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Organização Molecular do Espaço Intercelular na Sinalização do GM-CSF em Sistemas Hematopoéticos

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Kátia Denise de Souza Arcanjo e aprovada pela Comissão Julgadora.

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Dedico esta tese,

Aos meus pais,
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Ao meu "filhote" Jaime,
A "página" mais preciosa da minha vida, com quem aprendo muito,
todos os dias...

À você Filipe,
Pelo incentivo e por todos esses anos de felicidade ao teu lado.

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ABREVIATURAS UTILIZADAS

AH	ácido hialurônico
CS	condroitim sulfato
C-4S	condroitim 4-sulfato
C-6S	condroitim 6-sulfato
DS	dermatam sulfato
FGF-β	fator de crescimento fibroblástico
GAG	glicosaminoglicano
Gal	galactose
GM-CSF	fator estimulador de colônias de granulócitos e macrófagos
GPI	glicosilfosfatidilinositol
Hep	heparina
HGF	"hepatocyte growth factor"
HS	heparam sulfato
HSPG	proteoglicano de heparam sulfato
HSC	"stem cell" hematopoética
IL-3	interleucina-3
IL-5	interleucina-5
MEC	matriz extracelular
PG	proteoglicano
QS	queratam sulfato
TGF-β	fator de crescimento transformante
VEGF	fator de crescimento para endotélio vascular

ÍNDICE

I- Resumo.....01

II- Abstract.....03

III- Introdução.....05

1. Hematopoese.....05

2. Fatores de Crescimento.....06

3. Estroma Hematopoético.....07

4. Glicosaminoglicanos – GAGs.....08

4.1. Ácido Hialurônico (AH).....08

4.2. Condroitim Sulfato (CS) e Dermatam Sulfato(DS).....10

4.3. Queratam Sulfato(QS).....10

4.4. Heparam Sulfato (HS) e Heparina (Hep).....11

5. Proteoglicanos (PGs).....12

5.1. PGs de Grânulos Secretórios.....12

5.2. PGs de Superfície Celular.....14

a) Transmembranares.....14

b) Associados à Membrana via Glicosilfosfatidil inositol.....16

5.3. PGs de Matriz Extracelular.....17

a) PGs de Membrana Basal.....17

b) Hialectanos.....18

c) PGs Ricos em Leucina.....19

6.0. O Papel dos PGs, GAGs e Fatores de Crescimento no Ambiente Hematopoético.....20

IV- Objetivos.....24

V- Referências Bibliográficas.....25

VI- Artigos.....41

Anexo 1. Biochemical characterization of heparan sulfate derived from murine haemopoietic stromal cell lines: a bone-marrow-derived cell line S17 and a fetal liver-derived line AFT024. 42

Anexo 2. Stroma-mediated granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation of myelopoiesis: spacial organisation of intercellular interactions. 86

Anexo 3. The capacity of connective tissue stromas to sustain myelopoiesis depends both upon the growth factors and the local intercellular environment. 114

VII- Conclusões.....124

I- RESUMO

O microambiente da medula óssea desempenha importância fundamental na proliferação e diferenciação das células progenitoras hematopoéticas bem como no controle do egresso dessas células para o sangue periférico. Embora moléculas da matriz extracelular, juntamente com citocinas, sejam cruciais na compartimentalização dos microambientes da medula óssea, ainda não se conhecem os pré-requesitos para que um determinado estroma possa sustentar a hematopoese.

Usando uma linhagem celular (FDC-P1) dependente de fator de crescimento, comparamos modelos experimentais de estromas periféricos e de medula óssea, que sustentam ou não a proliferação mielóide "in vitro". Observamos que todos os estromas produzem um grupo similar de hemopoetinas, incluindo o fator estimulador de colônias de macrófagos e granulócitos (GM-CSF), o qual pode ser liberado das monocamadas estromais, através de altas concentrações de sais, numa forma biologicamente ativa. Por outro lado, glicosaminoglicanos (GAGs) obtidos a partir desses estromas, exibiram uma capacidade de modulação da atividade biológica do GM-CSF variável, dependendo da concentração utilizada. Fibroblastos de pele, que não são capazes de sustentar a mielopoese "in vitro", sintetizam GAGs que podem inibir a atividade mielopoética de GM-CSF. Concluímos que, a qualidade dos glicoconjugados pericelulares dos estromas, presentes no microambiente local, é determinante para que um dado estroma seja capaz de sustentar a mielopoese, mesmo quando hemopoetinas biologicamente ativas são localmente produzidas.

Os aspectos espaciais desse microambiente e as interações moleculares entre as células do estroma e progenitores mielóides foram investigados. Demonstramos que esse contato fisico pode disparar um "capping" de moléculas de superficie celular incluindo glicoconjugados de ácido siálico e proteoglicanos. Juntas, essas moléculas, potencialmente interativas com o GM-CSF, geram um ambiente intercelular esfecífico, promovendo condições fisico-químicas requeridas para essa interação.

A identificação da expressão dos HSPGs associados as superficies celulares foi monitorada através de RT-PCR. Os estromas analisados mostraram expressão de mRNA para glipicam, betaglicam e sindecam-4. O tratamento das monocamadas estromais com PI-PLC

(fosfolipase C) e tripsina branda, confirmou a presença de HSPGs de superfície celular (glipicam) e sindecam, respectivamente.

Aproximadamente, 20% dos HSPGs obtidos, estão associados à membrana através de âncoras de GPI enquanto o restante, 80%, permanece integrado à membrana plasmática.

Demonstramos que as moléculas marcadas com sulfato radioativo, deslocadas da superfície celular das células estromais após tratamento com tripsina branda, são capazes de aumentar a atividade biológica do GM-CSF, quando comparadas com aquelas deslocadas por PI-PLC as quais não apresentaram diferenças significativas se comparadas com o controle. Esses resultados sugerem a existência de HSPGs transmembranares e associados via âncora de GPI nas superfícies das células estromais, e que, a atividade biológica de GM-CSF na proliferação de FDC-P1 "in vitro", é aumentada através de uma possível interação física entre o fator de crescimento e um HSPG transmembranar.

II ABSTRACT

The bone marrow microenvironment plays an important role in promoting hematopoietic progenitor cell proliferation and differentiation, as well as their controlled release into the circulation. It has been shown that the extracellular matrix elements in combination with cytokines are crucial for compartmentalization of the bone marrow environment, but it is not clear which are the molecular and structural requirements for a given stroma to sustain hematopoiesis.

Using the growth factor-dependent cell line FDC-P1, we have compared *in vitro* experimental models of bone marrow and peripheral stromas, two of which sustain myeloid proliferation and one which does not. We have found that all the stromas produced a similar set of hemopoietins, including the GM-CSF, which could be released from the stromal cell layer in an active form by high-salt buffers. On the other hand, glycosaminoglycans (GAGs) obtained from stromas had variable concentration-dependent capacity to modulate the GM-CSF activity. The skin fibroblasts, which can not sustain myelopoieis *in vitro*, synthesized GAGs that could inhibit the myelopoiesis-promoting activity of GM-CSF produced by the same cells. We conclude that the quality of the stroma pericellular glycoconjugates, present in microenvironment, is determinant for the ability of a given stroma to sustain myelopoiesis, even when biologically active hemopoietins are locally produced.

The spatial organization of this microenvironment and molecular interactions among stroma cells and myeloid progenitors was investigated, and we have shown that the physical intercellular contact triggers the capping of cell surface molecules, including sialylated glycoconjugates and proteoglycans. Together, they generated the specific intercellular environment, rich in molecules potentially interactive with GM-CSF, and providing the physicochemical conditions required for this interaction.

We have partially assessed the identification of the core proteins of cell-associated HSPGs of the stromal cell lines monitoring their expression by RT-PCR. The assayed stromas expressed mRNA for glypican, syndecan-4 and betaglycan. The stromal layers treatment with PI-PLC and mild trypsin confirmed the presence of hydrophobic cell surface HSPG glypican and syndecan, respectively. About 20% of the HSPG are linked to membrane by

glycosilphosphatidylinositol (GPI) anchor while the remainder, 80%, are integrated in the plasma membrane. We have shown that the ^{35}-S labeled molecules dislodged from stroma cell membranes by mild treatment with trypsin increased the biological activity of the GM-CSF, whilst those dislodged by phospholipase-C did not. These results suggested the existence of both transmembrane HSPG and GPI-HSPG and that the biological activity of GM-CSF in FDC-P1 proliferation, *in vitro*, is increased by a physical interaction between growth factor and transmembranar HSPG.

III- INTRODUÇÃO

1. Hematopoese

A hematopoese representa uma cascata de eventos de proliferação e diferenciação celular precisamente regulados, onde uma população de células tronco totipotentes indiferenciadas da medula óssea (HSC – *hematopoietic stem cell*) pode proliferar, formando novas células totipotentes (auto-renovação), ou seguir processos de diferenciação, associados à proliferação, formando linhagens de células sangüíneas maduras **(HEYWORTH *et al.*, 1988; MORRISON *et al.*, 1996).** O fenômeno hematopoético compreende dois eventos distintos: a linfopoese e a mielopoese. A linfopoese é responsável pela diferenciação e maturação das linhagens B e T enquanto a mielopoese segue o caminho das demais linhagens hematopoéticas.

Durante o período embrionário, o primeiro sítio hematopoético pode ser localizado na parede do saco vitelino passando mais tarde a predominar no fígado e com menos intensidade no baço. A partir do desenvolvimento dos ossos longos principalmente, a hematopoese hepática e esplênica é deslocada para a medula óssea sendo este, na vida pós-embrionária, e em condições normais, o sítio de produção de células sangüíneas **(MULLER *et al.*, 1994; RICH, 1995; ZON, 1995).** Em algumas condições patológicas, na vida adulta, a hematopoese pode ser observada em sítios extramedulares, especialmente no fígado e no baço que demonstram assim reservar um potencial hematopoético. Este fenômeno é descrito como hematopoese extramedular, e pode estar associado às reações fibrogranulomatosas, como a esquistossomose mansônica **(STADECKER & WRIGHT, 1984; BOROJEVIC *et al.*, 1993).**

Experimentos *in vitro,* com populações hematopoéticas e fatores de crescimento purificados, contribuiram para o conhecimento sobre os fatores que regulariam a diferenciação hematopoética. A necessidade do estroma para a manutenção do fenômeno hematopoético sugere que as células do microambiente local produzam citocinas que permitam a auto-renovação e sobrevivência das células totipotentes e sua diferenciação terminal nas populações sangüíneas **(ARAI *et al.*, 1990)**.

2. Fatores de Crescimento

Glicoproteinas hematopoéticas regulatórias, conhecidas como fatores de crescimento são moléculas que podem ou não estar associadas a células e/ou matriz extracelular do microambiente em questão, exercendo seu papel biológico através de receptores específicos expressos nas superfícies das células alvo. Grande parte dessas moléculas apresenta um comportamento pleiotrópico e redundante. O pleiotropismo faz com que esses fatores se comportem multifuncionalmente podendo agir sobre diferentes tipos celulares apresentando, no entanto, efeitos biológicos distintos. Sua forma redundante permite que diferentes fatores atuem sobre o mesmo tipo celular, sinalizando efeitos semelhantes. Essa diversidade de comportamento, levando-se também em consideração sua capacidade de atuação sinergística ou antagonística, permite uma regulação coordenada e interativa da atividade celular **(KISHIMOTO *et al.*, 1992).**

A base molecular da organização dos receptores para os fatores de crescimento pode justificar seu mecanismo de ação. Os receptores, na maioria, são subunidades protéicas que interagem especificamente com o fator de crescimento, dando origem a um sinal molecular que alterará o padrão de expressão gênica da célula alvo. Alguns receptores compartilham domínios comuns na sua estrutura. Outras vezes, os receptores compartilham o mesmo mensageiro para a transdução do sinal, fazendo com que esses fatores exerçam efeitos semelhantes em diferentes tecidos **(KISHIMOTO *et al.*, 1994).**

Fatores como interleucina-3 (IL-3), interleucina-5 (IL-5) e fator estimulador de colônias de macrófagos-granulócitos (GM-CSF); produzidos por linfócitos T ativados e mastócitos, são responsáveis pela estimulação de várias linhagens hematopoéticas, e merecem particular atenção. Esses fatores apesar de não apresentarem qualquer homologia significativa nas suas seqüências de aminoácidos, exibem estruturas terciárias análogas, com um arranjo espacial formado por quatro alfa hélices e a transdução de seu sinal implica na fosforilação de moléculas análogas. Desta forma, a IL-3 e o GM-CSF podem competir com a IL-5 pela ligação com seu receptor, o que sugere que esses fatores possam compartilhar o mesmo receptor **(LOPEZ *et al.*, 1991).** Os receptores dessas citocinas são compostos por duas subunidades: as cadeias α e β. A cadeia α é específica para cada receptor enquanto a cadeia β, promíscua, é compartilhada por todos os receptores, sendo os domínios citoplasmáticos das duas subunidades

fundamentais para a transdução de sinal **(MIYAJIMA *et al.*, 1993; WOODCOCK *et al.*, 1996).** Ocorre assim, a interação do fator com as duas subunidades do receptor formando um complexo dimerizado que culmina com a sinalização.

Os requerimentos para a hematopoese são fornecidos pelas células do estroma medular e sua matriz extracelular que formam microambientes indutivos **(TRENTIN, 1978; DEXTER & TESTA, 1980).** O microambiente contém todos os elementos necessários para a sobrevivência, proliferação e diferenciação das células hematopoéticas. Entretanto, os mecanismos moleculares envolvidos na comunicação de células do estroma com células hematopoéticas são em grande parte desconhecidos.

3. Estroma Hematopoético

DEXTER e colaboradores (1977) , desenvolveram, *in vitro*, um modelo de cultivo de células hematopoéticas murinas de longa duração e obsevaram que a manutenção das populações hematopoéticas mais primitivas, bem como os eventos de proliferação e diferenciação celular, exigiam a formação de uma camada de células aderentes representativas: o estroma hematopoético. Na ausência do contato, precursores pluripotentes/estroma hematopoético, os precursores não teriam acesso aos sinais reguladores expressos pelas células do estroma **(BENTLEY &TRALKA, 1983).**

Inúmeros grupos de pesquisa têm demonstrado, ao longo dos anos, a importância da presença e organização das células do estroma de medula óssea no fenômeno de hematopoese **(GONG, 1978; LORD, 1990).** Investigações em animais e humanos, têm revelado que o contato das células estromais de medula óssea (fibroblastos reticulares, macrófagos, adipócitos, osteoblastos e células endoteliais) com as células sangüíneas e a produção de fatores solúveis **(HORUK & PEIPER, 1996; ALEXANDER, 1998; WHETTON & SPONCER, 1998)** bem como de elementos da matriz extracelular (MEC), **(SAELAND *et al.*, 1992; GUPTA *et al.*, 1998)** são pré-requisitos indispensáveis para a manutenção local das células hematopoéticas primitivas e no controle da diferenciação das linhagens específicas **(DEXTER *et al.*, 1990; OHTSUKI *et al.*, 1992).** Essa MEC, elaborada pelo estroma, é constituida por diversas proteínas estruturais e polissacarídeos, incluindo os colágenos, glicoproteínas não colagênicas, elastina,

proteoglicanos (PGs) e glicosaminoglicanos (GAGs) **(SCOTT, 1992; SCHUPPAN & RÜHL, 1994).** As células do estroma hematopoético depositam no meio extracelular colágeno tipo I e III **(BENTLEY *et al.*, 1984),** fibronectina, laminina **(CAMPBEL *et al*, 1985)**, trombospondina **(LONG & DIXIT, 1990),** hemonectina **(PETERS *et al.*, 1990),** ácido hialurônico e PGs **(KEATING & GORDON, 1988; SICZOWSKY *et al*, 1993).**

Apesar da diversidade desses componentes, a MEC é um sistema dinâmico e interativo, modulada em função do comportamento das células e de moléculas mensageiras. Esse repertório específico de moléculas secretadas para o meio extracelular ou associadas às superficies celulares, criam nichos específicos dentro do microambiente da medula óssea permitindo a regulação do fenômeno hematopoético.

4. Glicosaminoglicanos –GAGs

Os GAGs são heteropolissacarídeos carregados negativamente, com diferentes graus de complexidade, presentes na MEC e superficies celulares. As cadeias lineares de GAGs são constituidas por unidades dissacarídicas repetitivas onde está presente uma unidade de ácido hexurônico que pode ser o ácido idurônico ou ácido glicurônico e, invariavelmente, uma hexosamina, como a D-glicosamina ou D-galactosamina. O queratam sulfato (QS) pode ser considerado uma exceção pois o resíduo de ácido hexurônico é substituido por uma galactose. As sequências dissacarídicas podem apresentar substituições de grupamentos sulfato em várias posições **(CARNEY & MUIR, 1988).** De acordo com o tipo de unidade dissacarídica apresentada pelos GAGs, eles podem ser classificados como: ácido hialurônico (AH), condroitim sulfato (CS), dermatam sulfato (DS), queratam sulfato (QS) , heparina (Hep) e heparam sulfato (HS) (Tabela 1).

4.1.Ácido Hialurônico (AH)

O AH é o único GAG que não apresenta resíduos sulfatados, sendo constituído exclusivamente por dissacarídeos N-acetilados. Este polímero de alto peso molecular, não está

associado covalentemente as cadeias protéicas **(PREHM, 1988a)** sendo abundante nos tecidos conjuntivos onde associa-se aos PGs formando agregados de alto peso molecular **(NAOR *et al.*, 1997).** Está relacionado com os processos de proliferação, migração e reconhecimento celular **(KNUDSON *et al.*, 1984; LAURENT & FRASER, 1986; TURLEY *et al.*, 1990).**

Tabela 1: Estrutura dos dissacarídeos mais freqüentes encontrados nas diferentes classes de glicosaminoglicanos **(JEANLOZ, 1960) .**

GAG	Ácido Urônico	L.I.	Hexosamina	L.I.	Éster de Sulfato
AH	D- glicurônico	β (1-3)	N-acetil glicosamina	β (1-4)	-
C 0-S	D- glicurônico	β (1-3)	N-acetil galactosamina	β (1-4)	-
C 4-S	D- glicurônico	β (1-3)	N-acetil galactosamina	β (1-4)	C-4 (HX)
C 6-S	D- glicurônico	β (1-3)	N-acetil galactosamina	β (1-4)	C-6 (HX)
DS	L- idurônico	α (1-3)	N-acetil galactosamina	β (1-4)	C-4 (HX)
QS	D- galactose*	β (1-4)	N-acetil glicosamina	β (1-3)	C-6 (HX) [C-6(Gal)]
HS	D- glicurônico	β (1-4)	N-acetil glicosamina	α (1-4)	C-6 (HX) C-2 (HX) C-2,6 (HX)
Hep	L- idurônico	α (1-4)	glicosamina	α (1-4)	C-2,6 (HX) C-2 (AU)

AH (ácido hialurônico); C 0-S (condroitim 0-S); C 4-S (condroitim 4-S); C 6-S (condroitim 6-S); DS (dermatam sulfato); QS (queratam sulfato); HS (heparam sulfato); Hep (heparina).
L.I (ligação intra-dissacarídica), * D-galactose substitui o ácido urônico

4.2. Condroitim Sulfato (CS) e Dermatam Sulfato (DS)

CS e DS são considerados membros de uma mesma família de GAGs por apresentarem em sua estrutura unidades dissacarídicas contendo uma N-acetilgalactosamina associada ao ácido glicurônico (no caso do CS) ou, ao ácido idurônico (no caso do DS). A N-acetil galactosamina está sulfatada nas posições 4- ou 6- do CS, originando o condroitim 4- e 6-sulfato respectivamente. Não é comum encontrar todos os resíduos de hexosamina sulfatados na mesma posição; geralmente é encontrada uma estrutura híbrida, com grupos 4- e 6-sulfato presentes na mesma molécula, porém em diferentes resíduos da galactosamina **(SENNO *et al.*, 1975; MICHELACCI & DIETRICH, 1976; FAITYNEK & SILBERT, 1978; MICHELACCI *et al.*, 1979).**

O DS é uma forma isomérica do condroitim 4-sulfato o qual apresenta ácido idurônico, no lugar do ácido glicurônico, em grande parte de suas unidades dissacarídicas **(KOLSET & GALLAGHER, 1990)**. O padrão de sulfatação desses GAGs apresenta uma grande complexidade. Apesar de pouco freqüente, as unidades dissacarídicas podem apresentar uma sulfatação extra, originando unidades dissacarídicas dissulfatadas. A N-acetilgalactosamina do CS pode, ao mesmo tempo, ser sulfatada nas posições 4 e 6 enquanto que um sítio extra de sulfatação pode ocorrer na posição 2 do ácido idurônico do DS.

4.3. Queratam Sulfato (QS)

As cadeias de QS apresentam unidades dissacarídicas formadas por N-acetilglicosamina e galactose. As unidades monosulfatadas apresentam sulfatação na posição 6 da N-acetilglicosamina enquanto as unidades disulfatadas possuem sulfatação extra na posição 6 da galactose. As cadeias de QS podem ser tanto O- quanto N- ligadas à cadeia protéica dos PGs, apresentando como sítio de ligação um resíduo de serina ou de asparagina, respectivamente. **(FUNDERBURGH *et al.*, 1996).**

4.4. Heparam Sulfato (HS) e Heparina (Hep)

No HS e Hep é encontrada a glicosamina, que pode ser O-sulfatada na posição 6, para ambas e preferencialmente N-sulfatada na posição 2 da Hep. A Hep apresenta principalmente ácido idurônico, com quantidades variáveis de O-sulfatação na posição 2, e o HS apresenta proporções mais elevadas de ácido glicurônico, geralmente, não sulfatado.

GALLAGHER & WALKER (1985), admitem que todos os HS possuem aproximadamente 50% de seus resíduos N-sulfatados e que a razão de O-sulfatação para N-sulfatação deve ser menor ou igual a 1. Por outro lado, **LANE &LINDAHL (1989),** sugerem que aqueles compostos que possuam mais do que 80% dos seus resíduos de glicosamina N-sulfatados devem ser considerados como Hep desde que o número de resíduos O-sulfatados seja maior do que o número de N-sulfatados. Essas diferenças estruturais têm sido, ao longo dos últimos anos, bastante discutidas entre diferentes grupos de pesquisa, sendo uma análise mais complexa realizada pelo grupo de **NADER & DIETRICH (1989)**. Levando em consideração uma combinação de propriedades físico-químicas e biológicas dessas moléculas, puderam determinar diferenças entre HS e Hep. Toda Hep deve ser precipitada com acetato de potássio 2M, pH 5.7 a 4^0C. A migração eletroforética em gel de agarose deve ser similar ao padrão de Hep. Além disso, deve apresentar peso molecular variando entre 10.000 a 68.000 Da e ainda formar múltiplas bandas em gel de poliacrilamida de focalização isoelétrica. A razão molar de glicosamina/ácido hexurônico/sulfato deve ser compatível com a dos padrões de Hep, bem como ser suscetível ao tratamento com heparina liase I, uma enzima com grande atividade sobre heparinas. Por outro lado, HS é solúvel em acetato de potássio 2M, pH 5.7 a 4^0C, não forma várias bandas em gel de poliacrilamida de focalização isoelétrica e a razão de sulfato/glicosamina deve estar entre 0.8-1.8 além de ser pouco degradado pela heparina liase I.

Hep, em mamíferos, está presente nos grânulos citoplasmáticos de mastócitos do tecido conjuntivo e também em alguns tecidos de invertebrados. O HS tem uma distribuição mais ampla no reino animal, sendo encontrado na maioria das células e tecidos de mamíferos bem como em vertebrados inferiores e invertebrados **(NADER *et al.*, 1988)**. O HS tem sido relacionado com fenômenos de reconhecimento celular **(DIETRICH *et al.*, 1979; DIETRICH, 1984)** , adesão celular **(HOOK *et al.*,1984; HANGOG, *et al.*, 1993; COOMBE, *et al.*, 1994)**

e regulação do acesso de fatores de crescimento **(CARVALHO *et al.* ,2000; MODROWSKY *et al.*, 2000).**

5. Proteoglicanos (PGs)

Os GAGs, com exceção do AH, ocorrem nos tecidos covalentemente ligados a um esqueleto protéico (Fig. 1A) constituindo uma classe de moléculas conhecidas como PGs **(PREHM, 1988b).** A ligação ao esqueleto protéico, com exceção do QS, é mediada por uma unidade tetrassacarídica constituida de ácido glicurônico-galactosil-galactosil-xilose (GlcUA-Gal-Gal-Xil), onde a extremidade redutora (Xil) une-se com a hidroxila de um resíduo de serina da proteína, por uma ligação O-glicosídica. A extremidade não redutora (GluUA) dá continuidade à cadeia do GAG (Fig. 1B).

Os PGs são estruturas que revelam grande heterogeneidade em relação ao tamanho de seu esqueleto protéico e ao número e tipo de cadeias de GAGs. Essa variabilidade afeta, sensivelmente suas propriedades e funções biológicas, sendo capazes de interagir com várias moléculas, o que confere a estes compostos a participação em diversos mecanismos celulares **(IOZZO, 1998).** A interação dos PGs com outras moléculas pode ser mediada pela cadeia de GAG e/ou pelo esqueleto protéico. A ligação do PG às proteíinas é modificada pelo tipo, número, grau de sulfatação e tamanho das cadeias de GAGs, além de características da própria porção protéica.

Os PGs estão amplamente distribuídos nos tecidos de animais vertebrados e invertebrados **(GOMES & DIETRICH, 1982; NADER *et al.*, 1984)** e, de acordo com sua localização, podem ser classificados como intracelulares, pericelulares (os PGs de superfície) e extracelulares (aqueles secretados para o meio extracelular).

5.1. PGs de Grânulos Secretórios

Os PGs intracelulares são armazenados em grânulos secretórios sendo o serglicim o membro melhor caracterizado desta família. O serglicim possui no seu esqueleto protéico uma grande quantidade de resíduos de serina e glicina alternados, sendo

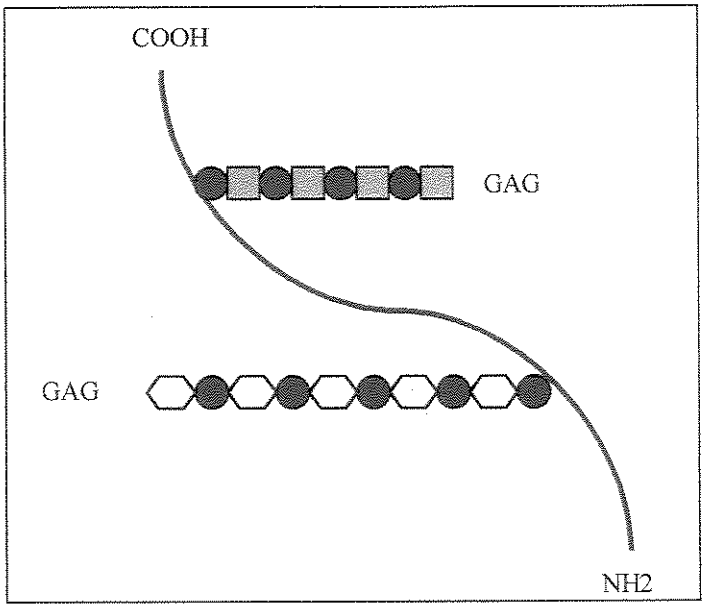

Figura 1A: Representação esquemática de uma molécula de proteoglicano. Núcleo proteico em azul associado a uma ou mais cadeias laterais de glicosaminoglicanos.

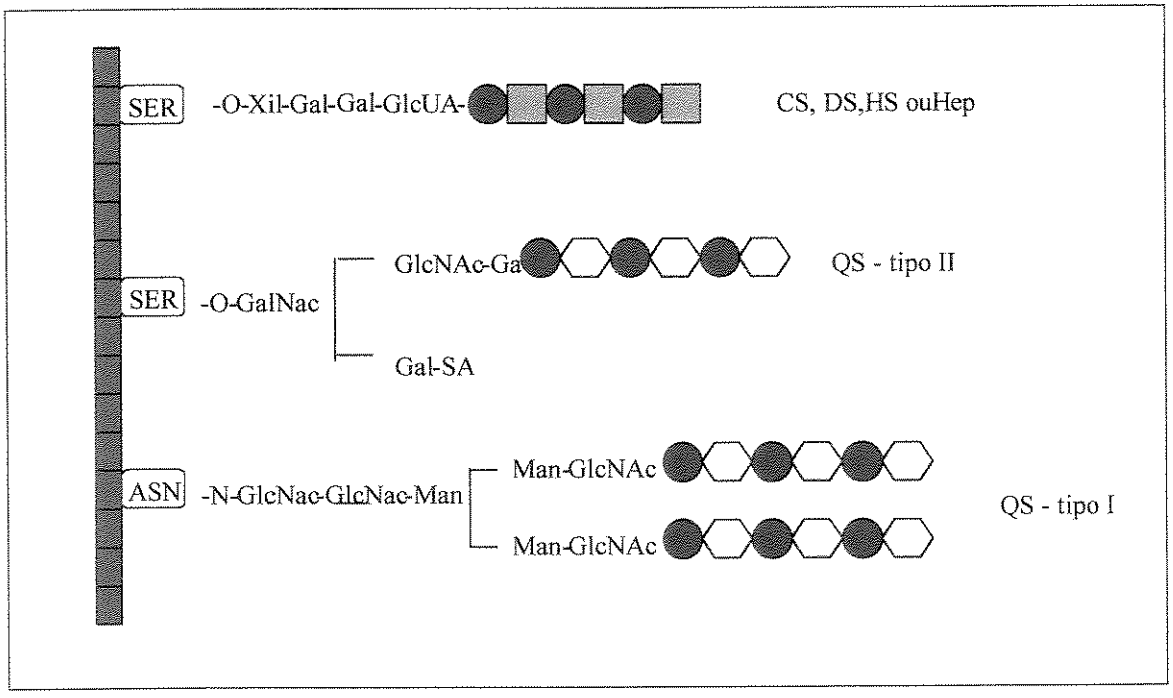

Figura 1B:.PROTEOGLICANO: REGIÕES DE LIGAÇÃO ENTRE AS CADEIAS DE GAGs E O NÚCLEO PROTEICO. GlcUA=ácido D- glicurônico; GalNAc=N-acetil-D-galactosamina; GlcNAc=N-acetil-D-glicosamina; AS=ácidosiálico; Man=D-manose; xil=D-xilose; SER=L-serina; ASN=L-asparagina

● =hexosamina ■ = ácidourônico ⬡ =D-galactose

Tersariol, 1995

Figura 1

constituido por cadeias de GAGs que podem ser heparina ou CS, frequentemente bastante sulfatado. Este PG é armazenado em grânulos secretórios de mastócitos, basófilos, eosinófilos, plaquetas e células "natural killer" (**KOLSET & GALLAGHER, 1990**). Pouco se conhece à respeito da importância biológica destes PGs, porém, acredita-se que sua função está indiretamente relacionada aos processos imunológicos, pelo fato de interagirem com proteases, carboxipeptidases e histaminas liberadas pelas células que contém esses PGs, em reações de defesa do organismo (**NADER & DIETRICH, 1989**).

5.2. PGs de Superfície Celular

Os PGs de superfície celular, presentes na maioria das células animais, apresentam principalmente cadeias de HS em sua estrutura. Esses PGs de HS (HSPGs) estão envolvidos em processos de comunicação e interação celular, seja com outras células ou com elementos da MEC. A interação com a superfície celular pode ocorrer de duas maneiras: através de proteínas integrais da membrana, via um domínio hidrofóbico, ou através de uma âncora de glicosilfosfatidilinositol (GPI) presente na camada externa da membrana plasmática (**KJÉLLEN *et al.*, 1981; DAVID *et al.*, 1990**).

a) PGs Transmembranares

Os PGs transmembranares costituem, principalmente , a família dos sindecans que possuem um segmento transmembranar e um pequeno domínio citoplasmático, ambos muito conservados. Estão presentes em diferentes espécies e se localizam na superfície das células epiteliais, endoteliais, nervosas, fibroblastos, adipócitos e musculatura lisa vascular (**BERNFIELD *et al.*, 1992**). Devido a grande homologia de suas sequências protéicas, principalmente nos domínios citoplasmáticos e transmembrânicos, são agrupados e diferenciados da seguinte maneira: sindecam 1 (o primeiro HSPG sequenciado) seguido pelos, sindecam 2 (fibroglicam), sindecam 3 (N-sindecam) e sindecam 4 (ruidocam, anfiglicam). O esqueleto protéico varia de 22 à 40 kDa ao qual estão associadas duas ou três cadeias da GAGs. Sindecans 1 e 3 apresentam cadeias de CS e HS, enquanto os sindecans 2 e 4 contêm somente cadeias de HS. (**BERNFIELD *et al.*, 1992**). Os membros da família dos sindecans são expressos em

estágios particulares do desenvolvimento embrionário. Nos tecidos adultos, o sidecam 1 está presente principalmente em células epiteliais, sidecam 2 é característico de células de origem mesenquimal, sindecan 3 é expresso em células do tecido nervoso e sindecam 4, ao contrário dos demais, é bastante comum e aparece na superfície de quase todos os tipos celulares **(TUMOVA & COUCHMAN, 2000).**

Esta família de PGs têm como característica, a interação com uma série de proteínas-sinal, como fatores de crescimento, citocinas, inibidores de proteases, enzimas e proteínas extracelulares, sendo assim estão envolvidos em funções como adesão celular, crescimento, divisão celular e controle de vias de degradação de proteínas e lipídios **(BERNFIELD *et al.*, 1992; DIETRICH, 1984; DAVID, 1993)**

Os sindecans se ligam aos colágenos dos tipos I, II, III, IV e V **(ELENIUS *et al.*, 1990; SAN ANTÔNIO *et al.*, 1994),** fibronectina **(SAUNDERS & BERNFIELD, 1998),** trombospondina, tenascina e laminina **(SALMIVIRTA *et al.*, 1991, 1992 e 1994),** além de se ligarem às moléculas que estão associadas ao crescimento celular como o fator básico de crescimento para fibroblasto (bFGF), fator de crescimento para endotélio vascular (VEGF), fator de crescimento para hepatócito (HGF) e citocinas **(MITSIADIS *et al.*, 1995; NELSON *et al.*, 1995; TAIPALE, 1997; FAHAM *et al.*, 1998).**

Outros PGs de superfície celular, que não possuem homologia entre si ou com os membros da família dos sindecans, são: betaglicam, trombomodulina, receptor de transferrina, cadeia invariante, neuroglican e CD44. CD44 teve sua estrutura determinada pelo processo de fragmentação alternativo ("splicing") das mais de dez regiões codificantes diferentes presentes na porção do gene que codifica o domínio extracelular, levando a uma glicosilação diferencial com oligossacarídeos, além das possíveis substituições com cadeias de GAGs **(JACKSON *et al.*, 1995; NAOR *et al.*, 1997).** Geralmente possui cadeias de CS, no entanto, dependendo da célula que o sintetiza pode apresentar cadeias de HS, como no caso dos queratinócitos, **(TANAKA *et al.*, 1993)** ou ainda QS em linhagens de células de carcinoma de cólon **(TAKAHASHI *et al*, 1996).** O principal ligante de CD44 é o AH, que desempenha importante papel no ancoramento das células à MEC **(LESLEY *et al.*, 1994)**, porém ele também pode participar de eventos como a ativação de linfócitos **(HAYNES *et al.*, 1989)** , adesão de linfócitos às vênulas de linfonodos **(JALKANEN *et al.*, 1987)** , a interação célula/célula **(St. JOHN *et al.*, 1990),** regulação da migração celular **(THOMAS *et al.*, 1992),** internalização e degradação de ácido hialurônico

(HUA *et al.*, 1993), desenvolvimento de tumor in vivo **(BARTOLAZZI *et al.*, 1994)** e metástase de tumores **(HOFMANN *et al.*, 1991; SANDERSON, 2001).**

O betaglicam (T beta RIII) é um exemplo de PG que atua como co-receptor para o fator de crescimento tansformante β (TGF-β) **(GEBKEN *et al.*, 2000, BLOBE *et al.*, 2001).** Este PG pode ser híbrido, ou seja, pode conter cadeias de CS e/ou HS podendo ainda ser secretado para o meio extracelular (forma solúvel do T beta RIII) **(ANDRES *et al.*, 1989, ZHANG *et al.*, 2001).** Neste modelo a cadeia protéica parece ser determinante na apresentação do TGF-β aos outros receptores, formando um complexo ternário de alta afinidade, não envolvendo diretamente as cadeias de GAGs **(LÓPEZ-CASILLAS *et al.*, 1994).**

b) PGs Associados à Membrana por Glicosilfosfatidilinositol

Os PGs podem estar ancorados covalentemente à membrana via âncora de glicosilfosfatidilinositol (GPI) **(CAREY & STAHAL, 1990).** Os membros desta família, cerebroglicam, OCI-5 e glipicam (seu representante mais estudado) têm associado como principal GAG, o HS. O cerebroglicam é encontrado freqüentemente em tecido nervoso **(STIPP *et al.*, 1994)**, o OCI-5 em tecido intestinal em desenvolvimento **(FILMUS *et al.*, 1995)** e o glipicam é expresso em diferentes linhagens celulares de origem fibroblástica e epitelial **(COUCHMAN & WOODS, 1993).**

A possibilidade da existência de uma população distinta de HSPGs que não seriam transmembranares nem associados perifericamente, foi reconhecida na década passada (**ISHIHARA *et al.*, 1987; CAREY & EVANS, 1989).** Esses experimentos inicais demonstraram que uma fração de HSPGs de superfície celular podia ser obtida após tratamento da monocamada celular com fosfolipases específicas para fosfatidilinositol.

Seis membros da família dos glipicans já foram identificados em mamíferos e apresentam de dois a cinco sítios de ligação com cadeias de HS próximo a extremidade C-terminal. Apresentam um grande domínio extracelular que inclui uma região globular com catorze resíduos de cisteína que formam pontes dissulfeto intramoleculares, conferindo aos glipicans uma estrutura tridimensional bastante conservada. Embora as abordagens funcionais sobre os glipicans ainda estejam se iniciando, é razoável especular, a partir da presença das cadeias de GAGs e da estrutura conservada do esqueleto protéico, que essas moléculas possam executar múltiplas funções celulares **(IOZZO, 2000).** Foi recentemente demonstrada uma super

expressão de glipicans em cânceres pancreáticos humanos e sugerida sua participação na resposta das células tumorais a determinados estímulos mitogênicos **(KLEEF et al., 1998)** . Grande parte dos estudos da participação dos HSPGs na resposta celular a fatores de crescimento, não identificaram o tipo de HSPG envolvido nessas interações. No entanto, existe alguma indicação de que, ao menos em alguns tipos celulares, os glipicans se associam ao fator de crescimento fibroblástico (FGF-β) e possam estimular a sinalização desses fatores de crescimento *in vitro*. **(BRUNNER et al., 1994; STEINFIELD *et al.*, 1996; KLEEF *et al.*, 1998).** Vale a pena ressaltar que nem sempre os glipicans tem um efeito estimulatório sobre a atividade dos fatores de crescimento. Já foi demonstrado que a presença do glipicam1 pode inibir a ativação do fator de crescimento do queratinócito (KGF), *in vitro*, em mioblastos de ratos **(BONNEH-BARKAY *et al.*, 1996).**

5.3. PGs de MEC

Os PGs secretados para a MEC, por diferentes tipos celulares, podem ser divididos em três grupos: os PGs de membrana basal, os hialectanos e os pequenos PGs ricos em leucina **(IOZZO, 1994)**.

a) PGs de Membrana Basal

Existem três PGs característicos de membranas basais de mamíferos: o perlecam **(NOONAN *et al.*, 1991),** agrim, um PG de HS presente nas membranas basais de junções neuromusculares **(RUEGG, 1996),** e o bacamam que apresenta cadeias de CS e está associado à estabilidade da membrana basal **(COUCHMAN & WOODS, 1993).**

O perlecam é o membro mais bem caracterizado, possuindo três cadeias de GAGs que podem ser de HS ou CS, dependendo, aparentemente, da especificidade celular **(KOKENYESI & SILBERT, 1995; GROFFEN *et al.*, 1996; DOLAN *et al.*, 1997; COSTELL *et al.*, 1997).** Muitas evidências apontam para uma possível participação do perlecam na diferenciação celular e morfogênese tecidual, sugerindo um importante desempenho na embriogênese. Um estudo sistemático da embriogênese murina demonstrou que a expressão de perlecan acontece em estágios primordiais de vasculogênese, como no coração primordial e nos principais vasos sangüíneos **(HANDLER *et al.*, 1997).** Neste mesmo estudo foi observado o acúmulo deste PG em tecidos mesenquimais, principalmente cartilagem em processo de

calcificação, onde persiste através dos vários estágios de desenvolvimento e na idade adulta. A expressão de perlecam está relacionada com o amadurecimento dos tecidos e é sempre proeminente na membrana basal das células endoteliais de todos os orgãos vascularizados, particularmente o fígado, pulmão, baço, pâncreas e rins **(IOZZO, 1998).**

A ditribuição de FGF-β em membranas basais de embriões de camundongos parece estar relacionada com a presença do perlecam **(HANDLER *et al.*, 1997; FRIEDL *et al.*, 1997).** Este PG pode atuar como regulador da sinalização do FGF-β e limitar o acesso do fator de crescimento às células alvo subjacentes **(FRIEDL *et al.*, 1997).** Função similar pode ser observada com outros fatores, tais como várias isoformas de TGF-β **(LYON *et al.*, 1997)** e o fator plaquetário 4 **(STRINGER & GALLGHER, 1997)** que se ligam às seqüências específicas de HS. Outra função conhecida do perlecam é a sua atuação como regulador da permeabilidade da membrana basal glomerular. Vários estudos comprovaram que a remoção das cadeias de HS aumenta a permeabilidade glomerular às proteínas levando à proteinúria **(KANWAR *et al.*, 1980)** A injeção de anticorpos monoclonais contra HS tem efeito nefritogênico podendo induzir uma proteinúria seletiva devido a uma neutralização dos sítios aniônicos do HS **(VAN DEN BORN *et al.*, 1992).** Além disto, alterações nos padrões das cadeias protéicas e/ou na estrutura do HS têm sido encontradas em várias glomerulopatias **(VAN DEN BORN *et al.*, 1993).**

b) Hialectanos

Os hialectanos são assim denominados por serem PGs de MEC capazes de interagir com AH e lectinas. Os quatro membros desta família, versicam, agrecam, neurocam e brevicam, possuem em sua estrutura três domínios bem definidos. O domínio N-terminal se liga ao AH, o domínio central apresenta as cadeias de GAGs geralmente de CS e/ou QS) e o domínio C-terminal que se liga a lectina, permitindo que essas moléculas executem associações entre a superfície celular e componentes da MEC **(IOZZO, 1998).**

Versicam é expresso em tecidos embrionários, que atuam como barreira para a migração de células da crista neural e parece estar envolvido na formação de lesões ateroscleróticas, sendo identificados nas camadas elástica interna e média dessas lesões **(WIGHT *et al.*, 1992).** Nas artérias normais a presença de versicam limita-se à túnica adventícia **(BODELESNIEWSKA *et al.*, 1996).**

As principais funções do agrecam estão associadas a duas de suas características estruturais: altas concentrações de cadeias laterais de CS e a formação de agregados supramoleculares com ácido hialurônico **(ROUGHLEY & LEEF, 1994).** Na cartilagem, cada monômero de agrecam ocupa um grande volume hidrodinâmico e quando submetida à forças compressivas, a água é deslocada de cada monômero. Quando essa compressão é suspensa, o tecido é reidratando **(IOOZO, 1998).**

O aspecto funcional dos PGs cerebrais são menos conhecidos. Neurocam associa-se, com grande afinidade, às moléculas de adesão celular NG-CAM e N-CAM, inibindo as interações homofílicas e bloqueando o processo de extensão de neuritos **(GRUMET *et al.*, 1993; FRIEDLANDER *et al.*, 1994).** A expressão de neurocam é regulada durante todo o desenvolvimento **(ENGEL *et al.*, 1996, MEYER-PUTTLITZ *et al.*, 1995)** sendo os astrócitos e neurônios responsáveis por sua expressão no cérebro **(WATANABE, *et al.*, 1995).** Estudos imunoquímicos e de hibridização *in sito* demonstram a presença de neurocam no cérebro pré- e pós- natal, mas não em outros orgãos **(MARGOLIS & MARGOLIS, 1994).**

O tipo de hialectano mais abundante no cérebro adulto é o brevicam. Dois tipos de moléculas são encontrados, uma de 145kDa e outra de 80kDa, representando a molécula inteira, e o brevicam na sua forma N- terminal truncada, respectivamente. A forma truncada torna-se mais freqüente em estágios mais tardios do desenvolvimento, provavelmente originando-se do processamento proteolítico da molécula original **(YAMAGUCHI, 1996).**

c) PGs Ricos em Leucina

A família dos PGs ricos em leucina apresentam no domínio central da cadeia protéica, sequências repetitivas, ricas em leucina. As cadeias de GAGs são encontradas na região amino-terminal da molécula **(IOZZO, 1998).** Os membros desta família foram caracterizados, de acordo com sua organização genômica e protéica, em três classes. A classe I representa os PGs decorim e biglicam que apresentam cadeias de CS e/ou DS. A classe II é formada, principalmente, por PGs que apresentam cadeias de QS como a fibromodulim, o lumicam, o queratocam, e a osteoaderina. A classe III engloba o epificam e osteoglicina, PGs que apresentam cadeias de DS/CS e QS, respectivamente **(IOZZO, 1998).**

Esta família de PGs teve sua importância reconhecida, no controle da fibrilogênese do colágeno, após a descoberta de que PGs de DS interagem com o colágeno,

aumentando a estabilidade das fibrilas e alterando sua solubilidade **(TOOLE & LOWTHER, 1968; TOOLE, 1969).** A partir daí, foram demonstradas interações específicas de vários membros desta família com as fibrilas colagênicas **(SCOTT, 1998 e 1991)** sendo a cadeia protéica o principal elemento envolvido **(VOGEL *et al.*, 1987; VOGEL & TROTTER, 1987).**

O decorim é um representante desta família que se destaca não só pela interação com o colágeno mas também por apresentar grande afinidade pelo TGF-β. Em condições patológicas como a cirrose hepática, fibrose pulmonar ou esclerose glomerular, ocorre um aumento significante na expressão desse fator. As isoformas TGF-β1, TGF-β2 e TGF-β3, ligam-se à cadeia protéica do decorim permitindo um estoque de fatores de crescimento no meio extracelular **(HILDEBRAND *et al.*, 1994)** .

O biglicam apesar de ser incapaz de estimular o crescimento de celulas mielóides, proporciona o aumento em número de células que são responsivas a IL-7 **(ORITANI & KINKADE, 1996).**

6. O Papel dos PGs, GAGs e Fatores de Crescimento no Ambiente Hematopoético

A importância dos PGs na hematopoese foi primeiramente demonstrada, de maneira indireta, por **SPONCER *et al*, 1983.** *In vitro,* demonstraram que após a adição de beta D-xilosídeos em culturas de medula óssea, havia um aumento no número das células tronco pluripotentes precursoras e também de células maduras. Esse incremento na hematopoese mostrou ser dose dependente, atingindo seu máximo na faixa de $5x10^{-4}M$ do xilosídeo. O xilosídeo atua como "promotor" artificial para a síntese da cadeia de GAG, que pode ser alongado na ausência da parte protéica da molécula. As cadeias de GAGs são secretadas em grandes quantidades sob a forma solúvel. Caso a síntese de PGs seja alterada na presença de xilosídeos, as interações célula-microambiente, nas quais os PGs parecem desempenhar um papel importante na regulação da hematopoese, também serão alteradas. **OGURI et al, 1987,** demonstraram que aproximadamente 79% do conteúdo de GAGs da medula óssea consiste de CS, localizado exclusivamente na MEC, 16% de AH e 5% de HS, sendo posteriormente confirmado por **BENTLEY *et al*, 1988, 1990.**

Existem na literatura poucos dados à respeito dos tipos de PGs e características estruturais dessas moléculas, sintetizados pelas linhagens celulares hematopoéticas **(KIRBY & BENTLEY, 1987; MINGUELL & TAVASSOLI, 1989; MORIS *et al.*, 1991; SHIROTA *et al.*, 1992).** No entanto, **DRZENIEK *et al.*, 1997 & SCHOFIELD, *et al.*, 1999,** identificaram, recentemente, membros dos dois principais grupos de PGs transmembranares, que contém cadeias de HS, presentes em estromas hematopoéticos de medula óssea murina e humana, respectivamente. Através de métodos imunoquímicos, usando anticorpos específicos contra HSPGs, demonstraram a síntese de sindecam, betaglicam, glipicam e perlecam por esses estromas.

Ao longo das três últimas décadas têm sido proposto o envolvimento dos PGs na interação das células progenitoras hematopoéticas com as células estromais de medula óssea. As observações de que fatores de crescimento, como IL-3 e/ou GM-CSF, podiam se ligar aos HSPGs das células estromais; conduziam à possibilidade de que esses fatores pudessem ser apresentados às células hematopoéticas na sua forma biologicamente ativa **(GORDON *et al.*; 1987; ROBERTS *et al.*, 1988; GORDON, 1991).** A importância do HS, presente no estroma hematopoético humano, na manutenção de precursores hematopoéticos, mediada por citocinas foi demonstrada por **GUPTA *et al*, 1996**.

Os mecanismos propostos para a modulação dos fatores de crescimento pelos HSPGs seriam: a) a proteção das citocinas da ação de proteases específicas, como no caso do FGF **(SAKSELA *et al.*, 1988)** e interferon-γ **(LORTAT-JACOB *et al.*, 1996);** b) a liberação dos fatores, a partir de complexos inativos, numa forma biologicamente ativa , como observado com o TGF-β **(McCAFFREY, 1992);** c) formação de complexos ternários de alta afinidade com o receptor celular correspondente, como observado na interação de GAGs com o FGF **(YAON, *et al.*, 1991; KAN *et al.*, 1993).**

Os FGFs (fatores de crescimento fibroblásticos) podem se ligar a heparina e HS que por sua vez modulam a atividade dos FGFs, às vezes potencializando um estímulo, outras, inibindo-o **(CONRAD, 1998).** Desta forma, a atividade biológica mitogênica do FGF-β (membro desta família mais bem caracterizado) está associada a sua interação com um PG de HS e seu receptor de alta afinidade situado na superfície da célula alvo, levando assim a formação de um complexo ternário. Tanto o FGF-β quanto o seu receptor celular apresentam domínios proteicos ricos em resíduos de aminoácidos básicos, necessários à interação com o HS/Hep. As

seqüências ligante da Hep com os FGFs já foram parcialmente caracterizadas. Seqüências de seis à oito resíduos de açúcar são suficientes para ligar o FGF isolado, no entanto para a formação de um complexo com o receptor celular, a fim de elicitar o sinal mitótico, são necessários deca, ou dodecassacarídeos **(YAYON *et al*, 1991; GAMBARINI *et al.*, 1996; FAHAM *et al.*1996; MOHAMMADI *et al*, 1996).**

A modulação da atividade mitogênica de GM-CSF bem como a de outros fatores de crescimento mielopoéticos (interleucinas –3 e -5) que atuam através da mesma família de receptores, demonstraram ser mediadas pela Hep ou HS, ambos presentes na medula óssea e no compartimento mielopoético extramedular **(GALLAGHER *et al.*, 1988; ALVAREZ-SILVA *et al*, 1993; ALVAREZ-SILVA *et al*, 1996; MODROWSKY *et al.*, 1998; CARVALHO *et al.*, 2000)).** Hep e HS solúveis, apresentam atividades biológicas similares àquelas correspondentes a dos PGs associados ao estroma para os três fatores de crescimento já mencionados, tanto nas culturas de células hematopoéticas humanas (tipo Verfaillie) **(GUPTA *et al*, 1996)** quanto na proliferação de linhagens celulares dependentes de fatores de crescimento **(ALVAREZ-SILVA *et al.*, 1996; LIPSCOMBE *et al.*, 1998).**

Esses achados sugerem que existam, *in vivo,* interações físicas entre o fator de crescimento e o HS podendo ser detectáveis em modelos experimentais usando moléculas solúveis purificadas.

WETTREICH *et al*, 1999, demonstraram que as interações físicas entre GM-CSF e HSPG, provavelmente responsável pela modulação da atividade do GM-CSF, depende de propriedades físico-químicas do microambiente. Na forma solúvel, a agregação dessas moléculas ocorre somente em níveis ótimos de pH entre 4 e 5. Provavelmente o ambiente intercelular, incluindo as cargas negativas dos glicoconjugados pericelulares, podem promover condições que induzam uma alteração conformacional na molécula de GM-CSF possibilitando sua interação com PGs. Este fato pode contribuir para uma apresentação mais efetiva do fator de crescimento ao seu receptor de alta afinidade.

Apesar de grande parte das interações moleculares estabelecidas pelos GAGs serem geralmente de natureza iônica, em vários sistemas biológicos, muitos ligantes para HS requerem sequências de GAGs com um comprimento e uma estrutura bastante definidos **(GUIMOND, *et al.*, 1993; LORTAT-JACOB *et al.*, 1995; ASHIKARI, *et al.*, 1995; SPILLMANN, *et al.*, 1998).** Vale ressaltar que processos celulares complexos como adesão,

motilidade, proliferação e diferenciação assim como a morfogênese dependem da estrutura integral do PG e, não apenas, da cadeia de GAG **(TURNBULL *et al.*, 2001)**.

Como podemos observar, os PGs são moléculas que apresentam uma diversidade estrutural muito evidente que culmina com sua abrangente atividade biológica. Acredita-se que essas diferenças estruturais sejam responsáveis por interações altamente específicas dos GAGs com fatores de crescimento locais, os apresentando à receptores de alta afinidade, gerando um mecanismo de sinalização celular que possa influenciar os mecanismos de proliferação e diferenciação celular dentro do compartimento hematopoético.

IV- Objetivos

1. Comparar a capacidade de diferentes estromas celulares murinos em sustentar a sobrevivência e proliferação de células mielóides e determinar seus mecanismos moleculares.

2. Caracterizar quantitativa e qualitativamente os glicosaminoglicanos sintetizados e secretados para o meio de cultura e presentes nas superficies celulares, pelas linhagens estromais hematopoéticas e não hematopoéticas.

3. Investigar a participação de moléculas de heparam sulfato (HS), purificadas a partir dos estromas, na atividade biológica do GM-CSF.

4. Analisar a estrutura dissacarídica do HS presente em estromas que sustentam a proliferação da linhagem mielóide e determinar, através de RT-PCR, a expressão de mRNA para os HSPGs.

5. Investigar a participação dos deslocados contendo moléculas transmembranares, obtidos após tratamento brando com tripsina, na atividade biológica do GM-CSF.

6. Caracterizar a organização espacial do microambiente pericelular estabelecido entre as células estromais e os progenitores mielóides.

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VI- ARTIGOS

ANEXO 1

Será submetido à Journal of Cell Biochemistry

Biochemical Characterization of Heparan Sulfate Derived from Murine Haemopoietic Stromal Cell lines: a Bone Marrow-Derived Cell Line S17 and a Fetal Liver-Derived Cell Line AFT024

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Abbreviated title: Heparan sulfate produced by haemopoietic stromal cells lines

Key words: Glycosaminoglycans, heparan sulfate, glypican, syndecan, haemopoiesis,

Summary

Heparan sulfate (HS) present on the surface of haemopoietic stromal cells has important roles in the control of adhesion and growth of haemopoietic stem and progenitor cells. Recent studies have characterized several different heparan sulfate proteoglycans (HSPGs) from both human and murine bone marrow stromal cells. In the present study, we have compared the molecular structure of HS, metabolically labeled with [^{35}S]-sulfate produced by two distinct preparations of murine haemopoietic stromal cell lines. These comprised a bone marrow-derived cell line S17 and a fetal liver-derived cell line AFT024. [^{35}S]-HS was examined in the cell layers and in the culture medium. We identified and measured the relative proportions of the various glycosaminoglycans (GAGs) in the two stromal cell lines. Chondroitin sulfate (CS) was preponderantly secreted by the stromal cell lines, while HS was relatively more abundant in the cell-associated fractions. The two types of stromal cells differ in their HS composition, mainly due to different patterns of *N*- and *O*-sulfation.

The two stromal cell lines expressed mRNA for different HSPGs. Data from reverse transcription PCR revealed that the two stromal cell lines expressed mRNA for glypican and syndecan-4. Only AFT024 cell line expressed mRNA for betaglycan. There was no evidence for expression of mRNA for both syndecan-1 and syndecan-2. [^{35}S]-sulfated macromolecules could be released from the cell surface of both stromal cell lines by phosphatidylinositol phospholipase C (PI-PLC), which is consistent with the expression of glypican detected by PCR experiments.

Introduction

In humans and others mammals, haemopoiesis takes place in the bone marrow and fetal liver. The development and maturation of the various blood cell types is dependent upon a complex interplay between different cytokines (Dexter et al, 1987; Metcalf, 1993) and the modulatory role of different microenvironments in the marrow stromas which include cell surface and extracellular matrix (ECM) proteoglycans (PGs) (Allen, 1990). Regional variation in these components within the haematopoietic microenvironment may create niches that are specifc for cells at a given state of differentiation (Hangoc et al, 1993). Heparan sulfate proteoglycans (HSPGs) present in bone marrow stromal cells and in the ECM have defined roles in the haemopoiesis, like the spacial organisation of the medullar microenvironment. Haemopoietic cytokines such as growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF) and interleukin-3 (IL-3) can bind to heparan sulfate and then be presented in a biologically active form to a haemopoietic progenitor cells (Yaon et al, 1991; Turnbull et al, 1992; Mason, 1994). Our previous study has shown that GAGs purified from stromal cell cultures, in apropriete concentrations, could be either stimulatory or inhibitory for GM-CSF activity (Alvarez-Silva and Borojevic, 1996, Carvalho et al, 2000). The ability of an HSPG to bind a variety of growth factors is dependent on the sulfation pattern of the composing disaccharides (Faham et al, 1996). It has been shown that specific sulfated domains within HS chain are a prerequisite for binding various

proteins such, fibroblast growth factor (FGF), interleukin-8 (IL-8) and others (Spillmann et al, 1998; Witt, 1994).

Structurally, HSPGs are heterogeneous macromolecules consisting of a core-protein connected via a link tetrasaccharide to a linear polysaccharide chains called glycosaminoglycans (GAGs). HS-GAGs consists of a disaccharide repeat unit (glucuronic acid and N-acetylglucosamine) which can be variously modified by N-sulfation, epimerization of glucuronic to iduronic acid, and O-sulfation at several differents sites. As a result, HS can present an extremely variable structure and it is believed that these differences are responsabile for highly specific interactions of GAGs with various macromolecules.

Cell-surface HSPGs from human bone marrow stromal cells have been extensively characterized. The molecular structure of the GAG chains of HS has been analyzed in the long-term bone marrow cultures (Morris et al. 1991). Recently, Schofield et al. (1999) have reported the expression of syndecan-3, syndecan-4 and glypican-1 in the surface of human stromal cells, together with perlecan in the extracellular matrix (ECM). A recent study has characterized several different HSPGs from the murine bone marrow stromal cell line MS-5 that can support the growth of human progenitor cell lines (Drzeniek et al. 1997). Their results have shown that MS-5 synthesizes HSPGs of syndecan family, glypican, betaglycan and perlecan. However, a detailed structural characterization of the HSPGs synthesized by murine stromal cell lines is still lacking.

UNICAMP

In the present study, we analyzed the fine structure of cell surface and secreted HS GAG chains from bone marrow-derived and fetal liver-derived stromas that sustain haematopoiesis. Additionally, we determined by RT-PCR the expression of mRNA for HSPGs core proteins in these cell lines and monitored the capacity of the HSPGs dislodgeds, obtained from the analysed stromas, to modulate the GM-CSF activity.

Materials and Methods

Materials

Dulbecco's minimum essential medium (DMEM), recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF), HEPES, EDTA, TRIS, thiazolyl blue (MTT), phosphatidylinositol phospholipase C (PI-PLC), chondroitin 4-sulfate (C-4S) from whale cartilage, chondroitin 6-sulfate (C-6S) from shark cartilage, dermatan sulfate (DS) from pig skin, and twice-crystallized papain (15 U/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chondroitin AC lyase (EC 4.2.2.5) from *Arthrobacter aurescens*, chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris*, heparin lyase (EC 4.2.2.7) and heparan sulfate lyase (EC 4.2.2.8), both from *Flavobacterium heparinum* were purchased from Seikagaku American Inc. (Rockville, MD, USA). HS was extracted from normal human aorta and purified as previously described (Cardoso and Mourão, 1994). Radiolabeled carrier-free ^{35}S-Na_2SO_4 was obtained from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, SP, Brazil). Standard disaccharides for analysis of HS composition, α-ΔUA-1→4-$GlcNSO_4$[1], α-ΔUA-1→4-GlcNAc($6SO_4$), α-ΔUA($2SO_4$)-1→4-GlcNAc, α-ΔUA-

[1] The abbreviations used were α-ΔUA, α-$\Delta^{4,5}$-unsaturated hexuronic acid; α-ΔUA($2SO_4$), α-$\Delta^{4,5}$-unsaturated hexuronic acid 2-sulfate; GlcNAc, *N*-acetylated glucosamine; $GlcNSO_4$, *N*-sulfated glucosamine; $GlcNSO_4$($6SO_4$), *N*-sulfated glucosamine 6-*O*-sulfated; GalNAc, *N*-acetylated galactosamine; GalNAc($4SO_4$), GalNAc($6SO_4$) and

1→4-GlcNSO$_4$(6SO$_4$), α-ΔUA(2SO$_4$)-1→4-GlcNSO$_4$, α-ΔUA(2SO$_4$)-1→4-GlcNAc(6SO$_4$), α-ΔUA(2SO$_4$)-1→4-GlcNSO$_4$(6SO$_4$), and of CS composition, α-ΔUA-1→3-GalNAc(4SO$_4$), α-ΔUA-1→3-GalNAc(6SO$_4$) were purchased from Seikagaku American Inc. (Rockville, MD). Oligo (dt) 12-18 primers, dATP, dCTP, dGTP, dTTP, and SuperScript II Reverse Transcriptase were obtained from GIBCO-BRL (Gaithersburg, MD, USA), and Taq DNA polymerase from CENTBIO (Porto Alegre, RS, Brasil).

Cells cultures

Permanent cell lines were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, RJ, Brazil). The murine hematopoietic bone marrow stroma cell line S17 is able to sustain both murine and human hematopoietic cells *in vitro* (Siczkowski et al. 1992). The murine fetal liver cell line AFTO24 was kindly provided by IR Lemischka, Princeton University, Princeton, NJ, USA. The multipotent myeloid precursor cell line FDC-P1 is dependent upon interleukin-3 (IL-3) or GM-CSF; it was routinely maintained in DMEN with 10% FBS, supplemented with supernatant of WeHi-3B cells that secret IL-3 constitutively (Ymer et al. 1985).

GalNAc(4,6-diSO$_4$) derivatives of *N*-acetylated galactosamine bearing a sulfate ester at position 4, at position 6, and at both positions, respectively.

In the co-culture assays, the FDC-P1 cells, previously washed with BSS in order to remove IL-3, were inoculated onto the confluent monolayers of S17 and AFTO24 cells in 24 –wells culture plates at 5x 10^4 cells per well. After 48 and 96h of co-incubation they were quantified by counting under the microscope equipped with phase contrast.

Preparation of ^{35}S-labeled GAGs

Labeling. The two stromal cell lines preparations were taken at 80% confluence and metabolically radiolabeled for 24 h with 40 μCi of [^{35}S] Na_2SO_4 per mL. Cells were then incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Isolation of the radiolabeled GAGs . The procedures used for isolation of the GAGs from the stromal cell cultures are similar to those previously described for other types of cells (Garcia-Abreu et al. 1996, DeCarvalho et al. 2000, Martins et al. 2000). Essentially, at the completion of the labeling period, conditioned media were removed and centrifuged (1000 rpm for 5 min) to remove any cell debris and stored at -20°C until required. The cells were rinsed with PBS (pH 7.4), being detached by incubation with 2 mL of 0.25% trypsin and 0.05% EDTA in PBS for 10 min at 37°C. Centrifugation of the trypsinate (2,000 *g* for 10 min at room temperature) separated the supernatant and cell pellet, which contained the *cell surface* and *intracellular* GAGs, respectively. Both fractions were incubated again for 12 h at 37°C with 2 mL of PBS (pH 7.4) containing 0.25% trypsin and 0.05% EDTA. The samples were further incubated with 10 mg papain in the presence of

5 mM EDTA and 5 mM cysteine at 60°C for 24 h. The ^{35}S-labelled GAGs were purified by DEAE-Sephacel, as described below.

For isolation of *extracellular* GAGs, the previously collected conditioned medium was first incubated with 10 mg papain in the presence of 5 mM EDTA and 5 mM cysteine at 60°C for 24 h. Then it was subjected to ion-exchange chromatography on a DEAE-Sephacel column (10.0 cm x 0.7 cm), equilibrated with 0.05 M sodium acetate (pH 5.0). The medium was manually loaded onto the column and eluted under gravity. The column was washed with 100 mL of 0.1 M NaCl in the same acetate buffer. The bound materials were eluted with 1.0 M NaCl in the same acetate buffer. Fractions containing the ^{35}S-labeled GAGs were exhaustively dialyzed against distilled water. The dialyzed sample was lyophilized, re-suspended in 0.5 mL of distilled water.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out as previously described (Martins et al. 2000). Approximately 1,000 cpm of ^{35}S-glycosaminoglycans from the three cellular compartments of stromal cell cultures, before and after chondroitin lyase digestion or deaminative cleavage with nitrous acid, as well as a mixture of standard C-4S, DS and HS (10 μg of each) were applied to 0.5% agarose gels in 0.05 M 1,3-diaminopropane:acetate (pH 9.0). After electrophoresis, GAGs were fixed in the gel with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide in water, and stained with 0.1% toluidine blue in

acetic acid:ethanol:water (0.1:5:5, v/v). The ^{35}S-labeled glycosaminoglycans were visualized by autoradiography of the stained gels.

Enzymatic and nitrous acid depolymerization of the GAGs

Digestion with chondroitin lyase. Digestions with chondroitin AC or ABC lyases were carried out according to Saito et al. (1968). Approximately 10,000 cpm of ^{35}S-labeled GAGs were incubated with 0.3 units of chondroitin AC lyase or chondroitin ABC lyase for 8 h at 37°C in 100 μL of 50 mM Tris:HCl (pH 8.0) containing 5 mM EDTA and 15 mM sodium acetate.

Digestion with heparin and heparan sulfate lyases. Exhaustive enzymatic digestion with a mixture of heparin and heparan sulfate lyases was performed with three additions of the enzymes mixture (1 mU each) in 100 μL of 100 mM sodium acetate and 10 mM calcium acetate (pH 7.0), over a 36 h-period at 37°C.

Deamination with nitrous acid. Deamination by nitrous acid at pH 1.5 was performed as described by Shively and Conrad (1976). Briefly, approximately 10,000 cpm of ^{35}S-labeled GAGs were incubated with 200 μL of fresh generated HNO_2 at room temperature for 10 min. The reaction mixtures were then neutralized with 1.0 M Na_2CO_3.

Analysis of the disaccharides formed by enzymatic depolymerization on a SAX-HPLC

Disaccharides from CS. Purified cell-surface and secreted radiolabeled GAGs were submitted to exhaustive digestion with chondroitin ABC lyase (see above). Disaccharides and chondroitin ABC lyase-resistant GAGs (composed of intact HS chains) were recovered by a Superdex peptide-column (Amersham Pharmacia Biotech) linked to a HPLC system from Shimadzu (Tokyo, Japan). The column was eluted with amonio bicarbonate 0.2 M pH 7.0 at a flow rate of 0.5 mL/min. Fractions of 0.25 mL were collected, monitored for UV absorbance at 232 nm and the radioactivity was counted in a liquid scintillation counter. Fractions corresponding to disaccharides and to the chondroitin ABC lyase-resistant GAGs (eluted at the void volume) were pooled, freeze dried, and stored at –20°C.

The lyase-derived radiolabeled disaccharides and standard compounds were subjected to a SAX-HPLC analytical column (250-mm x 4.6-mm, Sigma-Aldrich), as follow. After equilibration in the mobile phase (distilled water adjusted to pH 3.5 with HCl) at 0.5-mL/min, samples were injected and disaccharides eluted with a linear gradient of NaCl from 0,3 to 1.05M over 45 min in the same mobile phase. The eluant was collected in 0.250 mL fractions and monitored for ^{35}S-labeled disaccharide content for comparison with lyase derived disaccharide standards.

Disaccharides from HS. The radiolabeled HS, obtained after chondroitin ABC lyase digestion (void volume of the Superdex peptide HPLC, see above), and

was submitted to an exhaustive enzymatic digestion with a mixture of heparin and heparan sulfate lyases. The solution was then applied to a Superdex peptide-HPLC, as described above for the products obtained after chondroitin ABC lyase digestion. Fractions corresponding to disaccharides were pooled, freeze dried, and stored at –20°C. The lyase derived double-radiolabeled disaccharides and standard compounds were subjected to a SAX-HPLC system, eluted with a 0.3-1.05 M NaCl gradient, as described above for the disaccharides formed by chondroitin ABC lyase. Disaccharides were identified by comparison with the elution positions of known disaccharide standards.

Solubilization of the ^{35}S-labeled macromolecules of the cell surface

The two murine stromal cell lines were labeled overnight with [^{35}S]-Na_2SO_4, as described above. The cell layers were rinsed several times with ice-cold PBS and were incubated for 10 min in PBS supplemented with PI-PLC (0.4U/mL) (Brucato et al. 2001). The supernatant was collected and stored until dialyses. The cell layers were rinsed again with cold PBS (three times) and were incubated for 2 min in trypsin (50 μg/mL). Release of radioactivity from cell surfaces was monitored by liquid scintillation counting of small aliquots of the incubation medium. Fractions containing the ^{35}S-labeled dislodged molecules were exhaustively dialyzed against distilled water. The dialyzed samples were lyophilized, and resuspended in 1.0 mL of DMEN supplemented with 10% FBS before using.

FDC-P1 proliferation monitoring

FDC-P1 cells were plated in 96-well tissue culture plates, $3x10^4$ cells per well, in the presence of recombinant murine GM-CSF(1 ng/mL) (positive control), or in the presence of the same growth factor concentration, with ^{35}S-labeled dislodged molecules from the studied stromas. Cells were quantified after 24 h incubation using the thiazol blue MTT colorimetric assay (Mosmann, 1983).

RT-PCR analyses of HSPG core proteins

Total mRNA of S17 and AFTO24 cells was extracted using TRIzol® (GIBCO-BRL) following the manufacturer's instructions. Expression of β-actin, syndecan-1, syndecan-2, syndecan-4, glypican and betaglycan was monitored by the standard reverse transcripitase-polymerase chain reaction (RT-PCR) as previously described (Brito and Borojevic, 1997) using thirty amplification cycles.

Results

Cell proliferation monitoring

In previous works the capacity of haematopoietic tissue stroma to sustain myelopoiesis was monitored using the myeloid FDC-P1 cell line, which is dependent upon the myelopoietic gowth factors for both survival and proliferation (Siczkowski et al, 1992; Carvalho et al, 2000), Punzel et al, 1999 showed that murine fetal liver cell line AFT024 supports human long-term culture initiating cells (LTC-IC) as well as lymphoid progenitors when cultured in contact with the feeder. We now examine if AFT024 feeders supports the survival and proliferation of the myeloid FDCP-1 cell line when compared with bone marrow-derived cell line S17. Althought both cell lines sustained proliferation, the S17 stroma induced a more intense proliferation of FDC-P1 cells when compared with AFT024 cell line (Figure 1).

RT-PCR analysis of heparin-binding growth factors from the stromal cell lines

The biological activity of FDC-P1 cells depend upon haematopoietic growth factors principally GM-CSF and IL-3. The fetal liver derived-stroma showed expression, by RT-PCR, for the same set of citokines demonstrated in our previous work for S17 (Carvalho et al, 2000). GM-CSF, SCF and HGF are

growth factors with myelopoietic activity. AFT024 and S17 had no expression for IL-3 (Figure 2).

GAGs from stromal cell lines

We have previously showed that the HS-GAGs secreted by S17 are important for the biological activity of GM-CSF and consequently for FDC-P1 proliferation control. While heparan sulfate is involved in binding growth factors and probably in adhesion of myeloid cells to haematopoietic stroma we investigated and compared the structure of glycosaminoglycans, specifically HS, present in the cell surface and in the culture medium of S17 and AFT024 stromal cells.

Radiolabeled GAGs from the intracellular, cell surface and culture medium compartments of stromal cell cultures were exhaustively digested with papain. The GAG chains were then purified by anion-exchange chromatography and analyzed by agarose gel electrophoresis, before and after enzymatic and nitrous acid depolymerization (Figure 3). The structure features of the GAG chains were investigated using established protocols (Werneck et al. 1999, 2000), based on the disaccharides formed by exhaustive digestion with specific lyases.

Identification and relative proportions of the various GAGs

We analyzed and determined the total and relative amounts of stromal-derived ^{35}S-labeled GAGs found on the intracellular compartment, cell surface and those secreted into the culture medium, after a 24-h labelling period with [^{35}S] Na_2SO_4 added to the cell cultures. Our results revealed high amounts of ^{35}S-labeled CS. This was preponderantly secreted into the medium, but was also found at a lower proportion on the stromal cell surface. HS was relatively more abundant in the cell-associated fractions (Table 1).

Characterization of the ^{35}S-GAGs by agarose gel electrophoresis (Figure 3) revealed that culture medium of stromal cell lines have a preponderant electrophoretic band with a mobility between CS and DS standards. It totally disappears after chondroitin AC and ABC lyase digestions. Therefore, this major electrophoretic band is CS. The less intense band had the same mobility as the standard HS and totally disappeared from the gel after deaminative cleavage by nitrous acid. Agarose gel electrophoresis of the cell surface ^{35}S-GAGs revealed two major electrophoretic bands, one band had the same mobility as the standard HS, resisted to chondroitin AC and ABC lyase digestion, but totally disappeared after deaminative cleavage by nitrous acid and the other band had the same mobility as the CS/DS standard and totally disappeared from the gel after chondroitin AC and ABC lyase digestions. Therefore, the two major electrophoretic bands of the cell surface fraction correspond to HS and CS, respectively. For the intracellular fraction, it was found a major electrophoretic

band that had the same mobility as the standard HS and totally disappeared from the gel after deaminative cleavage by nitrous acid (Figure 3).

Analysis of the disaccharides formed by chondroitin ABC lyase digestion of the GAGs from stromal cell lines

The products formed by exhaustive action of chondroitin ABC lyase on the radiolabeled GAGs from the cell surface and culture medium of stromal cell lines were analyzed by gel filtration on a Superdex peptide-HPLC. The predominant products (~95%) were disaccharides (not shown). These disaccharide mixtures were then analyzed on a SAX-HPLC and the results are shown numerically (Table 2). This procedure separates the disaccharides formed by chondroitin ABC lyase, the two monosulfated α-ΔUA-1→3-GalNAc($6SO_4$) and α-ΔUA-1→3-GalNAc($4SO_4$), derived from chondroitin 6-sulfate (C-6S) and chondroitin 4-sulfate (C-4S), respectively. Stromal cells show the preponderant presence of the monosulfated disaccharide α-ΔUA-1→3-GalNAc($6SO_4$), but small amount α-ΔUA-1→3-GalNAc($4SO_4$) was observed as well (Table 2).

Disaccharide composition of the HS chains

The radiolabeled HS chains from the cell surface and culture medium fractions were depolymerized by exhaustive digestion with heparin + heparan sulfate lyases. The products formed were analyzed on a Superdex peptide-HPLC

(not shown). The disaccharides were analyzed on a SAX-HPLC, and the results are shown graphically (Figures 4 and 5) and numerically (Table 3). The major disaccharide was α-ΔUA-1→4-GlcNSO$_4$ (Figs. 4 and 5), which comprised ~60% of the total disaccharide units on the HS from both cell surface and culture medium (Table 3). Other monosulfated disaccharides were also constituents of the two HS fractions. The disulfated disaccharide α-ΔUA-1→4-GlcNSO$_4$(6SO$_4$) was only detected in HS chains from the culture medium of the two stromal cell lines (Table 3 and Figures 4 and 5). The trisulfated disaccharide α-ΔUA(6SO$_4$)-1→4-GlcNSO$_4$(6SO$_4$) was only detected in HS from the two cellular fractions of S17 cells (Figures 4 and 5 and Table 3).

Based on the proportions of disaccharides formed by exhaustive digestion with heparin and heparan sulfate lyases (Table 3) it was possible to determine the sulfation characteristics of the HS chains of the two stromal cell lines (Table 4). The average number of sulfate groups per 100 disaccharide units for the cell surface and culture medium HS were 120 and 123 for S17 and 100 and 102 for AFT024 cells, respectively. The higher sulfation of the cell surface and secreted HS from S17 was due to increase in N- and O-sulfation. When we compared HS from cell surface and culture medium of each stromal cell line, we noticed that the proportion of *N*-sulfation was nearly the same (Table 4). Conversely, the 6-*O*-sulfation increased from the cell surface to the culture medium. The proportions of 2-*O*-sulfation varied significantly between the two stromal cell lines, it was higher for S17 cells (Table 4).

RT-PCR analysis of HSPGs from the stromal cell lines

RT-PCR was performed using specific primer pairs for the different syndecans (1, 2 and 4), glypican and betaglycan. The specificity of PCR-amplification products was confirmed by sequencing of cloned amplification products. Figure 6 shows that mRNA for both syndecan-4 and glypican were detected in the two stromal cell lines. Only AFT024 expressed mRNA for betaglycan. Both syndecan-1 and syndecan-2 mRNAs were not detected in the two stromal cell lines.

Cell-layer trypsin and PI-PLC dislodgeds HSPGs

PI-PLC provides a useful experimental tool to demonstrate the presence of HSPGs intercalated into the plasma membrane through a phosphatidylinositol anchor (glypican type) (Brucato et al. 2001). Stromal cell lines were labeled with ^{35}S-sulfate for 24 h and then incubated in fresh medium with or without PI-PLC (0.4 U/mL) for up to 60 min, allowing a direct quantification of the GPI-anchored cell surface PGs. ^{35}S-radioactivity in the medium was several fold higher in PI-PLC-treated cells than in control cells (Figure 7). About 20% of the HSPG are linked to membrane by GPI anchor while the remainder (80%) are integrated in the plasma membrane (results not shown).

These results are consistent with the expression of HSPGs of glypican type by the two stromal cell lines evidenced by the RT-PCR experiments.

Modulation of the intrinsic growth factor activity by ^{35}-S labeled dislodged molecules

In our previous work we showed that S17-derived heparan sulfate had a low stimulatory activity on FDC-P1 cells proliferation in the presence of a constant quantity of recombinant murine GM-CSF. These results suggest that stroma-derived glycosaminoglycans could modulate the GM-CSF activity. We have now shown that the ^{35}-S labeled dislodged molecules obtained from the cell layers after treatment with trypsin increases the biological activity of the GM-CSF when compared with ^{35}-S labeled GPI-dislodged molecules which did not show significantly higher than in the control (Figure 8). These results suggested the existence of both transmembrane HSPG and GPI-HSPG and that the biological activity of GM-CSF in FDC-P1 proliferation, in vitro, is increased by a possible physical interation between growth factor and syndecan HSPG.

Discussion

We studied the synthesis of GAGs and their secretion to the cell surface and culture medium by two murine stromal cell lines: a bone marrow-derived cell line S17 and a fetal liver-derived cell line AFT024.

These two stromal cell lines produce HS and CS and show similar distribution of total GAGs among the intracellular, cell surface and extracellular compartments. In addition, no difference was observed on the relative proportions of HS and CS within each compartment. These cell lines secrete high amounts of CS into the culture medium, while HS was found to be more abundant in the cell-associated fractions. These results are in good agreement with previous reports on the type of GAG of these two stromal cell lines (Siczkowsk et al. 1992, Punzel et al. 1999, Carvalho et al. 2000). Drzeniek et al. (1997) have recently reported that a murine bone marrow stromal cell line MS-5 also synthesizes and secretes CS and HS in culture.

Although the GAG composition of murine stromal cell lines have been previously determined, as pointed out above, information regarding the fine structure of these GAG species, as well as, the identification of the core protein of the PG are still missing.

HS has a ubiquitous distribution on cell surfaces and in the extracellular matrix (ECM). HSPGs present on the cell surface of bone marrow stromal cells and in the ECM have important roles in the control of adhesion and growth of haemopoietic stem and progenitor cells. IL-3 and GM-CSF can be bound by

HSPGs from bone marrow stromal cells or their ECM and can be presented in a biologically active form to haemopoietic precursor cells (Gordon et al. 1987, Roberts et al. 1988, Alvarez-Silva et al. 1993, 1996).

Specific sulfation patterns of HS are required for binding and modulation of the activity of cytokines that have activity on haemopoietic progenitors (Lyon et al. 1994, Faham et al. 1998). Therefore, we investigated the fine structure of HS chains from S17 and AFT024 stromal cell lines. Comparison between HS from the cell surface and culture medium of the two stromal cell lines revealed that both stromal cell lines exhibited increasing proportions of sulfation in the HS from the cell surface to the culture medium, mostly due to 6-O-sulfation. When comparison is made among HS from the two cell lines, they differ in the proportions of N- and O-sulfation: S17 shows HS with higher levels of N-sulfation and 2-O-sulfation when compared with the respective compartments of AFT024 cells (Tables 3 and 4). Recent observations shows that the structure of HS from a haematopoieis-supportive cell line is more highly sulfated when compared with nonsupportive HS (Gupta et al, 1998). Interestingly, it was recently reported that specific 6-O-sulfated HS GAGs synthesized by AFT024 cell line are important for maintaining human long-term culture-initiating cells (LTC-IC) ex vivo (Gupta et al. 2000).

Data on the characterization of core proteins of HSPGs in haemopoietic stromal cells are scarce and controversial. Schofield et al. (1999) have reported that glypican-1, syndecan-3 and syndecan-4 are the major cell-membrane HSPG species in human marrow stroma and that perlecan is the major ECM PG.

Conversely, Drzenieck et al. (1997) have reported that the murine bone marrow stromal cell line MS-5 synthesizes several different HSPGs: syndecans-1-4, glypican, betaglycan and perlecan.

The PI-PLC and mild trypsinisation treatments strongly suggested that glypican and syndecan HSPGs are integral components of the membranes. We have partially assessed the identification of the core proteins of cell-associated HSPGs of the two murine stromal cell lines monitoring their expression by RT-PCR. The two assayed stromas expressed mRNA for glypican and syndecan-4. Only AFT024 expressed mRNA for betaglycan. There was no evidence for expression of syndecan-1 or syndecan-2 mRNA. RT-PCR analyses for syndecan-3 or perlecan were not performed. Therefore, we cannot exclude the possible expression of mRNA for these two PG cores in the two stromal cell lines. Since we have only partially approached the identification of the core protein of cell-associated HSPGs in the two stromal cell lines by using only RT-PCR experiments, a comparison with the published results mentioned above on the characterization of HSPGs in haemopoietic stromal cells is not possible at this stage. Further characterization of these molecules by more specific approaches will be focus of future studies.

The identification by RT-PCR of mRNA expression for glypican in the two stromal cell lines led us to a more specific study of glypican expression in these cells by the use of PI-PLC, which releases whole HSPG with the terminal phosphate group of inositol still attached, whereas diacylglycerol remains associated to the external leaflet of the membrane (Brucato et al. 2001). Addition

of the lipase to the stromal cell lines induced, in the incubation medium, a rapid and time-dependent accumulation of ^{35}S-material. These results together with the RT-PCR experiments strongly suggest the presence of glypican on the cell surface of these two stromal cell lines. However, the confirmation of glypican identity remains to be established by the use of specific monoclonal antibody.

About 80% of the HSPG are integrated in the plasma membrane of the studied stromas. These ^{35}-S labeled dislodged molecules obtained from the cell layers after treatment with trypsin, increases the biological activity of the GM-CSF when compared with ^{35}-S labeled GPI-dislodged molecules that did not show significantly higher than in the control. We have recently shown that the physical contact among the stroma and the myeloid cells triggers the capping of cell surface molecules, including sialyted glycoconjugates and proteoglycans. These molecules, including transmembrane HSPGs, can generate specific intercelular environment providing the physicochemical conditions for interaction with GM-CSF modulating their biological activity.

Acknowledgments

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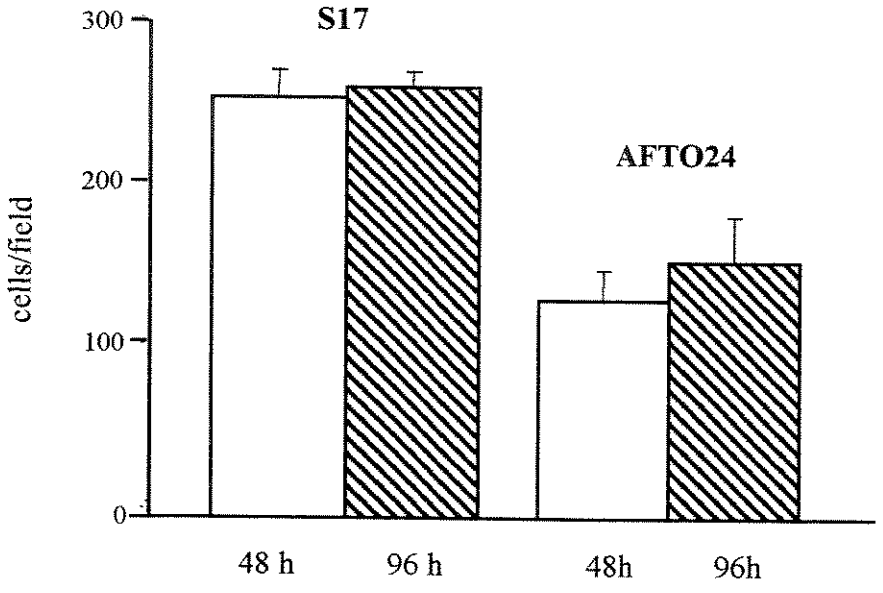

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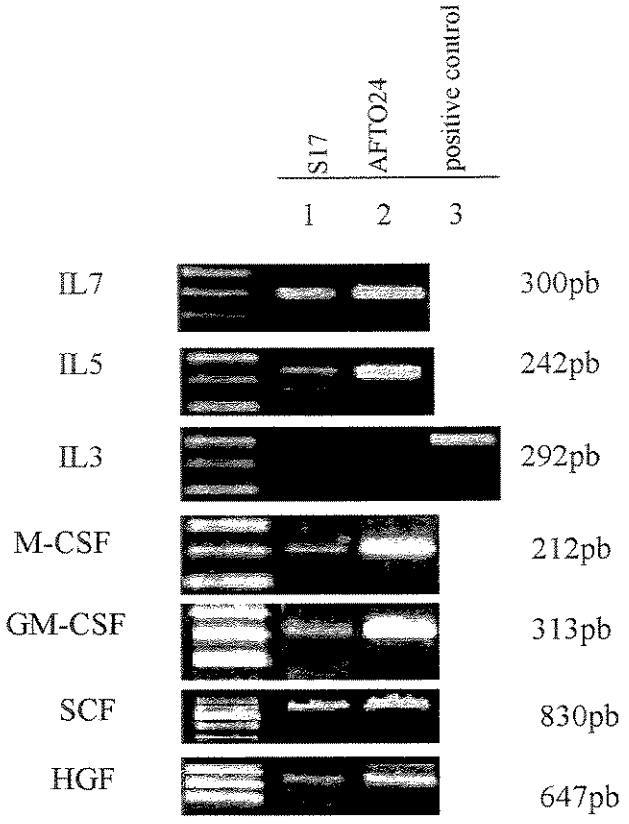
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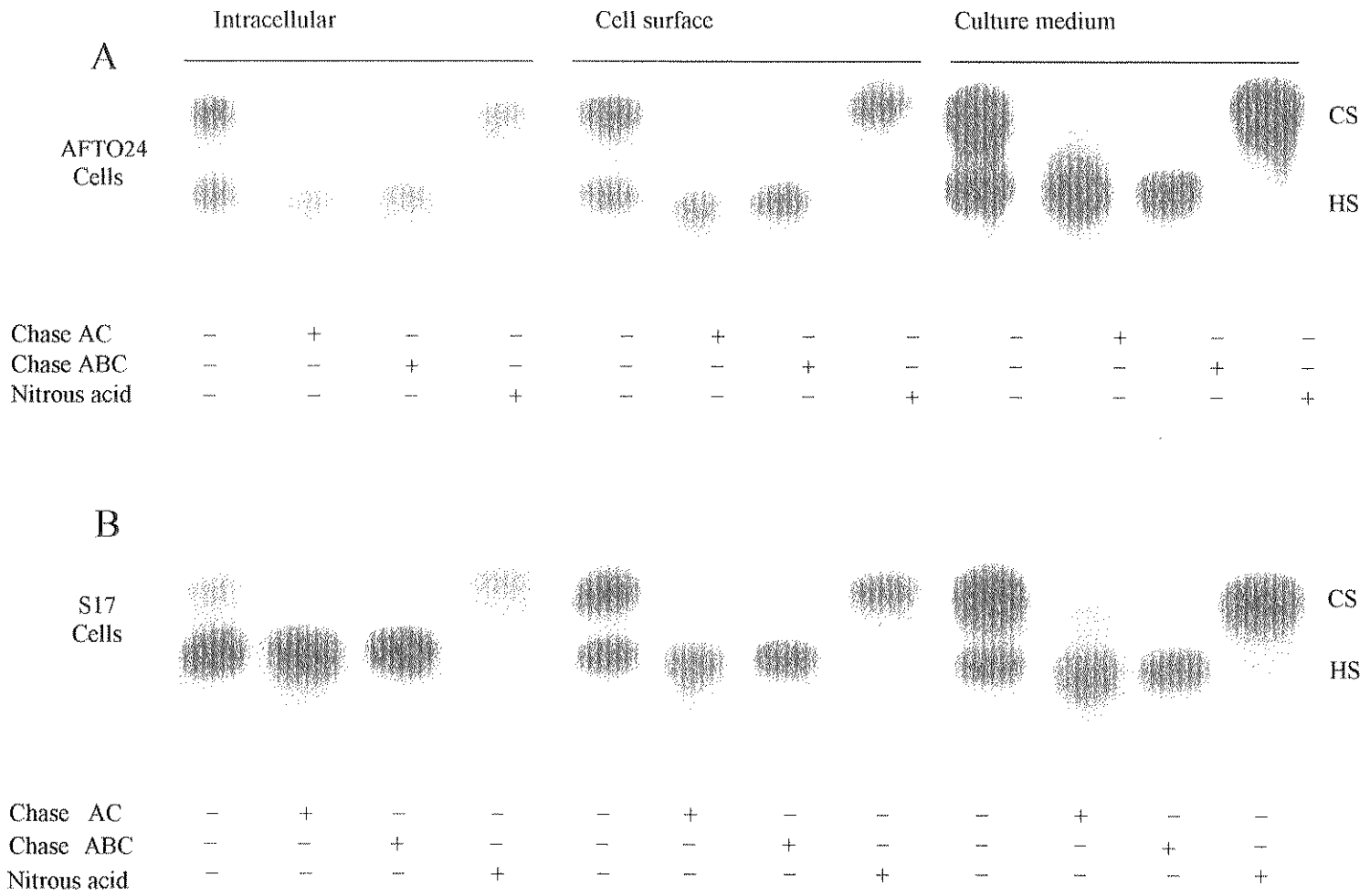
Figure 2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Intracellular
Cell surface
Culture medium
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B
S17
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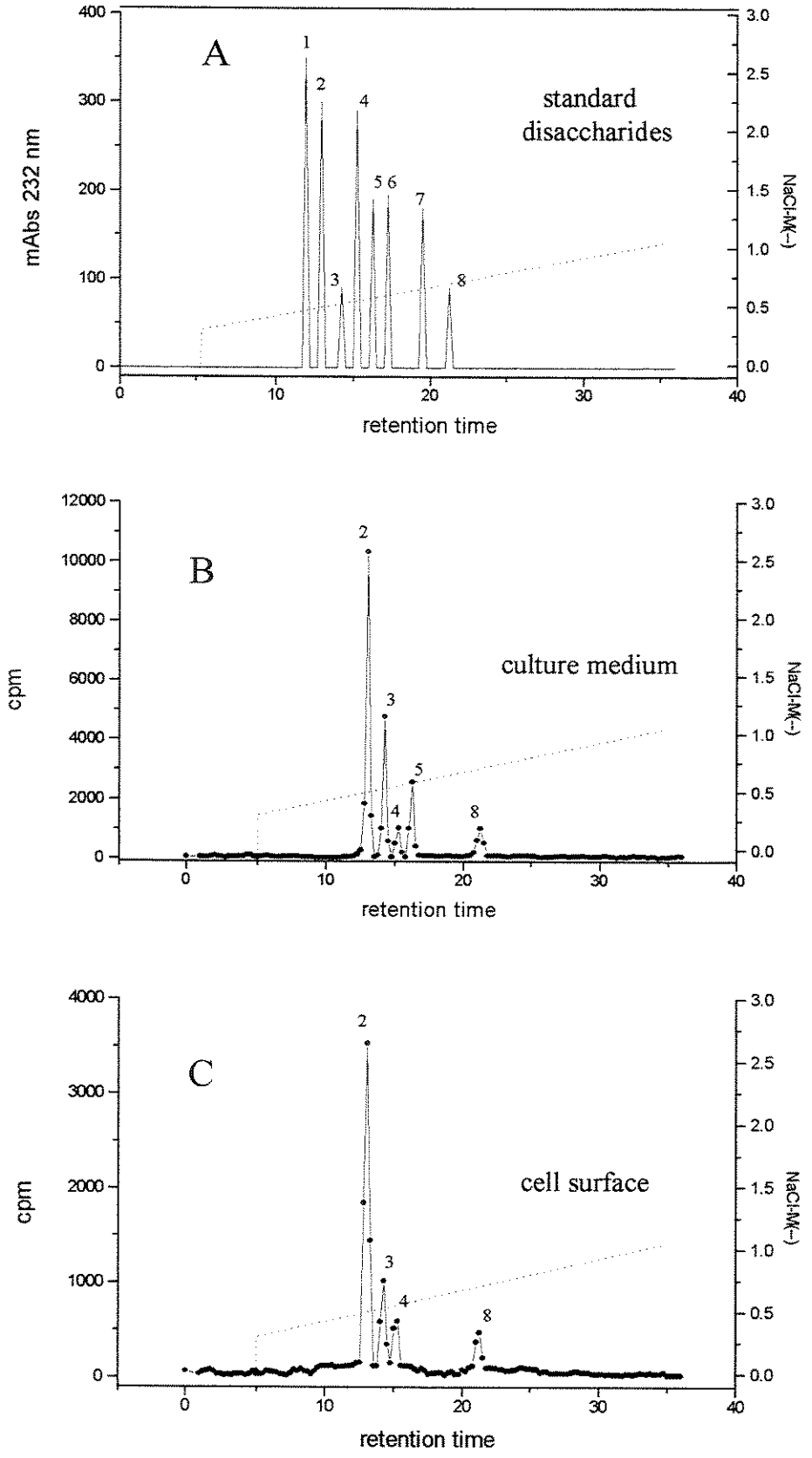
HS
Chase AC
Chase ABC
Nitrous acid

Figure 3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NaCl-Mk(--)
retention time

Figure 4

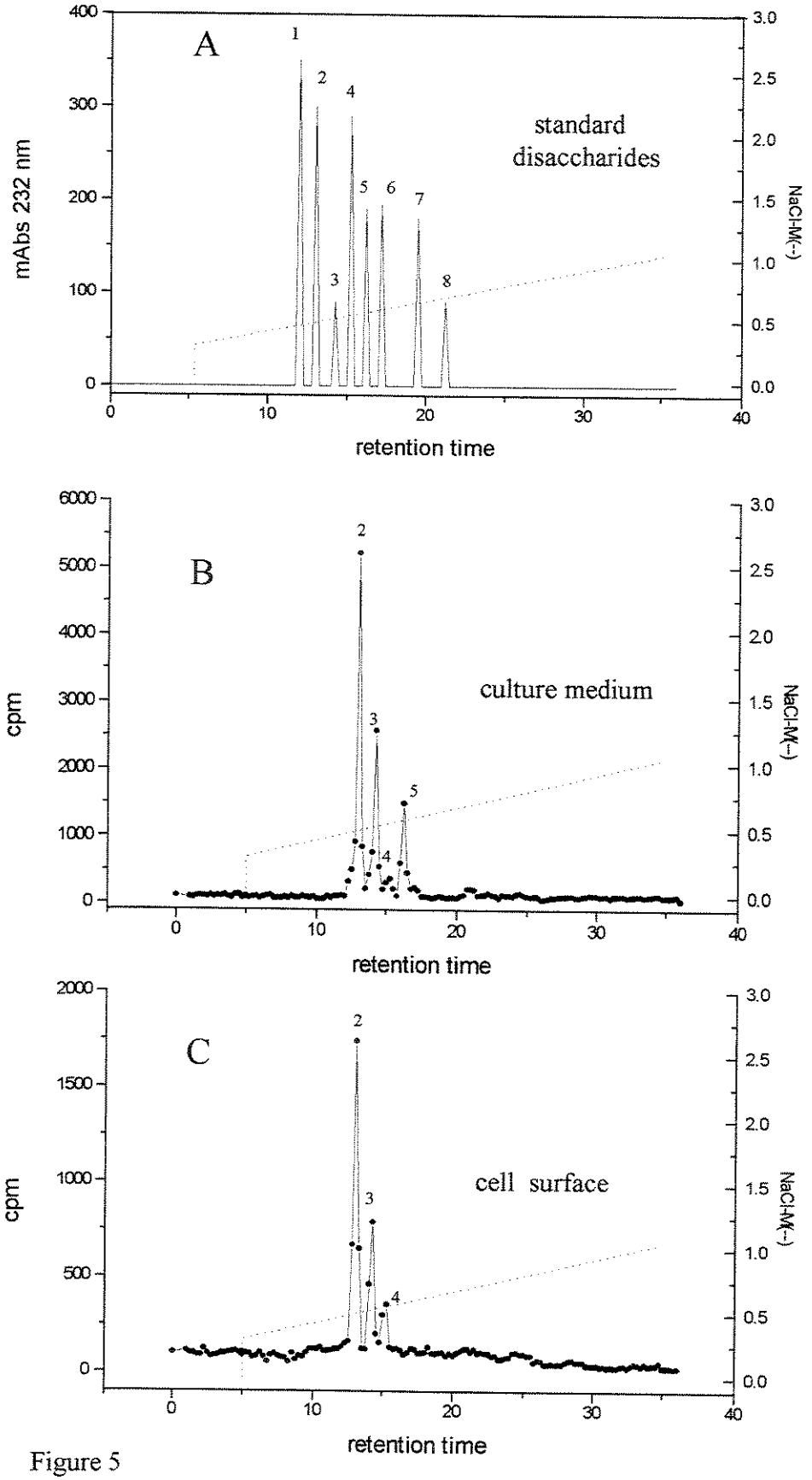
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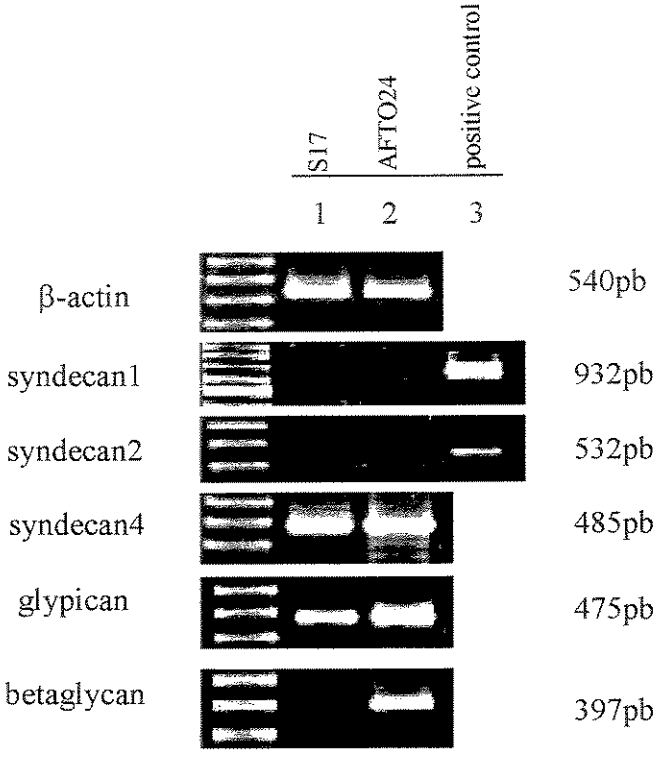
Figure 5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AFTO24
positive control
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2
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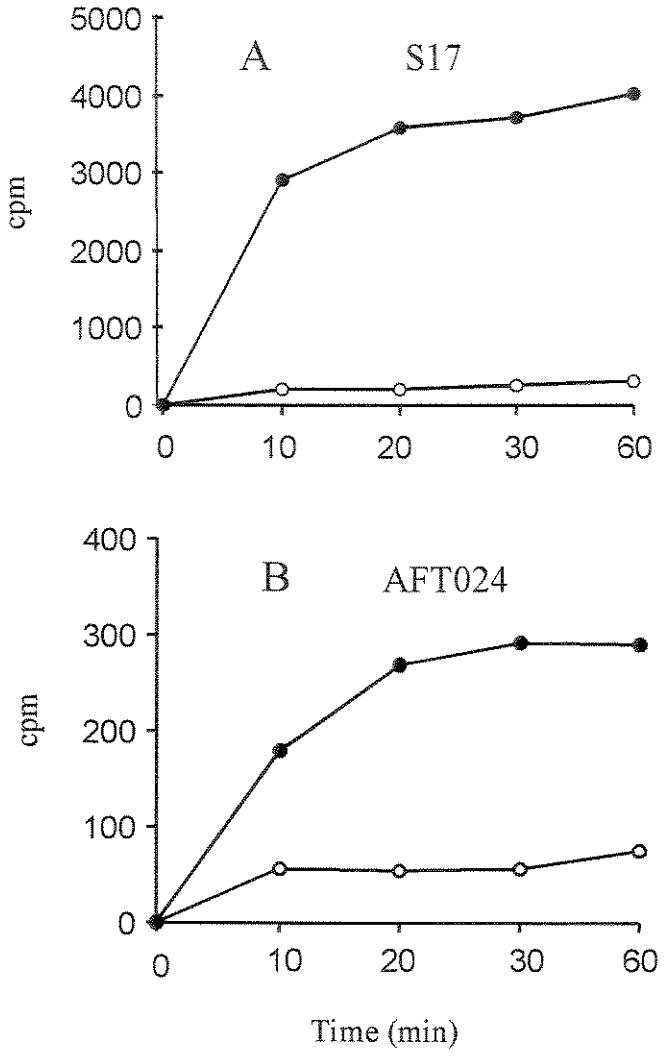
Figure 6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60
Time (min)

Figura 7

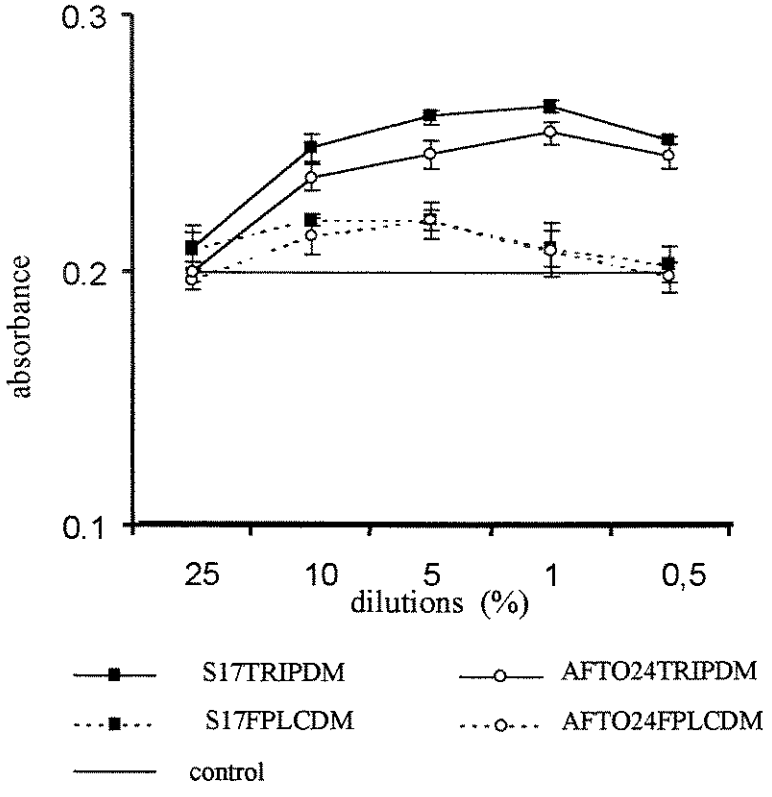
0.3
0.2
0.1
absorbance
25
10
5
1
0,5
dilutions (%)
S17TRIPDM
AFTO24TRIPDM
S17FPLCDM
AFTO24FPLCDM
control

Figura 8

Table 1 – Distribution of the ^{35}S-labeled GAGs in the different cellular compartments of stromal cell lines after a 24h labeling period.

Cell line	Compartment	Labeled GAGs*(cpm/10^6 cells)		
		Total	HS	CS
S17	Intracellular	308,250	283,590(92)	24,660 (08)
	Cell surface	417,344	208,672(50)	208,672(50)
	Culture medium	943,384	169,809(18)	773,575(82)
AFT024	Intracellular	126,063	57,989(46)	68,074(54)
	Cell surface	135,600	58,308(43)	77,292(57)
	Culture medium	691,932	159,144 (23)	532,788(77)

*The ^{35}S-GAGs were identified by agarose gel electrophoresis (see Fig.3). The radioactive bands having identical electrophoretic migration to HS and CS standards were scraped and radioactivity counted in 10 mL of 0,5% PPO/toluene solution. The values in parentheses are the percentages of HS or CS.

Table 2 - Disaccharides derived by chondroitin ABC lyase digestion of the radiolabeled GAGs from the cell surface and culture medium of stromal cell lines.

Cell line	Compartment	Disaccharide units* (cpm/10^6 cells)	
		α-ΔUA-1→3-GalNAc($6SO_4$)	α-ΔUA-1→3-GalNAc($4SO_4$)
S17	Cell surface	5,130(85)	904(15)
	Culture medium	15,512(85)	2,719(15)
AFT024	Cell surface	4,580(78)	1,271(22)
	Culture medium	35,317(84)	6,533(16)

*The values in parentheses are percentages of the ^{35}S-labeled disaccharides in each compartment.

Table 3 - Lyase-derived disaccharide composition of the radiolabeled HS from the cell surface and culture medium of stromal cell lines.

Disaccharide units	S17*		AFT024*	
	Cell surface	Culture medium	Cell surface	Culture medium
α-ΔUA-1→4-GlcN SO_4 (peak 2)‡	6,836(61)	13,644(52)	3,062(58)	7,481(60)
α-ΔUA-1→4-GlcNAc($6SO_4$) (peak 3)‡	1,962(18)	6,362(24)	1,452(27)	3,872(31)
α-ΔUA($2SO_4$)-1→4-GlcNAc (peak 4)‡	1,249(11)	1,764(07)	785(15)	869(07)
α-ΔUA-1→4-GlcNSO_4($6SO_4$) (peak 5)‡	n.d.	2,022(08)	n.d.	254(02)
α-ΔUA($2SO_4$)-1→4-GlcNSO_4($6SO_4$) (peak 8) ‡	1,070(10)	2,185(08)	n.d.	n.d.

* The radioactivity under the peaks in Figs. 4 and 5 was integrated to obtain the values shown in the table. The values in parentheses are percentages of the various ^{35}S-labeled disaccharides in each compartment.

‡ Standard peak number in order of elution, Figs. 4 and 5.

n.d., not detected.

Table 4 - Sulfation characteristics of disaccharides from HS obtained from cell surface and culture medium of stromal cell lines

	S17		AFT024	
Sulfation*	Cell surface	Culture medium	Cell surface	Culture medium
Total sulfation/100 disaccharides	120	123	100	102
N-sulfation	71	68	58	62
O-sulfation	49	55	42	40
6-O-sulfation	28	40	27	33
2-O-sulfation	21	15	15	07

* The calculations on sulfation characteristics shown are based on the overall disaccharide composition data (Table 3).

Figure legends

Figure 1 Co-culture assay-proliferation of FDC-P1 cells after 48h (empty bars) and 96h (full bars) culture over confluent monolayers of S17 and AFT024. Results represent viable cells counts/microscope field (x250); mean values of 50 fields, counted in two independent experiments.

Figure 2 RT-PCR analysis of expression of haemopoietins genes in S17 and AFT024 stromas. Amplification of cDNA for β-actin, IL-3, IL-5, IL-7, M-CSF, GM-CSF, SCF and HGF is shown in lane 1 (for S17) and lane 2 (for AFT024) . Lane 3 shown the positive control of WE-HI 3B cells for IL3.

Figure 3 Agarose gel electrophoresis of the ^{35}S-sulfated GAGs from the intracellular, cell surface and culture medium compartments of AFTO24 (A) and S17 (B), before and after chondroitin lyase digestion (chase AC and chase ABC) or deaminative cleavage by nitrous acid. After enzymatic incubation, the ^{35}S-GAGs were applied to 0,5% agarose gel and electrophoresis was carried out in 0,05M 1,3-diaminopropane:acetate buffer (pH9,0) for 1 h at 120 V. The GAGs in the gel were fixed with 0,1% N-cetyl-N,N,N-trimethylammonium bromide for 12 h and stained with 0,1% toluidine blue in acetic acid:ethanol:water (0,1:5:5, v/v). The radioactive bands corresponding to the ^{35}S-labeled GAGs were detected by autoradiography of the fixed and stained gel. Standard GAGs are a mixture

containing 10 μg each of chondroitin 4-sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS).

Figure 4 Strong anion-exchange HPLC analysis of the lyase-derived disaccharides from radiolabeled HS of S17 cells. A mixture of disaccharide standards (A) and the disaccharides formed by exhaustive action of heparin + heparan sulfate lyases on radiolabeled HS from the culture medium (B) and cell-surface (C) of S17 cells were analyzed on a SAXHPLC column. The numbered peaks correspond to the elution positions of known disaccharide standards as follows: *Peak 1*, α-ΔUA-1→4-GlcNAc; *Peak 2*, α-ΔUA-1→4-GlcNSO$_4$; *Peak 3*, α-ΔUA-1→4-GlcNAc(6SO$_4$); *Peak 4*, α-ΔUA(2SO$_4$)-1→4-GlcNAc; *Peak 5*, α-ΔUA-1→4-GlcNSO$_4$(6SO$_4$); *Peak 6*, α-ΔUA(2SO$_4$)-1→4-GlcNSO$_4$; *Peak 7*, α-ΔUA(2SO$_4$)-1→4-GlcNAc(6SO$_4$); *Peak 8*, α-ΔUA(2SO$_4$)-1→4-GlcNSO$_4$(6SO$_4$).

Figure 5 Strong anion-exchange HPLC analysis of the lyase-derived disaccharides from radiolabeled HS of AFT024 cells. A mixture of disaccharide standards (A) and the disaccharides formed by exhaustive action of heparin + heparan sulfate lyases on radiolabeled HS from the culture medium (B) and cell-surface (C) of AFT024 cells were analyzed on a SAXHPLC column. The numbered peaks correspond to the elution positions of known disaccharide standards as described in the legend of Fig. 4.

Figure 6 RT-PCR analysis of expression of HSPGs genes in S17 and AFT024 stromas. Amplification of cDNA for β-actin, syndecan-1, syndecan-2, syndecan-4, glypican and betaglycan is shown in lane 1 (for S-17) and lane 2 (for AFT024). Lane 3 shown the positive control of queratinocytes for syndecan 1.

Figure 7 Time course of labeled sulfated macromolecules release from cellular layer of S17 (A) and AFT024 (B) by PI-PLC treatment. Stromal cells were labeled with [^{35}S]sulfate. After removing the labeling medium, cells were incubated in fresh medium with 0.4 U/mL PI-PLC (•) or without (o, control) for up to 60 min. Aliquots of media were collected at each time point and the amount of radioactivity released was measured by liquid scintillation.

Figure 8 Myeloid cell proliferation assay in the presence of 1 ng rGM-CSF in standard culture medium supplemented with different dilutions of S17 and AFT024 cell-derived ($4x10^7$) dislodged with tripsyn (TRIPDM) and phospholipase C (FLPDM). Results are expressed in absorbance mediated by rGM-CSF alone (control). Data represent mean value of two experiments done in quadruplicate.

ANEXO 2

Submetido à Tissue & Cell Research

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Stroma-mediated granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation of myelopoiesis: spatial organisation of intercellular interactions

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Abstract

Granulocyte-macrophage colony stimulating factor (GM-CSF) is an important cytokine involved in the control of haematopoiesis both in bone marrow and in extramedullary sites. Its biological activity has been shown to depend on the molecular composition and on the physicochemical conditions of the microenvironment provided by the surface of the supporting stroma. In particular, GM-CSF signal is known to be modulated by the quality of stromal proteoglycans and its active conformation is favoured by pH acidification. Here, we have used electron microscopy and selective labelling to assess the morphological features of the microenvironment of the interface between stroma and haematopoietic cells. We present evidences that, upon interaction, the two cell types rearrange their surface both in shape and molecular composition. Haematopoietic cells extend thin cytoplasmic projections, which considerably increase the area of intercellular contact, while stromal cells, besides forming lamellipodia at the contact region, carry out a redistribution of membrane-associated sialilated glycoconjugates and proteoglycans. Such rearrangement leads to extensive capping of negatively-charged molecules at the interface between the supporting stroma and the haematopietic cells, probably associated to a local decrease in environmental pH. Results described here indicates that a distinguished distribution of negative charges at cellular interface may be responsible for the selectivity of the biological response to GM-CSF.

Key words

haematopoiesis, granulocyte-macrophage colony stimulating factor, stromal cells, cationic ferritin, proteoglycans

Introduction

Granulocyte-macrophage colony stimulating factor (GM-CSF) is one of the major haemopoietins controlling proliferation and differentiation of myeloid lineages. It is present in the bone marrow environment, as well as in peripheral tissues where (alone or in association with other haemopoietins) it can selectively mediate proliferation and/or activation of monocyte-derived lineages, including diverse types of tissue macrophages and osteoclasts, polymorphonuclear granulocytes, as well as of non-haemogenic normal or transformed cells such as osteoblasts, endothelial, endometrial, and prostate cancer cells (Griffin et al. 1990; Rokhlin et al. 1996; Zhao and Chegini 1999; Modrowski et al. 2000). The tissue background and its extracellular matrix composition are of key relevance for the modulation of GM-CSF biological activity. Accordingly, GM-CSF is one of many "heparin-binding growth factors" that bind to extracellular matrix components, most frequently to heparan-sulphate-containing proteoglycans (Alvarez-Silva and Borojevic, 1996). These compounds have specific patterns of distribution in normal tissues, which are in turn influenced by physiological transitions or pathologic alterations such as inflammatory reactions. Proteoglycan modulation of the biological activity of heparin-binding growth factors, which can be either stimulatory or inhibitory, follow different patterns: (a) factors may be sequestered in the matrix and released in an active form upon stimulation, (b) their activity can be enhanced by the presence of intercellular molecules that facilitate interaction with the corresponding receptors, and (c) binding to glycoconjugates can protect them from proteases, increasing their stability in the tissue environment (Taipaile and Keski-Oja 1997; Tumova et al. 1999).

In a previous study we have compared bone marrow and peripheral stromas, two of which sustain myeloid proliferation *in vitro* and one which does not, in order to identify the molecular requirements for a given stroma to sustain proliferation of myeloid lineages (Carvalho et al. 2000). We found that the three model stromas produced a similar set of haemopoietins, including GM-CSF, which could be released from the cell layer in an active form by high-

salt buffers. On the other hand, glycosaminoglycans (GAGs) isolated from each stroma presented a different capacity to modulate the activity of GM-CSF. In particular, skin fibroblasts, which do not sustain myelopoiesis *in vitro*, synthesised GAGs able to inhibit the proliferation of hematopoietic cells in response to soluble GM-CSF. We have thus proposed that the ability of a given stroma to sustain myelopoiesis depended both upon the locally produced growth factors and upon the quality of pericellular glycoconjugates. In a parallel study, we have shown that the physical interaction between GM-CSF and heparin, which is presumably required for the modulation of GM-CSF activity, depended upon the physicochemical properties of the environment (Wettreich et al. 1999). In soluble form, these two compounds interacted only at low pH, optimal levels being observed between pH 4 and pH 5. We proposed that the intercellular environment, which includes the negatively charged pericellular glycoconjugates, could provide conditions equivalent to low pH in the aqueous phase and trigger the functional interaction between GM-CSF and proteoglycans. In the present study we have characterised the spatial features, as well as the molecular interactions associated to the particular microenvironment confined between stromal cells and myeloid progenitors. We have used bone marrow-derived and liver inflammatory granuloma-derived stromas that sustain myelopoiesis, and skin fibroblasts that do not. We monitored interactions of these stroma with the FDC-P1 cell line, which is dependent upon GM-CSF both for survival and proliferation.

Materials and Methods

Cells and Cell Cultures

Cell lines were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro). The permanent murine bone marrow stromal cell line S-17, which can sustain proliferation of both murine and human bone marrow blood cell progenitors, was used following the authorisation given by

Dr. Keneth Dorshkind (Collins and Dorshkind 1987). Primary cultures of liver connective tissue GR cells that sustain extramedullar myelopoiesis were obtained from fibro-granulomatous inflammatory reactions elicited in murine liver by schistosomal infection, as previously described (Alvarez-Silva et al. 1993). Briefly, C3H/HeN mice, bred at the Rio de Janeiro Federal University, were infected by 30 cercariae of *Schistosoma mansoni* (BH strain, Instituto Oswaldo Cruz, RJ, Brazil). Mice were killed 90 days after infection, periovular granulomas were isolated by sedimentation from the homogenised liver tissue, and digested by collagenase. The harvested cells were seeded into 25 cm^2 tissue culture flasks and maintained routinely in Dulbecco's Minimum Essential Medium (DMEM – GIBCO, Gaithersbourg, MD, USA) supplemented with 10% foetal bovine serum (FBS – Cultilab, Campinas, SP, Brazil). In order to eliminate trypsin-resistant granuloma macrophages, cells were subcultured using a trypsinisation step. After the third passage, homogeneous connective tissue cell cultures were obtained. These cells have been fully described and characterised in previous studies (Boloukhère et al. 1993, Lazou et al. 1993), and their capacity to sustain myelopoiesis has been analysed in quantitative and qualitative terms (Alvarez-Silva and Borojevic 1996, Dutra et al. 1997). Primary cultures of skin fibroblasts that do not sustain myelopoiesis were obtained from new-born C3H/HeN mice as previously described (Carvalho et al. 2000). Briefly, mice were killed and their skin removed and sequentially digested by collagenase and trypsin. The obtained skin fibroblasts, referred to as "SF", were maintained in culture as described for GR cells. The myeloid progenitor cell line FDC-P1, dependent upon interleukin-3 (IL-3) or GM-CSF for both survival and proliferation, was maintained in DMEM with 10% FBS supplemented with supernatant of WeHi-3B cells that secrete IL-3 constitutively (Ymer et al. 1985).

For co-culture assays, FDC-P1 cells were extensively washed in order to remove IL-3 and plated onto confluent layers of stromal cells in 24-well culture plates (5×10^4 cells / well). Their survival and proliferation were monitored by direct counting under a phase contrast-equipped inverted microscope. When required, the assays were done in the presence of neutralising anti GM-CSF monoclonal antibody (mAb) obtained from

supernatants of 22E9 cells, originally supplied to the Rio de Janeiro Cell Bank by Robert Coffman (DNAX Institute for Cellular and Molecular Biology, Palo Alto, CA, USA). Purified mAb was prepared by Hélio S. Dutra (PABCAM). When used at 2 μg / mL concentration, the mAb fully neutralised 100 U / mL of recombinant murine GM-CSF (Carvalho et al. 2000). Stromal supernatants used for liquid cultures were harvested from confluent stroma layers after 24 h incubation in fresh medium.

For microscopy analyses, removable plastic coverslips were placed into the wells before cell seeding. Further processing will be described below.

Glycosaminoglycans purification

GAGs were purified from the studied stromas as previously described (Mourão et al. 1983; Silva et al. 1990). Briefly, confluent cell monolayers were detached by incubation with 0.125% trypsin and 0.05% EDTA (w/v) in calcium and magnesium free balanced salt solution. Cell pellets, obtained by centrifugation (2000 *g*, 10 min), were incubated with 0.25% trypsin and 0.05% EDTA for 12 h at 37°C. The GAGs were purified by anion exchange chromatography on a DEAE-Sephacel column equilibrated with 0.05 M sodium acetate (pH 5.0), washed with 0.1 M NaCl and eluted with 2.0 M NaCl in the same buffer, followed by extensive dialysis against distilled water. The dialysed samples were lyophilised. Purified heparan sulphate was obtained after digestion of the harvested GAGs with chondroitinase ABC.

Light scattering measurements

Formation of molecular aggregates between heparan sulphate and GM-CSF was monitored by light scattering using a spectrofluorometer as previously described (Wettreich et al. 1999). Briefly, recombinant murine GM-CSF (Amgen Inc, Thousand Oaks, CA, USA) was diluted to a final concentration of 5 μg / mL and transferred to a quartz cuvette. GAGs were added in increasing concentrations, and light scattering intensities were immediately recorded. The

data were corrected for scattering contribution from GAGs and for dilution caused by addition of GAGs stock solution.

Microscopy

For scanning electron microscopy (SEM), stroma layers with adjacent FDC-P1 cells were fixed in 3% glutaraldehyde in phosphate buffer, pH 7.2, washed and dehydrated in ethanol series. The samples were dried at critical point (CPD 030, Balzers Instruments, Liechtenstein) and gold sputtered (FL-9496 Balzers Union Coater). Scanning electron micrographs were obtained with a JEOL JSM-5310 scanning microscope.

For cytochemical studies in transmission electron microscopy (TEM), cell cultures were washed twice with phosphate-buffered saline (PBS), pH 7.2. One experimental group was incubated with neuraminidase (Type III from *Vibrio cholerae*, Sigma Chemical Company, St Louis, MO, USA), 0.15 U /mL, in Tyrode's solution, pH 6.0, for 60 min. In the other group, this treatment was omitted. Both were fixed in 3% glutaraldehyde in phosphate buffer, for 2 h. Cells were washed in PBS and incubated for 2 h with NH_4Cl in order to block aldehyde groups that could interact with ferritin (Danon et al. 1972). They were further washed twice and incubated for 1 h at room temperature with 100 μg / mL cationised ferritin (from horse spleen, Sigma) in PBS, pH 7.4. After washing, samples were post-fixed in 1% buffered OsO_4. Ultrathin sections were contrasted with 7% uranyl acetate and 1% lead citrate in water, and observed in a Zeiss EM-900 transmission electron microscope.

For determination of GAGs localisation, the cultures were fixed in 2.5% glutaraldehyde containing 0.2% cuprolinic blue and 0.2 M $MgCl_2$ in 0.05 M sodium acetate buffer, at pH 5.6 for 24 h. Post fixation was done in 1% sodium tungstate for 2 h (Scott et al. 1989). Alternatively, the glutaraldehyde solution was supplemented with 0.2% ruthenium red or 0.2% alcian blue in 0.1 M cacodylate buffer (pH 7.3) for 4 h (Tsuprun and Santi, 1996). Cells were post-fixed in 1% osmium tetroxide, and processed as above.

Results

Co-culture of the myeloid progenitor, FDC-P1, with different stromal layers clearly revealed differential cellular interactions (Figure 1). SFs did not sustain survival of FDC-P1 and no viable myeloid cells could be detected after 72 h in culture. As previously described (Carvalho et al. 2000), both GR and S-17 cells sustained FDC-P1 survival and proliferation, the density of myeloid cells reaching the highest levels in co-cultures with the S-17 stromal layer. In view of our previous observation that SF cells synthesised the message for GM-CSF, we questioned whether the growth factor could be produced and spontaneously released into the supernatant in an active form. When proliferation of FDC-P1 was monitored in liquid cultures, no growth stimulatory activity was detected in supernatants of bone marrow-derived S-17 cells (Figure 2). This result indicated that myeloid growth factors that sustained proliferation of FDC-P1 in co-culture with S-17 were retained on the surface of the latter and were directly presented to the target cells, a result compatible with our previous observation that treatment of S-17 layers with high ionic strength released fully active factors (Carvalho et al. 2000). Supernatants conditioned by peripheral GR and SF stromas sustained proliferation of FDC-P1 in liquid culture (Figure 2). Surprisingly, the SF supernatant was the most efficient, despite the fact that a SF stromal layer could not sustain FDC-P1 in co-culture (see Figure 1C). These data show that GR and SF spontaneously release biologically active myelopoietic growth factors into the supernatant. Addition of monoclonal anti-GM-CSF antibodies promoted only a slight inhibition of the proliferation level seen in the presence of the GR supernatant, whereas a decrease of approximately 70% was observed in the case of the supernatant of SF (Figure 2). This result indicated that only a small part of the stimulatory activity present in the GR supernatant was due to GM-CSF, while a significant one was due to this growth factor in the SF supernatant.

Identification of biologically active GM-CSF in supernatants of the SF stroma, which could not by itself sustain haemopoiesis, raised the question of why the factor in soluble form could not induce proliferation of FDC-P1 in the

co-culture assay. Since GAGs isolated from the three stromas modulated the GM-CSF activity and, in particular, those from SF presented a predominantly inhibitory effect on GM-CSF-induced proliferation of FDC-P1 (Carvalho et al., 2000), we considered the possibility that GAGs associated to each stroma could be responsible for a distinguished modulation of GM-CSF function. In order to test for this hypothesis, we have studied the interaction between the growth factor and GAGs isolated from each stroma in a cell-free assay. Aggregation was monitored by light scattering and no interaction was observed for GAGs of any of the three stromas when the solution pH was kept neutral (not shown). This result was not surprising because binding of GM-CSF to heparin in solution had been previously shown to occur only when the physicochemical conditions equivalent to low pH were provided (Wettreich et al., 1999). Accordingly, when interaction between GM-CSF and GAGs was measured at acidic pH a bell-shaped profile of aggregation was observed (Figure 3). Comparison of results obtained for the three samples revealed that GAGs isolated from the studied stromal layers interacted with GM-CSF in a similar fashion.

Since the lack of GM-CSF-induced proliferation of FDC-P1 in co-cultures with SF could not be ascribed to a specific blockage by SF-derived GAGs that were spontaneously released into the supernatant (Carvalho et al. 2000), we considered the possibility that GM-CSF present in supernatants could not be capable of stimulating stroma-attached FDC-P1 due to specific spatial features of the microenvironment between the stroma and the target cells in co-culture. We thus decided to characterise this particular region using electron microscopy assessment. When observed in phase-contrast microscopy FDC-P1 cells were round, and the major part of their surface was apparently free in contact with the culture medium (Figure 1). Hence, the stroma supernatant, which was stimulatory for FDC-P1 survival and growth in liquid cultures, had a free access to their surface and should have been able to stimulate the myeloid cells lying over the stroma. Observations in SEM indicated that the contact surface among the FDC-P1 cells and stroma were larger than observed in phase contrast microscopy (Figure 4). FDC-P1 cells extended thin cytoplasmic veils over stromal cells, establishing an extensive

surface of closely adjacent membranes. Moreover, FDC-P1 cells inserted frequently long cytoplasmic projections in between the layers of the stromal cells, that were in tight contact with the stroma. Consequently, narrow intercellular spaces delimited on one side by FDC-P1 cell surface and on the other by the stroma were often present in co-cultures (Figure 4B). Notwithstanding this close cell-cell contact, a large part of the FDC-P1 cells surface was free in a direct contact with the culture supernatant.

In view of the fact that interaction between isolated GM-CSF and GAGs occurred only under conditions equivalent to low pH, we monitored the distribution of negative charges in the pericellular environment. Cationic ferritin labelling of negatively-charged molecules on the surface of the studied cells disclosed different patterns. On the free surface facing the culture medium, FDC-P1 cells were either not labelled or sparsely labelled in irregularly dispersed points (Figure 5A). Closer inspection revealed that the observed sparse labelling was found either close to the cell membrane or being released into the supernatant, suggesting a shedding of the polar moieties of the glycocalyx into the supernatant (Figure 5B, arrows). Such a release occurred frequently on ruffling membranes that were observed on FDC-P1 when they entered in contact with the stroma (Figure 5A, arrows). The establishment of contacts among FDC-P1 cells and stromas was associated with formation of lamellipodia on both cell types (Figure 5C, asterisks). In contrast to the scarce and spotted ferritin labelling on FDC-P1 cells, labelling on the stromal cells formed a continuous thin layer in regions close to cell contacts (arrows), but it was divided in small patches of similar size in the more distant regions (Figures 5C and D, arrowheads). The distance among the patches decreased progressively, increasing the density of the ferritin labelling in the direction of the contact between FDC-P1 and stroma cell membranes. In the regions in which a tight apposition of the membranes occurred, all the intercellular space was heavily loaded with polar negative molecules (Figures 5D, E and F, arrows). These images suggested a capping of the membrane molecules carrying the negative charges at the interface between the two types of cells. Treatment with neuraminidase released nearly all the ferritin-labelled

molecules from the cell surface, indicating that labelling was due mainly to the presence of glycoconjugates containing terminal sialic acid groups (Figure 5G).

The distribution of proteoglycans on the studied cells was analysed by cuprolinic blue and ruthenium red staining. FDC-P1 cells showed a continuous and homogeneous labelling with ruthenium red, which was present in the glycocalyx (Figure 6A, arrows) and in thin thread-like molecules around the cell membranes (Figure 6A, arrowheads). Stromal cells showed two distinct patterns of proteoglycan distribution. Extracellular filamentous matrix was regularly decorated by short rods intensely stained in a pattern typical for collagen-associated proteoglycans (Figure 6B, arrowheads). The S-17 cells had intense cuprolinic blue staining in the large spherical cytoplasmic droplets which they form when reaching confluence (Figure 6C, arrowheads). Similar structures were observed on the external part of the glycocalyx, where polymerisation and organisation of the filamentous extracellular matrix takes place (Figure 6C, arrows). Simultaneously, a membrane associated labelling was observed, reminiscent of the pattern observed with the cationic ferritin (Figures 6D, arrowheads). Accordingly, when FDC-P1 cells entered in contact with stromal cells, an accumulation of the labelled material occurred at their junction, which is compatible with the idea of capping of membrane-associated proteoglycans. In closed intercellular spaces the accumulation of proteoglycans reached a remarkably high density, similar to that of the ferritin-labelled molecules (Figures 6E and F, arrowheads).

Taken together, the morphological data indicated a co-distribution of pericellular membrane-associated, but not matrix-associated, glycoconjugates containing sialic acid and GAGs. The contact between stromal and target cells elicited apparently a movement of membrane-bound molecules, generating a high concentration of the two types of glycoconjugates in the intercellular spaces.

Discussion

The biological activity of GM-CSF depends upon the tissue background in which it is acting. In bone marrow, it is one of the major haemopoietins. In peripheral tissues, it is produced by tissue macrophages or stromal cells early in inflammatory reactions, orchestrating regenerative and fibrotic responses (Andreutti et al. 1998). It may also be a co-factor of abnormal proliferation or neoplasic transformation (Rokhlin et al. 1996; Zhao and Chegini 1999). Consequently, its activity has to be tightly regulated in the context of each tissue. Our previous studies have indicated that the biological activity of GM-CSF heavily depended upon tissue cofactors, heparan-sulphate being one of the major ones (Alvarez-Silva and Borojevic 1996, Carvalho et al. 2000). We have also shown that the physicochemical properties of the pericellular environment were determinant for interaction among GM-CSF and GAGs, and we have proposed that sialylated glycolipids may be an essential element in generating the appropriate conditions for constitution of macromolecular complexes required to place the growth-factor message in the appropriate context (Metcalf, 1993). The present study has given a morphological support for both hypotheses. We have shown that the physical contact among the stroma and the myeloid cells triggers the capping of cell surface molecules, including both sialylated glycoconjugates and proteoglycans. This is an early response to the cell contact signal, since a gradient in concentration of polar surface molecules is observed already on thin lamellipodia formed after the approach of the two cell types, *i.e.* before full spread of haemopoietic cells over the stroma takes place (Figure 5C). Together, the two glycoconjugates generate a specific intercellular environment, very rich in molecules potentially interactive with GM-CSF, and providing the physicochemical conditions required for this interaction.

Surprisingly, we have found that the cell contact between myeloid cells and fibroblasts, which do not sustain myelopoiesis, is inhibitory for the overall myeloid cell responsiveness to GM-CSF. Even a relatively minor contact-surface is sufficient to turn myeloid cells unable to use the GM-CSF present in the supernatant liquid environment as a factor of survival or proliferation. In view of the described pattern of surface distribution of proteoglycans and sialylated glycoconjugates, such as observed in Figures 5E

and 6D, and their capping at the interface between the haemopoietic and stromal cells, it may be hypothesised that GM-CSF receptors are sequestered in lipid rafts, which are withdrawn from the free cell surface, turning the cell unresponsive to the growth factors present in the supernatant. Alternatively, the physical contact with the fibroblast membrane may generate intracellular signals that inhibit secondary messengers leading to cell proliferation and survival. These hypotheses are object of ongoing studies.

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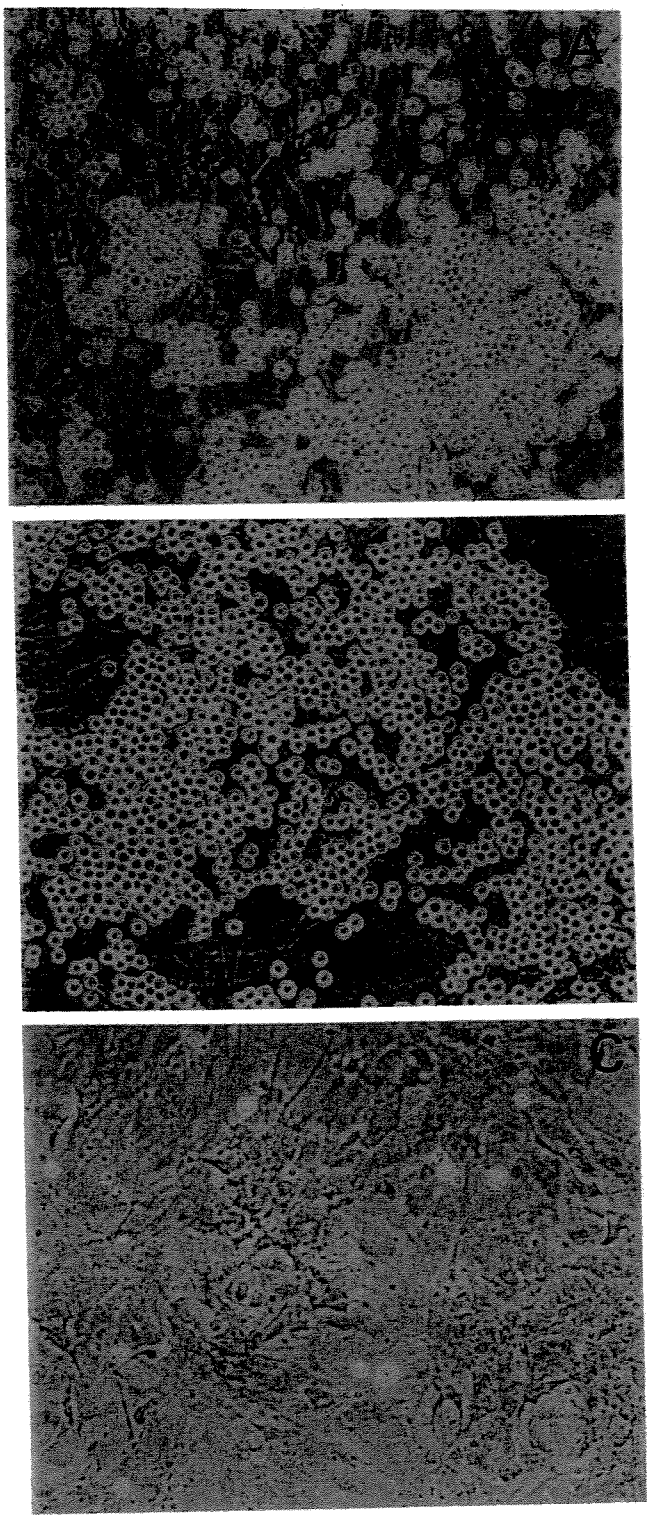

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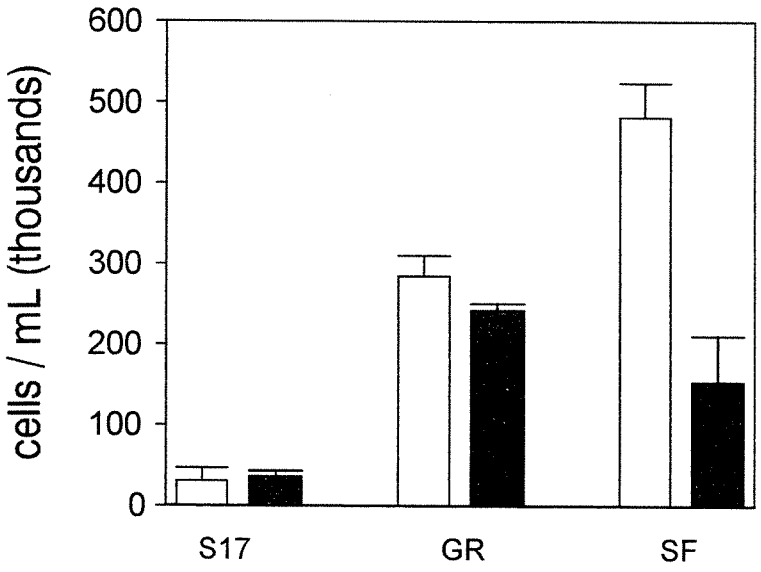

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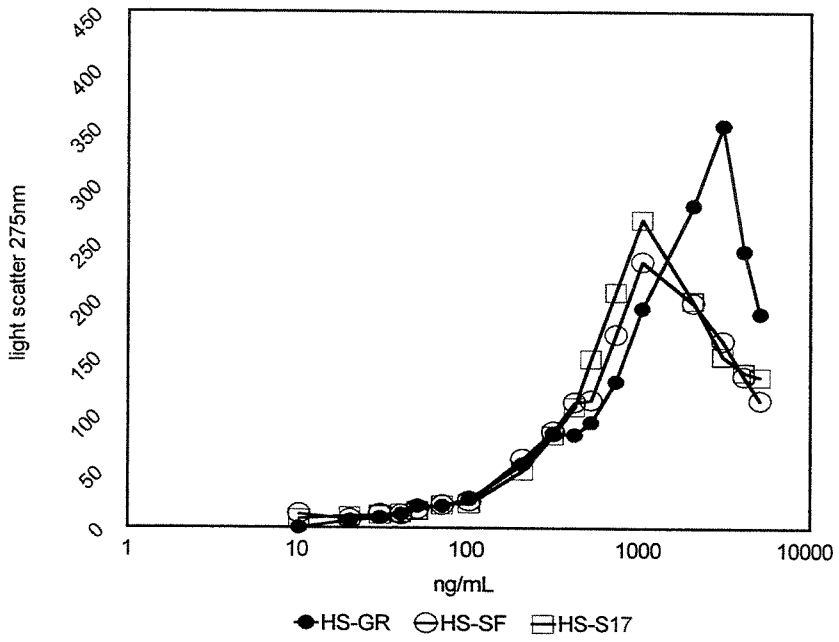
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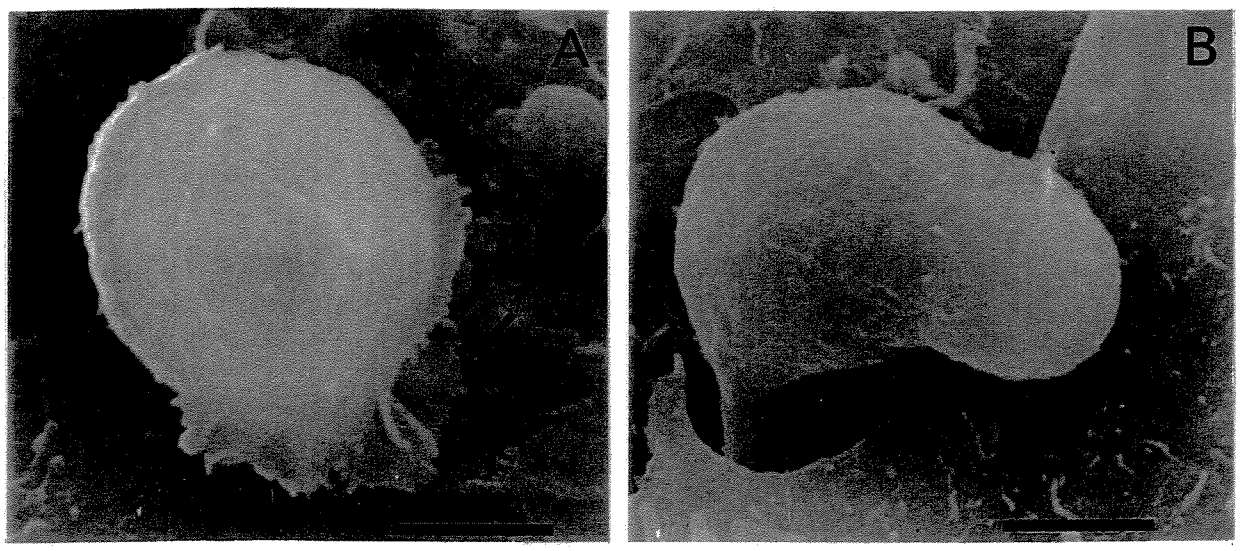
Figure 3



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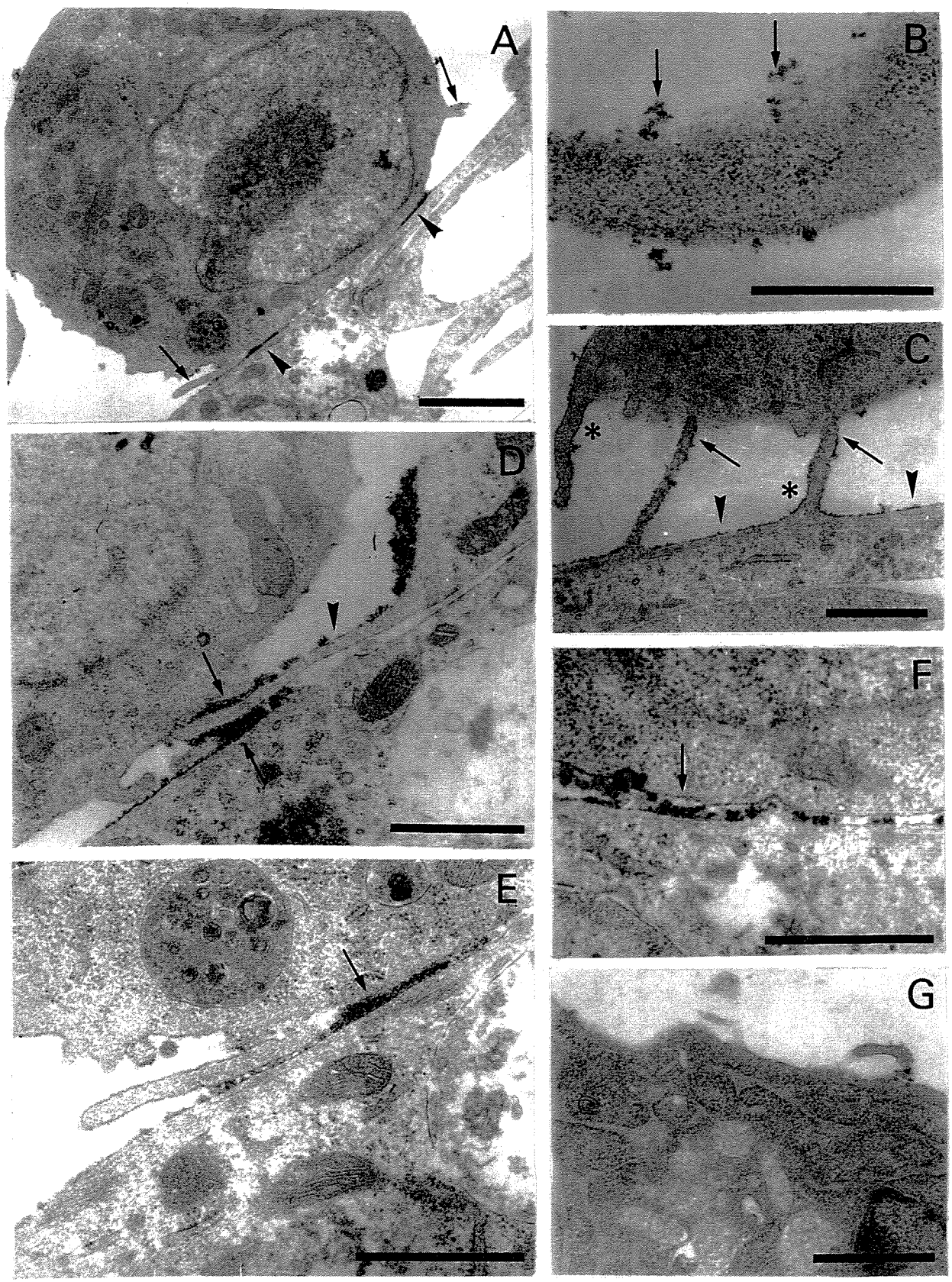

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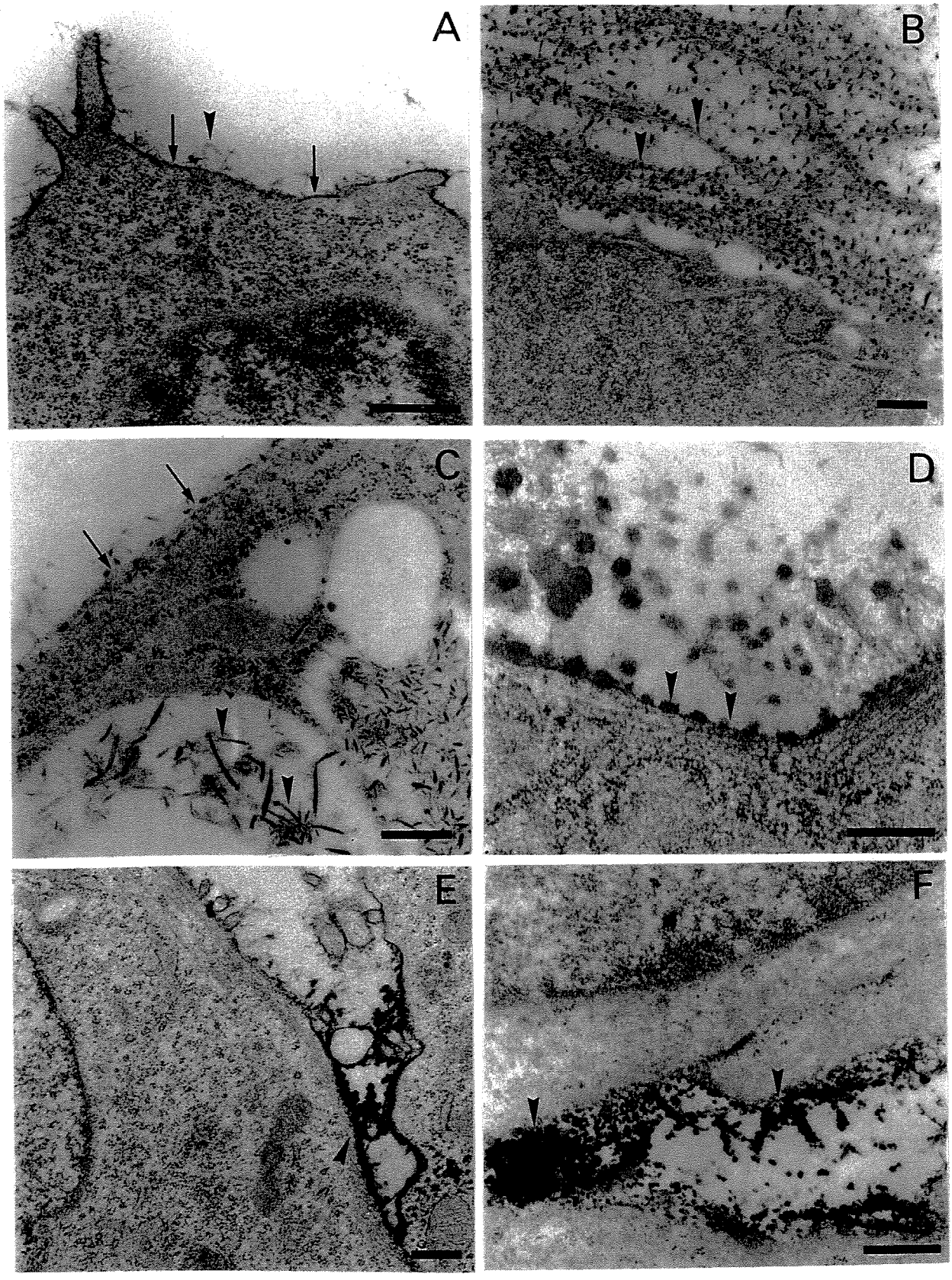

Figure 6

FIGURE LEGENDS

Figure 1.

FDC-P1 cells plated over confluent stromas of bone marrow stroma-derived S-17 cells (A), hepatic granuloma-derived GR cells (B) and skin fibroblasts SF cells (C), without addition of haemopoietins. Phase-contrast images represent cultures 48 h after plating. SF sustained neither survival nor proliferation of FDC-P1 cells.

Figure 2.

Quantification of FDC-P1 cells maintained for 48 h in the presence of supernatants harvested from stromal cultures of bone marrow-derived S-17 cells, granuloma-derived GR cells, and skin fibroblasts SF cells (empty bars) and corresponding supernatants supplemented with neutralising anti-GM-CSF monoclonal antibody (full bars). Results represent mean values of two independent experiments done in triplicate, and standard errors.

Figure 3.

Aggregation between isolated GM-CSF and cell-associated GAGs purified from bone marrow stroma-derived S-17 cells (□), hepatic granuloma-derived GR cells (●) or skin fibroblasts SF cells (O). Light scattering was measured in a spectrofluorometer by setting both excitation and emission monochromators at 275 nm.

Figure 4.

FDC-P1 cells plated over the GR stroma. Note in **A** the ruffling membrane on the lower surface of the cell (arrows) and the thin lamellipodia spreading over the stroma (arrowhead). In **B** the FDC-P1 cell extends a cytoplasmic projection between two underlying stromal cells. Bar = 3 μM.

Figure 5.

A. FDC-P1 cell on a GR stromal layer. Cationic ferritin labels negatively-polar molecules on the cell surface. Note the low labelling on free cell surfaces and the intense labelling at cells interface (arrowheads). Arrows point to sparse labelling on regions of membrane ruffling. Bar = 2 μM. **B**. A ruffling membrane of the FDC-P1 cell. Note the spotted labelling with cationic ferritin, and morphological indication of the shedding of polar molecules into the liquid medium (arrows). Bar = 1 μM. **C**. Establishment of the contact between FDC-P1 cell (upper) and GR stromal cell (lower). Lamellipodia are formed from both sides (asterisks). Note the uniform punctuate distribution of cationic ferritin on GR cell surface (arrowheads), and the increasing labelling in the region of contact with the FDC-P1 cell (arrows). Large but rare labelled moieties are seen on the FDC-P1 cell. Bar = 1 μM. **D** and **E** Interface between FDC-P1 cells and stroma. Note the increasing accumulation of polar molecules in the spaces delimited by the cell membranes (arrows). Polar molecules are not released into the supernatant, suggesting that they are anchored in membrane-associated molecular complexes. Bars = 1 μM. **F**. Thin intercellular space between FDC-P1 (upper) and stroma (lower) cells. Note that the space is filled with polar molecules (arrows). Bar = 1 μM. **G**. GR stroma treated with neuraminidase prior to labelling with cationic ferritin. Note the absence of polar moieties on the membrane. Bar = 1 μM.

Figure 6.

Proteoglycans labelling on the surface of FDC-P1 and stromal cells. **A**. FDC-P1 cell labelled with ruthenium red. Note the continuous labelling of the glycocalyx (arrows) and the thin thread-like molecules around the cell membranes (arrowheads). Bar = 0.5 μM. **B**. Ruthenium red labelled SF stroma with the *in vitro* secreted extracellular matrix. Note the regular distribution of the filamentous collagen-associated proteoglycans (arrowheads). Bar = 0.5 μM. **C**. Cuprolinic blue labelled S17 stromal layer. Note the large proteoglycan rods in the intracellular secretory vacuoles (arrowheads), and small proteoglycan moieties associated with the cell membrane (arrows). Bar = 0.5

μM. **D**. Ruthenium red labelled SF stroma showing the regular punctuate distribution of proteoglycans on the cell membrane (arrowheads). Similar patter in observed in appropriate sections of the other studied stromas. Bar = 0.5 μM. **E** and **F** Ruthenium red labelling of the interface between FDC-P1 cells and the S17 stroma. Note the intense accumulation of the labelled material between the cells (arrowheads). Bars = 0.5 μM.

ANEXO 3

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Original article

The capacity of connective tissue stromas to sustain myelopoiesis depends both upon the growth factors and the local intercellular environment

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In adults, haemopoiesis is located in the bone marrow, where it is tightly regulated by cytokines and by a physical association of haemopoietic progenitors with the stroma. However, in pathological situations, haemopoiesis can be partly or fully dislodged to peripheral tissues. It is not clear which are the requirements for a given peripheral stroma to sustain haemopoiesis. Using the growth factor-dependent cell line FDC-P1, we have compared the myelopoietic capacities of a murine bone marrow-derived cell line S17, a liver inflammatory granuloma-derived stroma (GR) that sustains haemopoiesis, and normal skin fibroblasts (SF) that sustain neither survival nor proliferation of myeloid cells. All three stromas expressed mRNA for major haemopoietins with the exception of IL-3. Despite the incapacity of SF to sustain FDC-P1 cells, the biologically active GM–CSF could be recovered from all the studied stromas by treatment with high-salt buffers that release non-covalently bound molecules from stroma cells. Glycosaminoglycans purified from stromas had distinct effect on the GM–CSF-mediated proliferation of FDC-P1 cells: those purified from S17 and GR cells were stimulatory, whereas those obtained from SF cells were slightly stimulatory at low concentration, but inhibitory at the higher ones. We conclude that the quality of the stroma pericellular glycoconjugates is determinant for the ability of a given stroma to sustain myelopoiesis, even when biologically active haemopoietins are locally produced. © 2000 Éditions scientifiques et médicales Elsevier SAS

haemopoiesis / cytokines / glycosaminoglycans / FDC-P1 cells / S17 cells / liver connective tissue

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1. INTRODUCTION

The haemopoietic system maintains the basal homeostasis of blood cells and can respond to the specific peripheral demands by modifications of the rate and the pattern of production of one or several blood cell lineages. The tightly regulated proliferation, commitment, and terminal differentiation of haemopoietic precursors occurs normally within the bone marrow microenvironment composed of stroma cells, the associated extracellular matrix, and the growth factors or cytokines that can be locally produced or brought into the bone marrow by blood circulation. However, in contrast to the tight regulation of haemopoiesis in the bone marrow stands the fact that it can also occur in extramedullar sites. This is observed during the foetal development, when haemopoiesis moves from yolk sac to the para-aortic region, liver and spleen (Delassus and Cumano, 1996, Tavian et al., 1999). In the adult life, a partial of full haemopoiesis can also occur in peripheral tissues, such as spleen or liver, being frequently associated with inflammatory reactions (Borojevic and Carvalho, 1981; Borojevic et al., 1983, 1989; Geuskens et al., 1991). These observations infer that connective tissue stromas other than that of the bone marrow can substitute, at least partially, for the haemopoietic medullar stroma. The molecular properties of a given stroma that are required to sustain haemopoiesis are not clear.

At each stage of the production of blood cells, the proliferation and differentiation of haemopoietic precursors are strictly dependent upon the presence of the appropriate set of growth factors. Consequently, one of the caveats of the capacity of a given stroma to sustain haemopoiesis is the production of the required cytokines (Metcalf, 1993). The other one is the fact that growth factors have to be presented to the target cells in the context of the appropriate intercellular environment, since it has been well established that the long term production of blood cells depends upon the cell–cell contacts of blood cell progenitors with stroma cells and with the associated matrix (Allen et al. 1990).

More than 20 years ago, Dexter and his colleagues have developed an in vitro system of long-term bone marrow cultures, in which cellular interactions are amenable to experimental analysis. Early studies of this experimental model have shown that proteoglycans of bone marrow extracellular matrix are involved in the spatial organisation of the medullar haemopoietic environment, as well as in stabilisation and presentation of specific growth factors to the corresponding myeloid precursors (Roberts et al. 1988; Gallagher and Dexter, 1988). The same type of molecule is considered to participate in adhesion of cells inside the bone marrow, and in the control of their release into blood circulation after maturation (Kolset and Gallagher, 1990). Accordingly, recent in vitro studies have shown that an adequate combination of glycosaminoglycans and growth factors is required and sufficient for a relatively long-term survival and proliferation of early human blood cell precursors (Gupta et al. 1998, 2000).

In the present study, we have compared the haemopoiesis in the context of bone marrow-derived stroma and of two stromas derived from extramedullar sites, one of which can sustain in vivo peripheral haemopoiesis, and the other one that cannot (Dutra et al., 1997). We have questioned whether the presence of appropriate proteoglycans together with the production of the required growth factors may be determinant for the capacity of a given stroma to sustain haemopoiesis. Previous studies have shown that functional association of the major haemopoietic growth factors to stroma involves heparan sulphate proteoglycans, which can modulate their mitogenic activity (Alvarez-Silva and Borojevic 1996; Modrowski et al., 2000). Moreover, soluble heparin and heparan sulphate were shown to have biological activities similar to the corresponding stroma-associated proteoglycans, either in Verfaillie-type cultures of human haemopoietic cells (Gupta et al., 1996) or in the proliferation of growth factor-dependent cell lines (Alvarez-Silva and Borojevic, 1996; Lipscombe et al., 1998). These findings suggested that a physical interaction between growth factors and heparan sulphate occurs in vivo, and may be determinant for the growth factor's biological activity. We could show now that both the appropriate set of cytokines and the quality of glycosaminoglycans are determinant for the myelopoietic capacity of a given connective tissue stroma.

2. MATERIALS AND METHODS

2.1. Materials

Dulbecco's minimum essential medium (DMEM), collagenase (type IA), streptomycin, penicillin, recombinant murine granulocyte–macrophage colony stimulating factor (GM–CSF), HEPES, EDTA, TRIS, glutaraldehyde, thiazolyl blue (MTT) and trypsin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Foetal bovine serum (FBS) was obtained from Cultilab (Campinas, SP, Brazil) and plastic tissue culture dishes from Nunc (Roskilde, Denmark). Oligo(dt) 12–18 primers, dATP, dCTP, dGTP, dTTP, and Super-Script II Reverse Transcriptase were obtained from GIBCO–BRL (Gaithersburg, MD, USA), and Taq DNA polymerase from CENTBIO (Porto Alegre, RS, Brazil).

2.2. Cells and cell cultures

Permanent cell lines were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio

de Janeiro, RJ, Brazil). The murine haemopoietic bone marrow stroma cell line S17 is able to sustain both murine and human haemopoietic cells in vitro (Siczkowski et al. 1992). The multipotent myeloid precursor cell line FDC-P1 is dependent upon interleukin-3 (IL-3) or granulocyte–macrophage colony stimulating factor (GM–CSF); it was routinely maintained in DMEM with 10% FBS, supplemented with supernatant of WeHi-3B cells that secrete IL-3 constitutively (Ymer et al. 1985).

Liver connective tissue cells that sustain extramedullar myelopoiesis were isolated from hepatic fibrous granulomas elicited in mouse livers by schistosomal infection, as previously described (Alvarez-Silva et al. 1993). Briefly, C3H/HeN mice were infected by 30 cercariae of *Schistosoma mansoni* (BH strain, Instituto Oswaldo Cruz, RJ). Mice were killed 90 days after infection, periovular granulomas were isolated from the homogenised liver tissue by sedimentation and digested by collagenase. The harvested cells were seeded into 25 cm^2 tissue culture flask in DMEM with 10% FBS. Cells were subcultured by trypsinisation that eliminated the trypsin-resistant granuloma macrophages. After the third passage, homogeneous connective tissue cell cultures were obtained, named 'GR' cells.

Primary cultures of skin fibroblasts that do not sustain myelopoiesis were obtained from newborn C3H/HeN mice. Mice were killed, their skin harvested and digested by collagenase (1 $mg \cdot mL^{-1}$ in DMEM) for 1 h, at 37°C, under stirring. This treatment was followed by trypsin (0.125% w/v in calcium- and magnesium–free balanced salt solution (BSS) supplemented with 0.05% EDTA), for 30 min at 37°C. The obtained skin fibroblasts, referred to as 'SF', were maintained in culture as described for GR cells.

In the co-culture assays, the FDC-P1 cells, previously washed with BSS in order to remove IL-3, were inoculated onto the confluent monolayers of SF, GR and S17 cells in 24 or 96-wells culture plates, at 5×10^4 and 5×10^3 cells per well, respectively. After 48 and 96 h of co-incubation they were quantified by counting under the microscope equipped with phase contrast.

2.3. Harvesting of cell supernatants and 'high ionic force dislodged molecules' (HIFDM)

WeHi-3B supernatants were harvested from 72 h cultures of cells originally seeded at 10^5 $cell \cdot mL^{-1}$ in DMEM with 10% FBS.

Confluent stroma cell layers were washed with BSS, and incubated with 2 M NaCl for 30 min. The supernatants containing the molecules dislodged by high ionic force were dialysed against bi-distilled water and concentrated (×10) before using.

2.4. Labelling and isolation of sulphated glycosaminoglycans from stromal cell cultures

Stromal cell cultures that reached confluence were treated with 30 µCi of [^{35}S]-Na_2SO_4 in culture medium. Cultures were incubated in this medium at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Sulphated glycosaminoglycans were isolated from the intracellular, pericellular (containing cell-surface glycosaminoglycans) and extracellular compartments of stromal cultures as previously described (Silva et al. 1992a,b). Briefly, at the completion of the labelling period, stroma-conditioned media containing 'extracellular' glycosaminoglycans were removed, cleared by centrifugation and stored at –20°C until required. Stroma cells were rinsed with PBS (pH 7.4), detached by treatment with 0.25% trypsin and 0.05% EDTA in PBS, for 10 min at 37°C. Centrifugation of the trypsinate ($2{,}000 \times g$ for 10 min at room temperature) separated the supernatant and the cell pellet, containing the 'pericellular' and 'intracellular' glycosaminoglycans, respectively. These fractions obtained from stromal cultures were incubated for 12 h at 37°C with PBS (pH 7.4) containing 0.25% trypsin and 0.05% EDTA, and dialyzed against distilled water. The samples were further incubated with 10 mg papain in the presence of 5 mM EDTA and 5 mM cysteine at 60°C for 24 h. The [^{35}S]-labelled glycosaminoglycans were purified by anion exchange chromatography on a DEAE column as described below.

For isolation of extracellular glycosaminoglycans, the previously collected stromal supernatants were incubated with 10 mg papain in the presence of 5 mM EDTA and 5 mM cysteine at 60°C for 24 h, then dialysed against distilled water and applied to a DEAE–cellulose column (3.5 cm × 2.5 cm), equilibrated with 0.05 M sodium acetate (pH 5.0). The column was washed with 100 mL of the same buffer. The [^{35}S]-glycosaminoglycans were eluted from the column with 1.0 M NaCl and exhaustively dialysed against distilled water. The dialysed glycosaminoglycan samples from stromal media were lyophilised, and dissolved in 0.2 mL distilled water.

For cell proliferation assays, cell membrane-associated gycosaminoglycans were purified from the studied stromas as previously described (Alvarez-Silva and Borojevic, 1996).

2.5. Identification of the [^{35}S]-labelled glycosaminoglycans

[^{35}S]-labelled glycosaminoglycans were identified by their mobility in agarose gel electrophoresis alongside with known standards, before and after enzymatic degradation with chondroitin AC or ABC lyases, or deaminative cleavage with nitrous acid. Agarose gel

Table I. RT–PCR primers used for the cytokine expression monitoring assay.

Cytokine	*Primer sequence*	*Reference*
β-actin	5' GTGGGCCGCTCTAGGCACCA 3' 5' CTCTTTGATGTCACGCACGATTTC 3'	Alonso et al. 1986
IL-3	5' GAAGTGGATCCTGAGGACAGATACG 3' 5' GACCATGGGCCATGAGGAACATTC 3'	Yokota et al. 1984
GM–CSF	5' AGAAGCTAACATGTGTGCAGACCCG 3' 5' ATTCCAAGTTCCTGGCTCATTACGC 3'	Gough et al. 1984
SCF	5' CCGGATCCTGGAGCTCCAGAACAGCTAA 3' 5' GGCTGCAGTCCACAATTACACCTCTTGAA 3'	Flanagan et al. 1991
HGF	5' TACGCCCACGCCAAAGAATGGC 3' 5' CGCAAACCAGGTCGAGATGTGAAGC 3'	Degen et al. 1991

electrophoresis was carried out as previously described (Silva et al. 1992a). After electrophoresis, glycosaminoglycans were fixed in the gel with 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide in water, and stained with 0.1% toluidine blue in acetic acid:ethanol:water (0.1:5:5, v:v). The [^{35}S]-labelled glycosaminoglycans were visualised by autoradiography of the stained gels. The radioactive bands having identical electrophoretic migration as standard glycosaminoglycans were carefully scraped into 10 mL 0.5% PPO:toluene solution and counted in a liquid scintillation counter.

2.6. FDC-P1 proliferation monitoring

FDC-P1 cells were plated in 96-well tissue culture plates, 5×10^4 cells per well, in the presence of recombinant murine GM–CSF or supernatants of WEHI-3B cells (positive controls), or in the presence of HIFDM from the studied stromas. Cells were quantified after 24 h incubation using the thiazol blue MTT colorimetric assay (Mosmann, 1983). The assays were made in presence and absence of neutralising anti GM–CSF monoclonal antibody (mAb) obtained from supernatants of 22E9 cells, originally supplied to the Rio de Janeiro Cell Bank by Robert Coffman (DNAX Institute for Cellular and Molecular Biology, Palo Alto, CA, USA). Purified mAb were prepared by Hélio S. Dutra (PABCAM). Alternatively, FDC-P1 cells were incubated with GM–CSF (25 U·mL^{-1}), and the proliferation was monitored by MTT assay after 96 h, in the presence of different concentrations of purified heparins (generously supplied by Karl P. Dietrich and Helena Nader, Universidade Federal de São Paulo, Brazil), commercial bovine kidney heparan sulphate (Sigma), or heparan sulphate purified from the assayed stromas.

2.7. Cytokine expression monitoring assay

Total RNA of WeHi-3B, FDC-P1, SF, GR and S17 cells was extracted using TRIzol® (Gibco–BRL) following the manufacturer's instructions. Expression of β-actin, IL-3, GM–CSF, hepatocyte growth factor (HGF) and stem cell factor (SCF) was monitored by the standard reverse transcriptase–polymerase chain reaction (RT–PCR) as previously described (Brito and Borojevic, 1997) using thirty amplification cycles. The sequence of the primers used are listed in *table I*.

3. RESULTS

The capacity of the studied connective tissue stromas to sustain myelopoiesis was monitored using the myeloid FDC-P1 cell line, which is dependent upon the myelopoietic growth factors for both survival and proliferation. FDC-P1 cells were plated over confluent stroma layers and their survival and/or proliferation were monitored during three days (*figure 1*). In co-

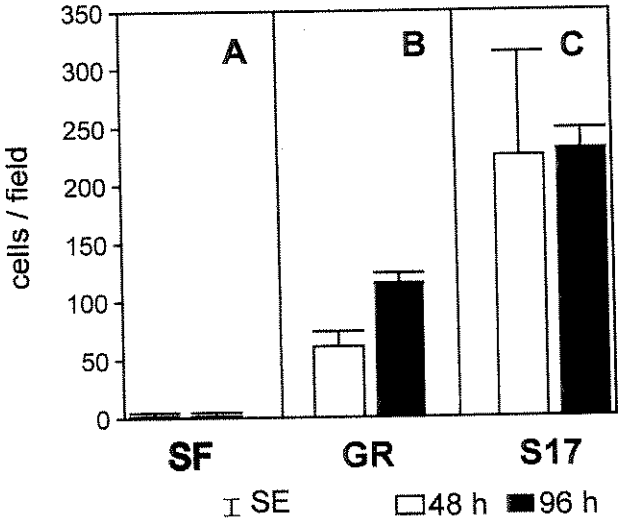

Figure 1. Co-culture assay. Proliferation of FDC-P1 cells after 48 h (open bars) or 96 h (black bars) culture over confluent monolayers of SF (**A**), GR (**B**) or S17 (**C**) stromas. Results represent viable cell counts per microscope field (×250); mean values of 50 fields, counted in two independent experiments, and standard errors (SE) are shown.

cultures, the skin fibroblasts (SF) could sustain neither the proliferation nor the survival of FDC-P1 cells, and after 48 h no myeloid cells could be found any more in the culture (*figure 1A*). In accordance with previous results (Siczkowski et al. 1992; Alvarez-Silva and Borojevic, 1996), the bone marrow-derived (S17) and inflammatory granuloma-derived (GR) cells sustained the proliferation; the S17 stroma induced a more rapid and intense proliferation as compared to GR cells (*figure 1B, C*). The proliferating FDC-P1 cells adhered to the stroma and occasionally migrated into the stroma cell layer.

Since FDC-P1 cells depend upon haemopoietins (principally IL-3 and GM–CSF), we have questioned whether the stromas produced the required growth factors, and we monitored their expression by RT–PCR (*figure 2*). All the assayed stromas expressed GM–CSF, and no one expressed IL-3. All the stromas expressed SCF that has also been reported to stimulate FDC-P1 cells (Caruana et al. 1993), and HGF that is known to have a myelopoietic activity (Nishino et al. 1995). When comparing the stroma monolayers alone with those co-incubated with myeloid cells, we could not observe any modulation of growth factor transcription induced by the presence of FDC-P1 cells, such as observed for mast cell-mediated induction of SCF expression in liver connective tissue stromas, reported previously (Brito and Borojevic, 1997; Gaça et al. 1999) (*figure 2*).

Since SF expressed the same set of cytokines as the two stromas that sustained efficiently myelopoeisis, we questioned whether these cytokines were produced and functional in the used assays. The stroma-dependent growth stimulatory activity has been related to the non-covalent binding of growth factors with cell membrane-associated molecules or with the adjacent extracellular matrix components, and in particular with heparan sulphate, suggesting that growth factors might be retained on the stroma layer and presented to the target cells (Gallagher and Dexter,1988). In accordance with this proposal and with our previous results for the GR cells (Alvarez-Silva and Borojevic, 1996), we have dislodged the non-covalently bound molecules from the cell layer by a high ionic strength solution, and monitored their biological activity (*figure 3*). The dislodged molecules (HIFDM) from all the three stromas had indeed a stimulatory activity for FDC-P1 cell proliferation (*figure 3C*, open bars). Neutralising anti-GM–CSF antibodies, used in conditions under which they fully inhibited the GM–CSF activity (*figure 3A, B*), inactivated a large part, but not all the stimulatory activity of stroma-derived HIFDM, indicating the presence of biologically active GM–CSF together with other growth factors (*figure 3C*, black bars). The total amount of GM–CSF activity released from SF was smaller as compared to GR and S-17 cells,

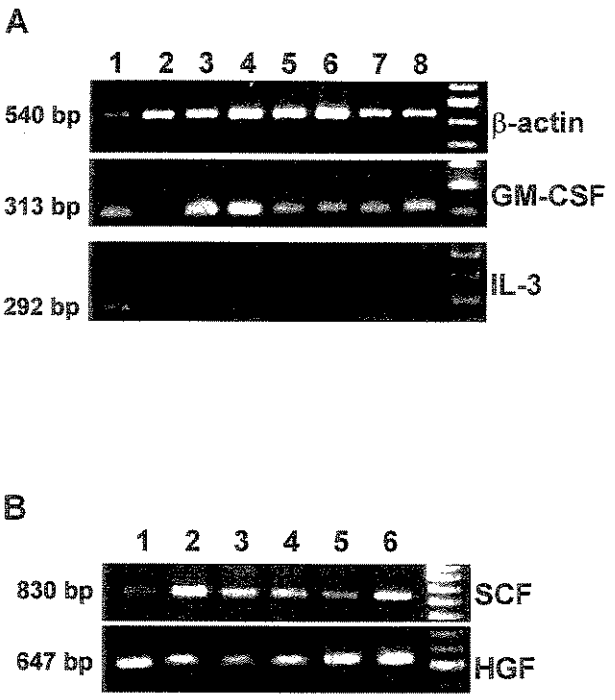

Figure 2. RT–PCR analysis of expression of haemopoietin genes in S17, GR and SF stromas. Amplification of cDNA for β-actin, GM–CSF, IL-3 is shown in (**A**), for WeHi-3B cells (**1**), FDC-P1 cells (**2**), S17 cells (**3**), S17 cells in co-culture with FDC-P1 cells (**4**), GR cells (**5**), GR cells in co-culture with FDC-P1 cells (**6**), SF (**7**) and SF in co-culture with FDC-P1 cells (**8**). Amplification of cDNA for stem cell factor (SCF) and hepatocyte growth factor (HGF) is shown in (**B**) for S17 cells (**1**), S17 cells in co-culture with FDC-P1 cells (**2**), GR cells (**3**), GR cells in co-culture with FDC-P1 cells (**4**), SF (**5**) and SF in co-culture with FDC-P1 cells (**6**).

but it was significantly higher than in the controls ($p < 0.05$). This result was in accordance with the observed presence of mRNA for the selected set of growth factors, indicating that the corresponding proteins were indeed produced in a biologically active form.

Taken together, these results indicated that: a) the myelopoietic capacity of the assayed stromas depended upon the stimulation mediated by growth factors associated with the cell membrane, b) the growth factors could be either retained on the stroma layer or dislodged by high salt concentrations, and c) the biological activity of growth factors released in the soluble form was distinct from that of the membrane-bound factors.

GM–CSF and HGF are known to belong to the group of heparin-binding growth factors, and cell membrane-bound or the extracellular matrix heparan-containing proteoglycans can modulate the growth factors' biological activity (Alvarez-Silva and Borojevic, 1996; Sakata et al., 1997). Consequently, we determined the

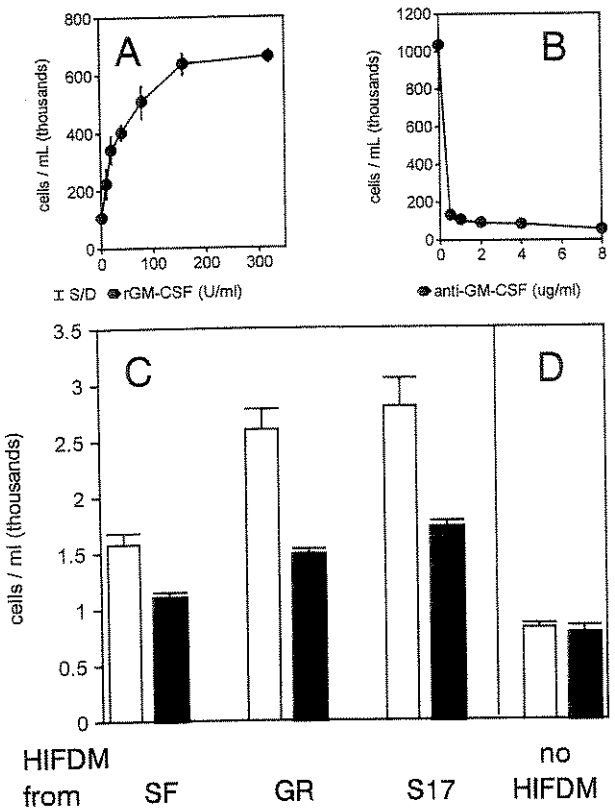

Figure 3. FDC-P1 cell proliferation in presence of increasing quantities of recombinant murine GM–CSF (**A**), 200 U·mL^{-1} rGM–CSF and increasing quantities of neutralising monoclonal antibody for murine GM–CSF(**B**), molecules dislodged from the confluent stromas by high ionic force (HIFDM) (**C**,**D**). FDC-P1 cells were quantified after 24 h incubation with the assayed molecules. HIDFM from SF, GR or S17 cell layers (open bars) is shown in (**C**), as compared to proliferation in the control condition without addition of any supplement (**D**). Partial neutralisation of the growth stimulatory activity by monoclonal antibody for murine GM–CSF is shown by black bars. Mean values of two independent experiments done in sextuplicate and the standard deviation in (**A**) and errors in (**C**,**D**) are shown. Cell numbers in all the cultures stimulated with HIFDM (**C**) were significantly different from that of cultures without HIFDM (**D**) ($p < 0.05$, Mann Whitney *U* test), as well as all the assays of proliferation inhibition with the neutralising monoclonal antibody (open versus black bars).

amounts of [^{35}S]-labelled glycosaminoglycans found in the intracellular, pericellular and extracellular compartments of the studied stroma cells in vitro after a 24 h labelling period with [^{35}S]-Na_2SO_4. These glycosaminoglycans were characterised following their migration in agarose gel and digestion with chondroitin lyases or deamination with nitrous acid. One glycosaminoglycan migrated in agarose gel as heparan sulphate standard, and disappeared totally from the gel after deaminative cleavage with nitrous acid. Another [^{35}S]-glycosaminoglycan migrated as chondroitin sulphate standard. It was totally digested by chondroitin lyases (*figure 4*). These experiments characterised these two glycosaminoglycans as heparan sulphate and chondroitin sulphate, respectively.

Based on these experiments we calculated the relative proportions [^{35}S]-heparan sulphate and [^{35}S]-chondroitin sulphate in the various compartments of the three types of stromal cells (*figure 5A–C*). The stromal cells differed significantly in the distribution of [^{35}S]-labelled glycosaminoglycans in the intracellular compartment. However, only slight differences were observed in relative proportions of heparan sulphate and chondroitin sulphate within the fractions that potentially participated in interactions with growth factors, such as those retained in the glycocalyx (pericellular compartment), and no differences were found among those produced and released into the culture medium that potentially became associated with the adjacent extracellular matrix (extracellular compartment). These results indicated that quantitative differences in glycosaminoglycans were probably not responsible for wide differences in the biological activity of stroma-associated haemopoietins.

In view of previous results on the control of GM–CSF activity by heparin and heparan-sulphate (Alvarez-Silva and Borojevic, 1996; Gupta et al., 1998), and the fact that SF cells produced the GM–CSF in an active form, but could not sustain FDC-P1 survival and proliferation in the direct cell–cell contact, we monitored the FDC-P1 proliferation in the presence of a constant quantity of recombinant murine GM–CSF and increasing quantities of glycosaminoglycans. In the assay using two heparins and a commercial bovine kidney-derived heparan sulphate, only the 12 kDa heparin (Hep12) showed a significant stimulatory activity in the assayed range of concentrations: the stimulatory activity reached the plateau in low concentrations and it did not increase further in a broad range of concentrations (*figure 6*). Conversely, the heparan sulphates purified from the studied stroma cell layers and used in soluble form showed distinct patterns of modifications of the GM–CSF activity, specific of each cell line. The S17-derived heparan sulphate had a low stimulatory activity over a broad range of concentrations, the GR-derived one had a distinct stimulatory effect in a narrow range of low concentrations, whilst the heparan sulphate obtained from SF had only a slight stimulatory activity in low and a clear inhibitory activity in the higher concentrations (*figure 7*).

Taken together these results indicated that a) the cell layer glycosaminoglycans could either stimulate or inhibit the growth factors' biological activity in appropriate concentrations, and b) under the used experimental conditions the stroma-derived gly-

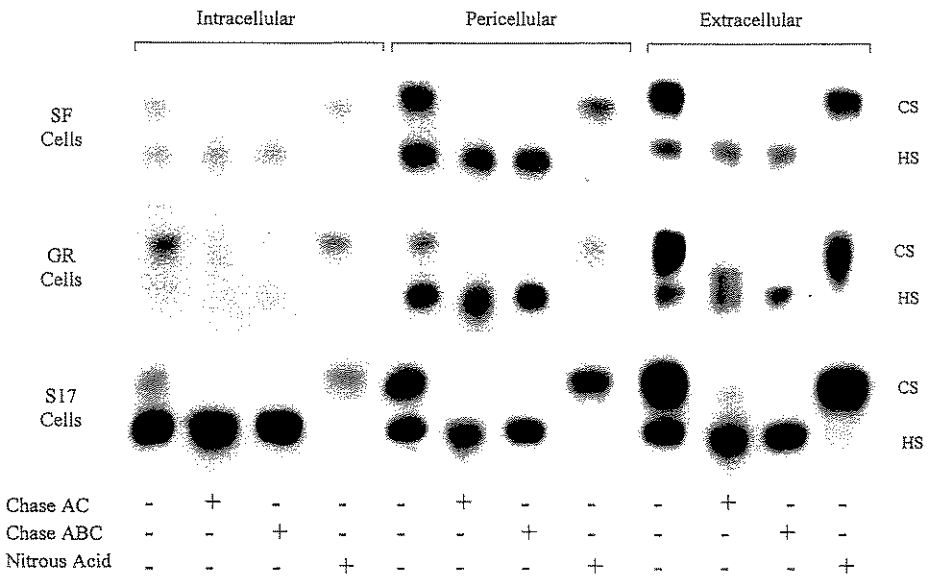

Figure 4. Agarose gel electrophoresis analysis of [^{35}S]-glycosaminoglycans from the studied stromas. Autoradiograms of the [^{35}S]-glycosaminoglycans from the intracellular, pericellular and extracellular compartments of SF, GR and S17 stromal cells before (–) and after enzymatic degradation with chondroitin AC lyase (+), chondroitin ABC lyase (+), or deaminative cleavage by nitrous acid (+). Chase AC: chondroitin AC lyase; Chase ABC: chondroitin ABC lyase; HS: heparan sulphate; CS: chondroitin 4/6 sulphate.

cosaminoglycans modulated the GM–CSF activity in a cell-type-specific pattern.

4. DISCUSSION

The present study has shown that functional interactions between the connective tissue stroma and myelopoietic cell lines depended upon the juxtacrine interactions among the cells, such as those occurring in the haemopoietic tissues, in which a close and extensive apposition among the membranes of stromal and myeloid cells is observed (Allen et al. 1990). The same set of myelopoietic growth factors, endogenously produced by all three cell stromas studied, were either fully active or completely inactive, depending upon the intercellular microenvironment in which they were acting. Heparan sulphate, which has been shown to modulate the studied growth factors' activity, was also relevant for the cell communication in our experimental model (Alvarez-Silva and Borojevic, 1996; Modrowski et al., 2000; Gupta et al. 2000).

It is noteworthy that all three stromas expressed GM–CSF, and produced the protein, which could be released in a biologically active form by treatment with high salt solutions. In particular, the SF stroma, which could sustain neither survival nor proliferation of FDC-P1 cells, produced the active growth factors, indicating that the SF pericellular environment was not permissive or was inhibitory for myeloid cell proliferation. The activity of haemopoietins, which are widely present in tissues and body fluids, and in particular that of GM–CSF, is known to be both redundant and pleiotropic (Metcalf, 1993). These characteristics entail a risk of an illicit stimulation of cell proliferation, which should be normally limited to the haemopoietic environment. GM–CSF is known to trigger responses in a broad range of myeloid precursors ranging from early progenitors to mature macrophages. The biological responses to GM–CSF involve essentially the proliferation in the former and activation in the latter ones. The overall response of cells to external stimuli reflects the interaction among different signalling pathways from cytokine and chemokine receptors, as well as from the adhesion molecules. Notwithstanding this overall internal integration of cell information, it is tempting to assume that the tissue background can fully redirect the stimulatory activity of a given cytokine. In the present work, we have monitored the proliferation of a myeloid progenitor line, which occurred on stromas that are myelopoietic in vivo, but not in the environment in which myelopoiesis does not occur, such as dermis. Accordingly, in this tissue GM–CSF is expected to elicit activation of cells of the mono–macrophagic lineage, but not their proliferation.

We have now shown that the control of the GM–CSF on a given cell type is due, at least in part, to the heparan sulphate proteoglycans present in the stroma cells glycocalyx, which can have either a stimulatory or a fully inhibitory action on the GM–CSF biological activity. On the other hand, our previous studies have shown that the studied cell lines release the heparan sulphate into the culture supernatant (Silva et al., 1992a, b). The GM–CSF is also known to be secreted into the supernatant, being possibly free or associated with other released molecules including the heparan sulphate. Hence, the functional association among heparan sulphate and the growth factors in the intercellular space is biologically different from that occur-

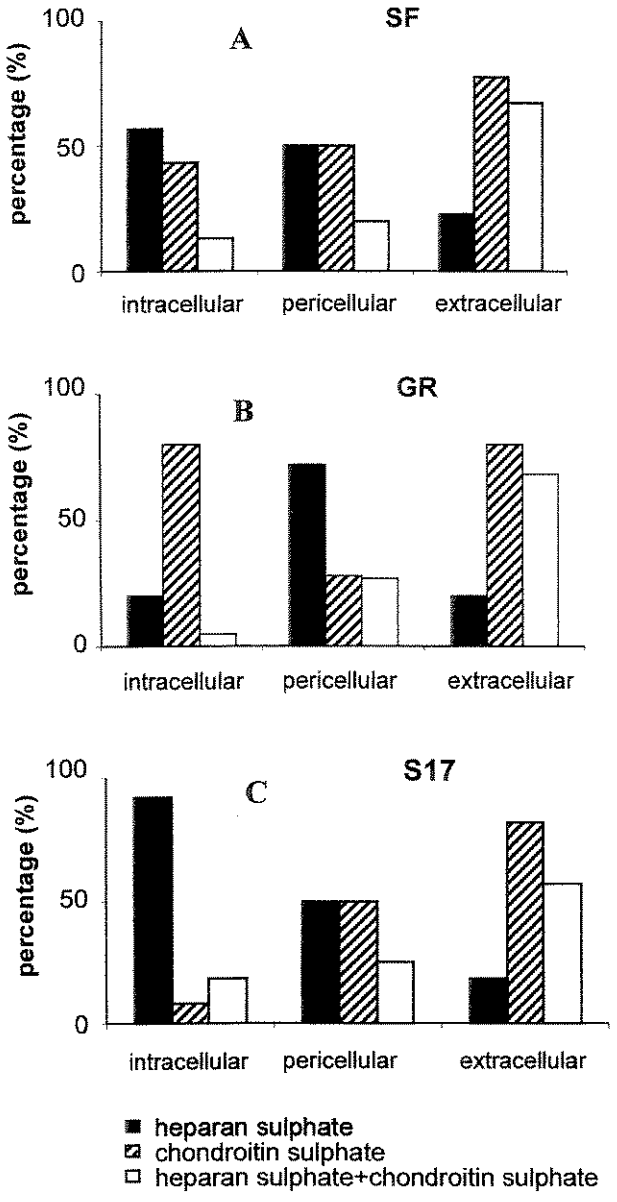

Figure 5. Histograms showing the relative proportions of total sulphated glycosaminoglycans (heparan sulphate + chondroitin sulphate), heparan sulphate and chondroitin sulphate from intracellular, pericellular and extracellular compartments of SF (**A**), GR (**B**) and S17 (**C**) cells, respectively. The percentage values were calculated from sulphate incorporation expressed in cpm, as described in material and methods. Results represent mean values of three experiments.

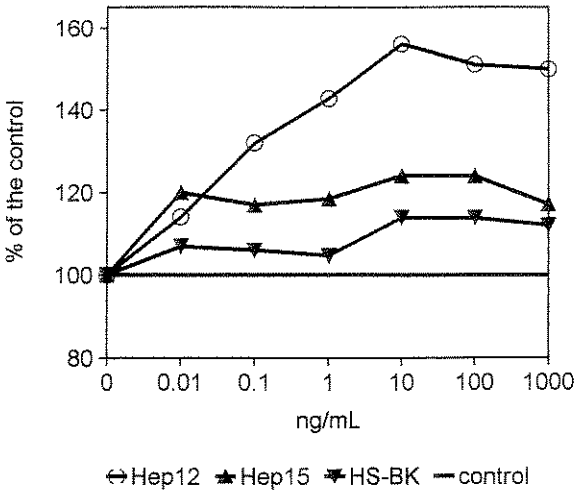

Figure 6. Myeloid cell proliferation assay in the presence of GM–CSF and glycosaminoglycans. Proliferation of FDC-P1 cells in the presence of 25 U·mL^{-1} of rGM–CSF in standard culture medium supplemented with increasing quantities of heparins of different molecular size (Hep12, Hep15) and bovine kidney heparan sulphate (HS-BK). Results are expressed as percentage (%) of the cell proliferation mediated by GM–CSF alone (control). Data represent mean values of two experiments done in sextuplicate.

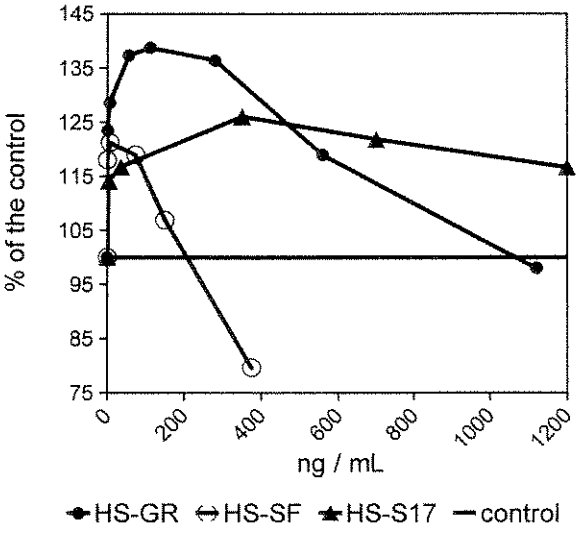

Figure 7. Soluble factor assay in the presence of heparan sulphate purified from the assayed stromas. Proliferation of FDC-P1 cells in the presence of 25 U·mL^{-1} of rGM–CSF in standard culture medium supplemented with increasing quantities of SF, GR and S17 cell-derived heparan sulphate (HS). Results are expressed as percentage (%) of cell proliferation mediated by rGM–CSF alone (control). Data represent mean values of two experiments done in sextuplicate.

ring in the culture supernatant. This may be a consequence of the ratio between heparan sulphate and GM–CSF, since the titration of the growth-stimulatory activity of GM–CSF versus the quantity of stroma-derived heparan sulphate has shown that the

product of each cell line has a specific pattern of interaction, ranging from a stimulatory to an inhibitory activity as a function of the relative concentration of the two molecules (*figure 7*). The local concentration of glycosaminoglycans in the intercellular space is difficult to estimate, and may be much higher than that observed in cell culture supernatants, which would be consistent with the inhibitory activity of SF-derived heparan sulphate in higher concentrations. On the other hand, we have recently shown that the quality of interactions among GM–CSF and glycosaminoglycans was dependent upon the polarity of the intercellular microenvironment, the negatively charged environment being required for functional association between GM–CSF and glycosaminoglycans (Wettreich et al., 1999). Molecular interactions among the growth factors, the receptors, and the pericellular glycoconjugates in the juxtacrine context can be consequently quite different from that observed for free molecules in the liquid environment.

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VII- Conclusões

1. Todos os estromas investigados produzem constitutivamente GM-CSF e, com exceção do estroma da linhagem de SF, todos sustentam a proliferação de precursores mielóides através de um mecanismo de sinalização justácrina.

2. Apesar do condroitim sulfato ser o glicosaminoglicano preponderantemente secretado pelas linhagens celulares estromais, o heparam sulfato (HS) está presente em maior quantidade nas frações associadas às superfícies celulares.

3. O HS presente no espaço pericelular dos estromas estudados, modula a atividade biológica do GM-CSF; positivamente, no caso dos HS derivados dos estromas das linhagens GR e S17 e negativamente no caso do HS derivado do estroma da linhagem SF.

4. A análise da estrutura do HS presente em estromas que sustentam a sobrevivência e proliferação da linhagem mielóide, revela um expressivo percentual de dissacarídeos N-sulfatados no compartimento pericelular. A investigação da expressão de HSPGs pelas células estromais, através de RT-PCR, indica mensagem para sindecam, betaglicam e glipicam.

5. Os "deslocados" contendo moléculas transmembranares, obtidos após tratamento brando com tripsina, são responsáveis por um aumento na atividade biológica do GM-CSF enquanto os GAGs dos PGs ancorados à membrana via âncora de GPI não demonstram a mesma atividade.

6. O contato físico entre o estroma e os progenitores mielóides promove, possivelmente, um "capping"de moléculas de superfície celular, que inclui glicoconjugados contendo ácido siálico e proteoglicanos (PGs). Esse microambiente criado na interface do contato celular pode proporcionar condições adequadas para disparar a interação funcional do fator de crescimento com o PG.

No nosso modelo de estudo, mostramos que a composição molecular e as propriedades fisicoquímicas do microambiente pericelular estabelecido entre o estroma e os progenitores hematopoéticos são determinantes para a atividade biológica do GM-CSF. A organização espacial desse microcompartimento é determinada pela presença de glicoconjugados sialilados aos quais se encontram associados os proteoglicanos transmembranares, responsáveis pela interação com o fator de crescimento.

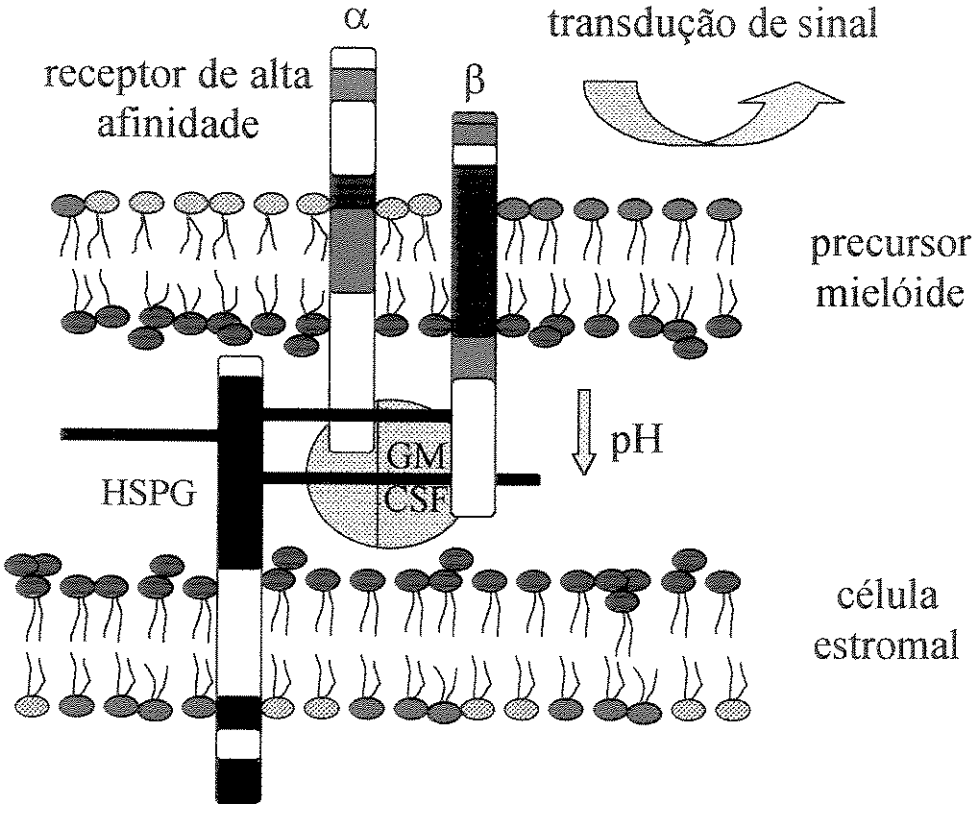
A movimentação das membranas celulares durante o contato entre as células promoveria um "capping" dessas moléculas, carregadas negativamente, que parecem ficar inseridas em

pequenas regiões da membrana formando "rafts". Os receptores para GM-CSF podem também ser deslocados da superfície celular livre em direção ao microambiente interativo, sendo seqüestrados para o interior dos "rafts" encontrando um ambiente favorável para disparar a cascata de sinalização.

PERSPECTIVAS

Essas propostas abrem caminhos para investigações sobre a composição molecular e organização dos "rafts", presentes no microambiente pericelular, aos quais os receptores de alta afinidade para GM-CSF estariam também associados.

Outra perspectiva seria a determinação das vias de sinalização que promoveriam a ativação dos receptores de alta afinadade para GM-CSF.



Modelo esquemático que sugere a organização molecular do microambiente intercelular na sinalização do GM-CSF em sistemas hematopoéticos.