytokinu v krvi.

Jedné skupině myší byly aplikovány buňky 12B1/GM-CSF/cl-5 a druhé mateřské buňky 12B1. Dvě myši ze skupin se pak odebíraly ve dvoudenním nebo čtyřdenním

intervalu pro histologické a imunologické vyšetření. V obou skupinách se začaly objevovat nádory kolem 10. dne a rostly stejně rychle i v následujících dnech. Myši inokulované buňkami 12B1/GM-CSF/cl-5 začaly být už od 8. dne ježaté a apatické a začaly ztrácet na váze. Ještě před objevením se nádoru u nich byly při pitvě pozorovány orgánové změny a detekována zvýšená hladina GM-CSF v krvi.

Křivka hladiny GM-CSF v krvi měla exponenciální charakter se svým maximem v 16. den. Tuto křivku kopírovala i křivka zvětšení slezin a nárůstu MDSC buněk v populaci splenocytů. Slezina se během osmnácti dnů zvětšila skoro 20x, zatímco slezina myší inokulovaných mateřskými buňkami 12B1se zvětšila jen 6x. Zvětšení sleziny u myší inokulovaných buňkami 12B1/GM-CSF/cl-5 bylo způsobeno hlavně infiltrací nezralými myeloidními buňkami. Červená pulpa nahradila bílou pulpu a docházelo v ní k silné extramedulární hematopoéze. Přes 50% splenocytů mělo na závěr pokusu znak myeloidních buněk CD11b+, 30% bylo CD11b+Gr-1+, což jsou markery myších MDSC. Sleziny byly infiltrovány také nádorovými buňkami, ty ale tvořily v závěru pokusu jen 5% buněčné populace. Sleziny myší inokulovaných buňkami 12B1 obsahovaly na konci pokusu 40% nádorových buněk a žádné nápadné změny v jejich strukturách jsme nepozorovali. Jejich zvětšení tudíž vysvětlujeme infiltrací nádorovými buňkami.

Jedním z mechanismů, kterým MDSC potlačují imunitní odpověď, je indukce Treg. Několik prací poukazuje na souvislost mezi vysokou koncentrací GM-CSF a nárůstem Treg (Vasu *et al*, 2003;Bhattacharya *et al*, 2011). Předpokládali jsme proto, že v našem systému také dojde k podobnému jevu, a že se zvyšující se hladinou GM-CSF poroste i jejich populace. To se nám nepotvrdilo. Zvýšení Treg lymfocytů ve slezině se projevilo až 18. den a došlo k němu jak u myší inokulovaných 12B1/GM-CSF/cl-5, tak i u myší inokulovaných mateřskými buňkami. Nicméně je třeba si uvědomit, že přítomnost Treg lymfocytů jsme vyjádřili jejich procentuelním zastoupením v populaci splenocytů. Vzhledem k rozdílným velikostem slezin jich v absolutních počtech bylo větší množství u myší inokulovaných 12B1/GM-CSF/cl-5. Korelace mezi hladinou GM-CSF a MDSC, ale ne mezi GM-CSF a Treg, byla také zjištěna u myší, kterým byly aplikovány ozářené melanomové nádorové buňky s vysokou expresí GM-CSF (1500 a 6000 ng/24 hod). Nejvíc MDSC bylo detekováno ve slezinách 3. den po inokulaci buněk a bylo jich až 20% (Serafini *et al*, 2004).

Nádory myší z buněk sekretujících GM-CSF byly velmi dobře vaskularizované na rozdíl od nádorů vyvolaných mateřskými buňkami 12B1, ve kterých byla nalezena nekrotická ložiska. Jedním z možných vysvětlení je aktivace endoteliálních buněk

prostřednictvím receptoru pro GM-CSF a působení MDSC, které podporují angiogenezi (Murdoch *et al*, 2008).

Markantní poškození bylo nalezeno u plic. Došlo k destrukci intraalveolárních sept a k překrvení s ohnisky krvácení. Mezi dalšími poškozenými orgány byly játra a ledviny. Oba orgány byly překrvené s hemoragickými ložisky. V játrech byla patrná extramedulární hematopoéza, v ledvinách byly pozorovány hyalinní kapky v epiteliích proximálních tubulů. Výše popsané změny v plicích, játrech a ledvinách nebyly pozorovány u myší očkovaných mateřskými buňkami.

Patologické změny u transgenních myší s indukovanou nadprodukcí GM-CSF popsali Burke a spol. (Burke *et al*, 2004). Uvádějí úbytek svalů, masivně zvětšenou slezinu s převládající červenou pulpou a zvětšená játra s extramedulární hematopoézou. Pozorovali též, že myším se v očních bulbech akumulovaly makrofágy, což vedlo až k jejich slepotě. O poškození plic a ledvin se ale nezmiňují. S GM-CSF-transgenními myšmi pracovala i Metcalfova skupina (Metcalf *et al*, 1998). Samice měly v séru 4 - 16 ng GM-CSF/ml. Zmínění autoři analyzovali intraabdominální buněčnou populaci a zjistili, že většinu buněk tvořily makrofágy. Během 2-3 měsíců také pozorovali destrukci retiny a její infiltraci makrofágy, místní záněty ve svalech a bulky na peritoneu a pleuře a ve stěně močového měchýře. Myši také ztrácely na váze a u některých se objevila slabost až paralýza zadních končetin. Metcalf i Burke tak popisují spíše „chronickou otravu cytokinem GM-CSF“, kterou sledovali několik měsíců. V našem pokusu šlo spíš o „akutní otravu cytokinem“ komplikovanou růstem zhoubného nádoru, díky čemuž myši nepřežily ani tři týdny po aplikaci buněk.

Abychom se přesvědčili, že poškození orgánů bylo způsobené vysokou koncentrací GM-CSF v organizmu, provedli jsme pokus s neutralizačními protilátkami proti GM-CSF. Myším jsme aplikovali buď jen protilátky, nebo protilátky a buňky 12B1/GM-CSF/cl-5 a nebo jen buňky 12B1/GM-CSF/cl-5. Orgánové změny jsme porovnávali u myší, které měly zhruba stejnou velikost nádoru. Bylo patrné, že ve skupině myší, kterým byly kromě buněk aplikovány i neutralizační protilátky, je daleko menší rozsah poškození všech zkoumaných orgánů než u myší, které dostaly jen buňky. Samotné neutralizační protilátky neměly žádné účinky na strukturu zkoumaných orgánů. Pro dokumentaci těchto rozdílů ve stupni poškození orgánů jsme použili histologické preparáty plic.

Lze tedy shrnout, že pokud je hladina GM-CSF zvýšená lokálně a přechodně, hraje tento cytokin nezastupitelnou roli v indukci imunitní odpovědi. Přílišné množství GM-CSF

má však imunosupresivní účinek a pokud je mu organizmus vystaven delší dobu a cytokin působí systémově, může vyvolat rozsáhlé orgánové poškození.

Jsem si vědoma toho, že náš experimentální myší systém jen částečně napodobuje CML u lidí. Je založen na lymfoblastoidních buňkách, které se objevují hlavně v terminálním stádiu nemoci nebo u pacientů s akutní lymfoblastoidní leukémií pozitivních na bcr-abl. Bylo by zajímavé vyzkoušet podobný experimentální systém založený na buňkách transformovaných bcr-abl s myeloidním fenotypem. Buňky myeloidní řady mají na rozdíl od lymfoidních buněk receptor pro GM-CSF, což by mohlo mít dopad na onkogenní i imunogenní potenciál buněk exprimujících GM-CSF. Ovlivněna výsledky našich experimentů soudím, že buněčné vakcíny odvozené od leukemických buněk produkujících IL-2 by mohly být vhodné k imunoterapii CML.

Domnívám se, že v budoucnosti bude vhodné nadále kombinovat námi připravené buněčné vakcíny s chemoterapií a/nebo se substancemi, které potlačují účinek produktů, o kterých se ví, že působí imunosupresivně a proangiogenně. V naší laboratoři se již provedly předběžné experimenty s 1-methyltryptofanem, inhibitorem IDO, a se substancí nor-NOHA, inhibitorem arginázy, a výsledky jsou povzbudivé. Určitě by se mělo vyzkoušet současné podání vakcín a monoklonálních protilátek, jako jsou anti-CTLA-4 nebo anti-CD25. Zároveň je třeba monitorovat imunologické reakce zvířat, imunosupresivní buněčné populace a molekuly.

Současně by mělo probíhat i rozšiřování znalostí o povaze imunitních reakcí u pacientů s CML (Chen *et al*, 2008a;Zamarron & Chen, 2011;Rohon *et al*, 2010;Humlova *et al*, 2010;Kreutzman *et al*, 2011) a se v tomto kontextu podrobně zkoumat leukemické kmenové buňky (Gerber *et al*, 2011;Corbin *et al*, 2011;Hamilton *et al*, 2012). I na našem oddělení probíhají projekty zaměřené tímto směrem. Každá nově získaná informace může změnit pohled na imunoterapii CML a posunout ji o krok dopředu.

5. ZÁVĚR

Imunoterapii v CML se v posledních letech věnuje velká pozornost. K vakcinaci se používá kombinace peptidů odvozených od proteinu BCR-ABL, peptidy odvozené od LAA jako jsou WT1 a PR3, autologní dendritické buňky a alogenní buněčná vakcína exprimující GM-CSF.

Předložená práce se věnuje přípravě experimentálních buněčných vakcín proti CML založených na genově modifikovaných myších buňkách. Vakcíny byly odvozeny od buněk B210 a 12B1, transformovaných genem bcr-abl. Pro jejich transfekci byla optimalizována metoda elektroporace. Z transfekovaných buněk byly odvozeny klony exprimující stabilně různé množství daného cytokinu a pro následné pokusy byly vybrány převážně klony s nejvyšší produkcí.

Vnesením genu pro GM-CSF nebo IL-2, případně IL-12, se podařilo zvýšit imunogenní potenciál buněk B210 a 12B1. Modifikované buňky B210 produkující IL-2, GM-CSF nebo IL-12 byly neonkogenní a v pokusech na myších se používaly jako živé vakcíny. Buňky 12B1 produkující IL-2 měly onkogenní potenciál výrazně nižší než buňky mateřské. Pokud po jejich inokulaci myš onemocněla nebo měla nádor, nádorové buňky tvořily heterogenní populaci, jejíž velká část ztratila schopnost produkce IL-2 a rezistenci k blasticidinu. Buňky 12B1 produkující vysoké množství GM-CSF byly stejně onkogenní jako buňky mateřské a po subkutánním podání se u všech myší vyvinuly nádory. Se zvětšujícím se nádorem se významně zvyšovala i hladina GM-CSF v organizmu zvířete. To vedlo jednak k fatálnímu poškození důležitých orgánů (nejvíce sleziny, plic, jater a ledvin), a jednak ke zmnožení MDSC. Vzhledem k zachování onkogennosti se buněčné vakcíny odvozené od buněk 12B1 používaly ozářené.

Pro oboje buňky platilo, že v terapeutickém uspořádání pokusů nejlépe účinkovaly buňky exprimující IL-2 a v profylaktickém uspořádání buňky exprimující GM-CSF. Lepších terapeutických výsledků s buňkami B210 bylo dosaženo při kombinaci buněčných vakcín produkujících IL-2 s chemoterapií. To jsou nadějné výsledky, zvlášť když si uvědomíme, že na myších se nemoc vyvolaná buňkami 12B1 projevuje formou, která je typická pro terminální fázi nemoci, při níž je manévrovací prostor pro jakoukoliv léčbu velmi omezený.

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