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Biochemical Characterization of Heparan Sulfate Derived From Murine Hemopoietic Stromal Cell Lines: A Bone Marrow-Derived Cell Line S17 and a Fetal Liver-Derived Cell Line AFT024

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Abstract Heparan sulfate (HS) present on the surface of hemopoietic stromal cells has important roles in the control of adhesion and growth of hemopoietic 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 hemopoietic 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 syndecan4. Only AFT024 cell line expressed mRNA for betaglycan. There was no evidence for expression of mRNA for both syndecan1 and syndecan2. [^{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. J. Cell. Biochem. 87: 160–172, 2002. © 2002 Wiley-Liss, Inc.

Key words: glycosaminoglycans; heparan sulfate; glypican; syndecan; hematopoiesis

Abbreviations used: 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) and GalNAc(6SO_4) derivatives of *N*-acetylated galactosamine bearing a sulfate ester at position 4 and 6, respectively.

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In humans and in others mammals, hematopoiesis takes place in the bone marrow and fetal liver. The development and maturation of the various blood cell types is dependent upon circulating or locally produced cytokines and the modulatory role of different microenvironments in the marrow stromas, which include cell surface and extracellular matrix proteoglycans (PGs) [Dexter, 1987; Allen et al., 1990; Metcalf, 1993]. Regional variation in these components within the hematopoietic microenvironment may create niches that are specific for cells at a given state of their differentiation [Hangoc et al., 1993]. Heparan sulfate proteoglycans (HSPGs) present in bone marrow stromal cells and in the extracellular matrix have relevant functions in the hematopoiesis, such as spatial organization of the medullar microenvironment and modulation of the biological activity of several cytokines. Hematopoietic cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor (FGF), and interleukin-3 (IL-3) can bind to heparan sulfate (HS), which actively participates in their interaction with the corresponding cell receptors [Gordon et al., 1987; Roberts et al., 1988; Yayon et al., 1991; Turnbull et al., 1992]. Our previous studies have shown that glycosaminoglycans (GAGs) purified from stromal cell cultures could be either stimulatory or inhibitory for GM-CSF activity, depending upon their concentration [Alvarez-Silva and Borojevic, 1996; Carvalho et al., 2000]. The ability of HSPGs to bind a variety of growth factors is dependent upon the sulfation pattern of the composing disaccharides, which is a prerequisite for specific binding of various proteins [Witt and Lander, 1994; Faham et al., 1998; Spillmann et al., 1998].

Structurally, HSPGs are heterogeneous macromolecules consisting of a core-protein connected via a link tetrasaccharide to a linear polysaccharide GAG chains. HS-GAGs consists of a disaccharide repeat unit (glucuronic acid and *N*-acetylglucosamine) which can be modified by *N*-sulfation, epimerization of glucuronic to iduronic acid, and *O*-sulfation at several different sites. As a result, HS can have extremely variable structure, and it is believed that structural differences are responsible for highly specific interactions of GAGs with other 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]. Expression of syndecan3, syndecan4, and glypican1 was recently reported at the surface of bone marrow stromal cells, together with perlecan in the extracellular matrix [Schofield et al., 1999]. The murine bone marrow stromal cell line MS-5 that can support the growth of human progenitor cell lines was reported to synthesize HSPGs of the syndecan family, glypican, betaglycan, and perlecan [Drzeniek et al., 1997].

In the present study, we have addressed the question of the potential correlation among the structure of PGs and the capacity of stromas to sustain hematopoiesis. We monitored proliferation of the myeloid progenitor cell line FDC-P1, which is dependent upon the myelopoietic growth factors for both survival and proliferation [Siczkowski et al., 1992; Carvalho et al., 2000]. The S17 murine bone marrow-derived stroma cell line sustains both human and murine hematopoiesis [Collins and Dorshkind, 1987]. The AFT024 cell line was derived from fetal liver, which is the site of intense proliferation of embryo hematopoiesis. Punzel et al. [1999] showed that the cell line sustains human long-term culture initiating cells as well as lymphoid progenitors when cultured in contact with the feeder layer. We determined by RT-PCR, the expression of mRNA for HSPGs core proteins and analyzed the fine structure of cell surface and secreted HS GAG chains. We also monitored the capacity of the HSPGs dislodged by differential treatments from the studied stromas to modulate the GM-CSF activity in order to identify the HS-nearing PGs that interact preferentially with the signaling pathway of central hematopoietins in the stroma environment.

MATERIALS AND METHODS

Materials

Dulbecco's minimum essential medium (DMEM), recombinant murine GM-CSF, HEPES, EDTA, TRIS, thiazolyl blue (MTT), phosphatidylinositol phospholipase C (PI-PLC), chondroitin 4-sulfate (C-4S), chondroitin 6-sulfate (C-6S), dermatan sulfate (DS), heparan sulfate, and twice-crystallized papain (15 U/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO). 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). Radiolabeled carrier-free $^{35}S\text{-}Na_2SO_4$ was obtained from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, SP, Brazil). Standard disaccharides for analysis of HS composition, α-ΔUA-1 → 4-GlcNAc, α-ΔUA-1 → 4-GlcNSO$_4$, α-ΔUA-1 → 4-GlcNAc(6SO$_4$), α-ΔUA (2SO$_4$)-1 → 4-GlcNAc, α-ΔUA-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 chondroitin sulfate (CS) composition, α-ΔUA-1 → 3-GalNAc(4SO$_4$), α-ΔUA-1 → 3-GalNAc(6SO$_4$) were purchase 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), and Taq DNA polymerase from CENTBIO (Porto Alegre, RS, Brazil).

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, able to sustain both murine and human hematopoietic cells in vitro, was used after the authorization given by Kenneth Dorshkind [Collins and Dorshkind, 1987]. The murine fetal liver cell line AFTO24 was kindly provided by IR Lemischka, Princeton University, Princeton, NJ. The multipotent myeloid precursor cell line FDC-P1 is dependent upon interleukin-3 (IL-3) or 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].

In the co-culture assays, the FDC-P1 cells, previously washed with balanced salt solution (BSS) in order to remove IL-3, were inoculated onto the confluent monolayers of S17 or AFTO24 cells in 24-well tissue culture plates at 5×10^4 cells per well (Nunclon, Roskilde, Denmark). After 48 and 96 h of co-incubation, they were quantified by counting under the microscope equipped with phase contrast.

Preparation of ^{35}S-Labeled GAGs

Labeling. The two stroma cell lines preparations were harvested at 80% confluence and metabolically radiolabeled for 24 h with 40 μCi [^{35}S] Na_2SO_4 per milliliter. 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 were similar to those previously described for other types of cells [Werneck et al., 1999, 2000; Carvalho et al., 2000]. Briefly, at the completion of the labeling period, conditioned media were removed and centrifuged (1,000 rpm for 5 min) to remove cell debris, and stored at −20°C until required. The cells were rinsed with phosphate-buffered saline (PBS) (pH 7.4) being detached by incubation with 2 ml 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 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-labeled GAGs were purified by DEAE-Sephacel, as described below.

For purification 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. It was further subjected to ion-exchange chromatography on a DEAE-Sephacel column (10.0 cm × 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 material was 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 and 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 10,000 cpm of ^{35}S-GAGs 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 GAGs 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 U 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 [Werneck et al., 2000].

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 Strong Anion-Exchange (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 distilled water:acetonitrile:trifluoroacetic acid (80:20:0.1,v/v) 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 × 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 0.3–1.05 M 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) 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 radiolabeled disaccharides and standard compounds were subjected to a SAX-HPLC system, eluted with a 0–1.5 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 [Werneck et al., 2000].

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 (0.4 U/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, 3×10^4 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, interleukin 3 (IL3), interleukin 5 (IL5), interleukin 7 (IL7), macrophage colony-stimulating factor (M-CSF), GM-CSF, hepatocyte growth factor (HGF), stem cell factor (SCF), syndecan1, syndecan2, syndecan4, 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. The sequences of the primers used are listed in Table I.

RESULTS

FDC-P1 Cell Proliferation

Both stroma cell lines sustained in co-culture, the proliferation of FDC-P1 cells. The S17 stroma induced a rapid and intense proliferation that reached the saturation by 48 h of culture, while AFT024 cells sustained a slower growth of myeloid cells (Fig. 1).

RT-PCR Analysis of Heparin-Binding Growth Factors From the Stromal Cell Lines

Since in the co-culture, the hematopoiesis is dependent upon the locally produced cytokines, we monitored the expression of the major hematopoietins in the two stromas (Fig. 2). No one expressed IL-3, and both expressed the major growth myelopoietic factors: SCF, HGF, GM-CSF, and M-CSF. IL-5, which is considered to be essentially produced by Th2 lymphocytes, was also expressed, as well as the major cytokine that controls the bone marrow B-lymphopoiesis, the IL-7.

GAGS From Stromal Cell Lines

We have previously show that the HS-GAGs secreted by S17 are important for the biological

TABLE I. RT-PCR Primers Used for the Cytokines and HSPGs Expression Monitoring Assay

Primer	Sequence	Reference
Syndecan1	5′ATGAGACGCGCGGCGCTCTGGC3′ 5′GGCGTAGAACTCCTCCTGCTTGGT3′	Drzeniek et al. [1997]
Syndecan2	5′GCAGCCAAGCCACCAGTCTAG3′ 5′TGTAGAGTGCTGTACTCCAG3′	Drzeniek et al. [1997]
Syndecan4	5′CGAGAGACTGAGGTCATCGAC3′ 5′CGCGTAGAACTCATTGGTGG3′	Drzeniek et al. [1997]
Betaglycan	5′GAGCTGTATAACACAGACCTC3′ 5′CGTCGTCAGGAGTCACACAC3′	Drzeniek et al. [1997]
Glypican	5′CGCCAGATCTACGGAGCCAAG3′ 5′GAACTTGTCGGTGATGAGCAC3′	Drzeniek et al. [1997]
β-actin	5′GTGGGCCCTCTAGGCACCA3′ 5′CTCTTTGATGTCACGCACGATTTC3′	Alonso et al. [1986]
IL3	5′GAAGTGGATCCTGAGGACAGATACG3′ 5′GACCATGGGCCATGAGGAACATTC3′	Yokota et al. [1984]
IL5	5′ATGACTGTGCCTCTGTGCCTGGAGC3′ 5′CTGTTTTTCCTGGAGTAAACTGGGG3′	Cardell and Sander [1990]
IL7	5′GAGATGAGGACACAGAAATGCAGC3′ 5′CAAGAGCATTATCCATCACCGTAGG3′	Sakata et al. [1990]
M-CSF	5′TGCCCTTCTTCGACATGGCTGGGC3′ 5′GAACAGCTGGATGATCCTGTTTGC3′	Rajavashisth et al. [1987]
GM-CSF	5′AGAAGCTAACATGTGTGCAGACCCG3′ 5′ATTCCAAGTTCCTGGCTCATTACGC3′	Gough et al. [1984]
SCF	5′CCGGATCCTGGAGCTCCAGAACAGCTAA3′ 5′GGCTGCAGTCCACAATTACACCTCTTGAA3′	Flanagan et al. [1991]
HGF	5′TACGCCACGCCAAAGAATGGC3′ 5′CGCAAACCAGGTCGAGATGTGAAGC3′	Degen et al. [1991]

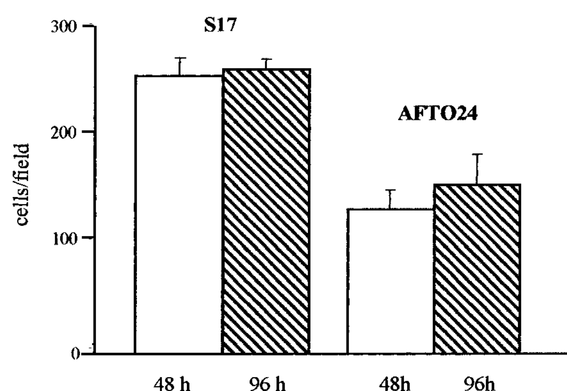

Fig. 1. Co-culture assay-proliferation of FDCP-1 cells after 48 h (open bars) and 96 h (striped bars) culture over confluent monolayers of S17 and AFT024. Results represent viable cells counts/microscope field (250 ×); mean values of 50 fields, counted in two independent experiments.

activity of GM-CSF and consequently for FDC-P1 proliferation [Carvalho et al., 2000]. Since HS is involved in binding growth factors, and probably in adhesion of myeloid cells to hematopoietic stroma, we investigated and compared the structure of GAGs present in the

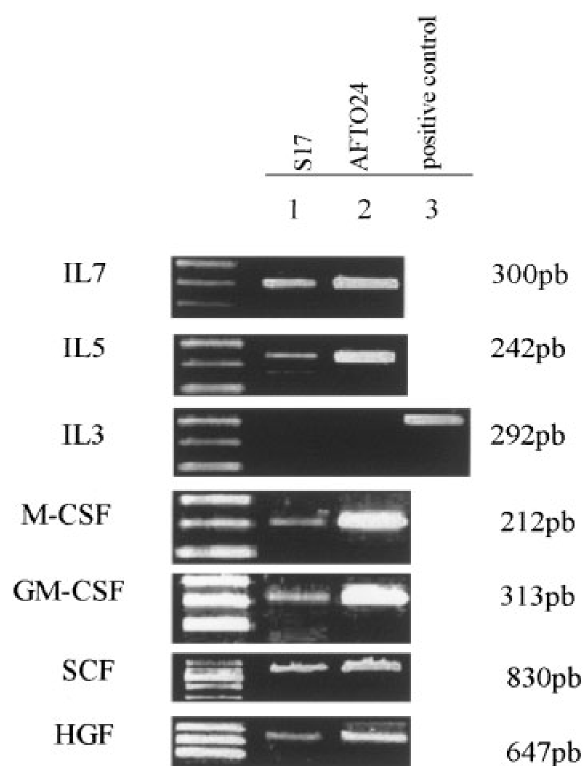

Fig. 2. RT-PCR analysis of expression of hemopoietin genes in S17 and AFT024 stromas. Amplification of cDNA for IL-7, IL-5, IL-3, M-CSF, GM-CSF, SCF, and HGF is shown in **lane 1** for S-17 and in **lane 2** for AFT024. **Lane 3** shown the positive control of WE-HI 3B cells for IL-3.

cell surface and in the culture medium of S17 and AFT024 stromas.

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 labeling period with [^{35}S] Na_2SO_4 added to the cell cultures. We detected high amounts of ^{35}S-labeled CS, which was mainly secreted into the culture 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 II).

Characterization of the ^{35}S-GAGs by agarose gel electrophoresis (Fig. 3) revealed that culture medium of stromal cell lines had a major electrophoretic band with a mobility between CS and DS standards. It totally disappeared after chondroitin AC and ABC lyase digestions. Therefore, this major electrophoretic band was identified as 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 standard CS/DS standards 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, we found that a major band had the same mobility as the standard HS and totally disappeared from the gel after deaminative cleavage by nitrous acid (Fig. 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 ($\sim$95%) were disaccharides (not shown). These disaccharide mixtures were then analyzed on a SAX-HPLC and the

TABLE II. Distribution of the ^{35}S-Labeled GAGs in the Different Cellular Compartments of Stromal Cell Lines After a 24-h Labeling Period

Cell line	Compartment	Labeled GAGs[a] (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)

[a]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.

results are shown numerically (Table III). 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 C-6S and 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 III).

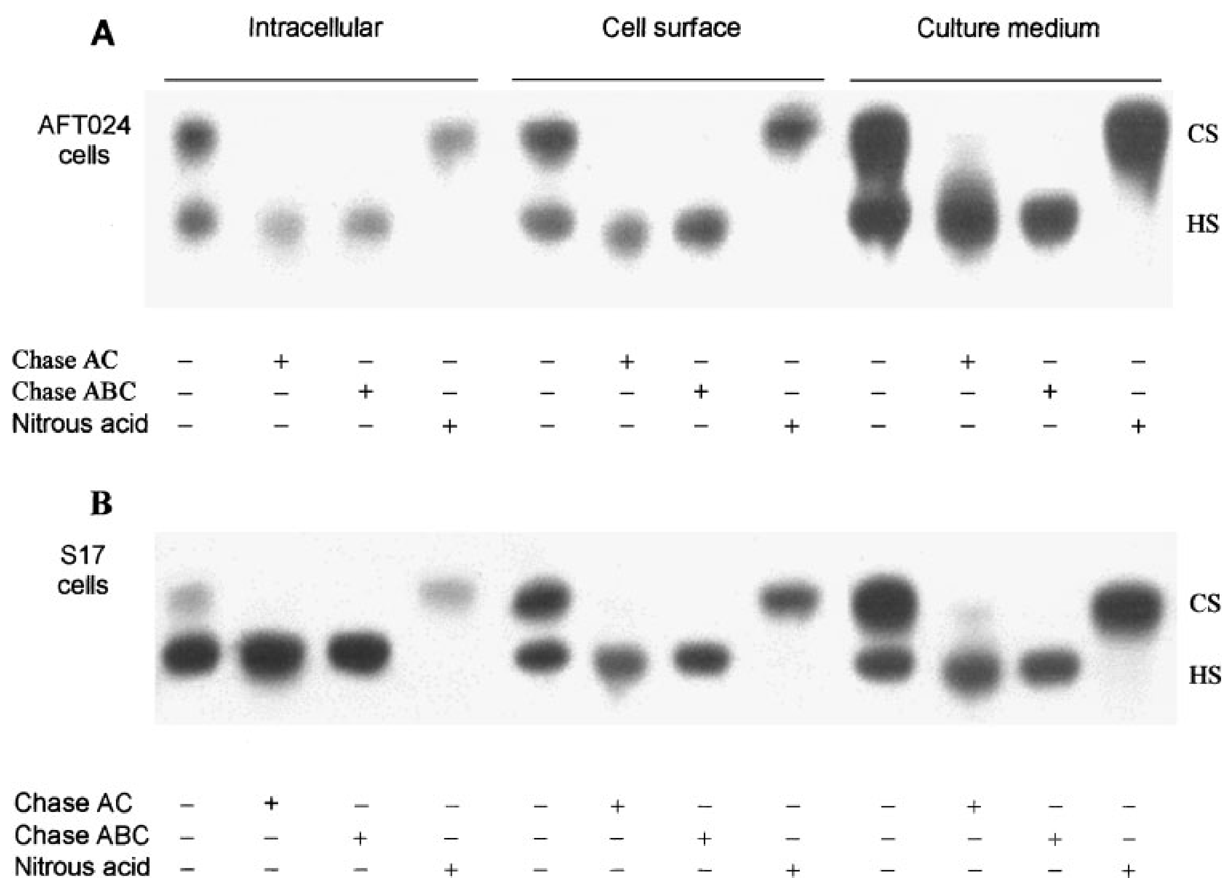

Fig. 3. Agarose gel electrophoresis of the ^{35}S-sulfated glycosaminoglycans (GAGs) from the intracellular, cell surface, and culture medium compartments of AFTO24 (**A**) and S17 (**B**), before and after chondroitin lyase digestion (chase AC and chase ABC) or deaminative cleavage by nitrous acid. After enzymatic incubation, the ^{35}S-GAGs were applied to 0.5% agarose gel and electrophoresis was carried out in 0.05 M 1,3-diaminopropane:acetate buffer (pH 9.0) 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. CS, chondroitin 4/6-sulfate; HS, heparan sulfate.

TABLE III. Disaccharides Derived by Chondroitin ABC Lyase Digestion of the Radiolabeled GAGs From the Cell Surface and Culture Medium of Stromal Cell Lines

		Disaccharide units[a] (cpm/10^6 cells)	
Cell line	Compartment	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)

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

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 (Figs. 4 and 5) and numerically (Table IV). The major sulfated disaccharide was α-ΔUA-1 → 4-GlcNSO_4 (Figs. 4 and 5), which comprised ~60% of the total sulfated disaccharide units on the HS from both cell surface and culture medium (Table IV). 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 IV; Figs. 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 (Figs. 4 and 5; Table IV).

Based on the proportions of sulfated disaccharides formed by exhaustive digestion with heparin and heparan sulfate lyases (Table IV), it was possible to determine the sulfation characteristics of the HS chains of the two stromal cell lines (Table V). 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

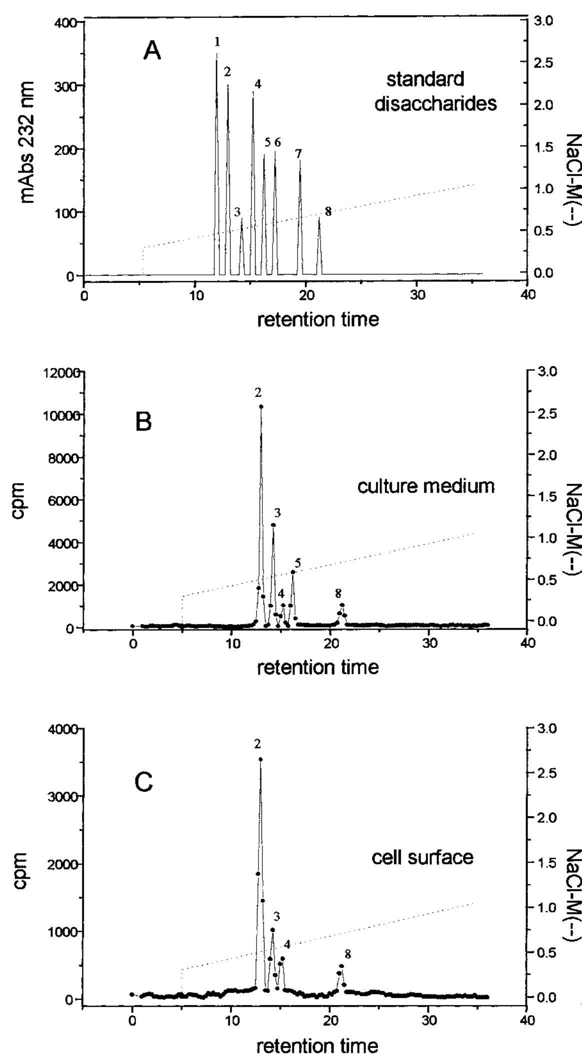

Fig. 4. Strong anion-exchange HPLC analysis of the lyase-derived disaccharides from radiolabeled HS of S17 cells. A mixture of disaccharide standards (**A**) and the disaccharides formed by exhaustive action of heparin + heparan sulfate lyases on radiolabeled HS from the culture medium (**B**) and cell-surface (**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($6SO_4$).

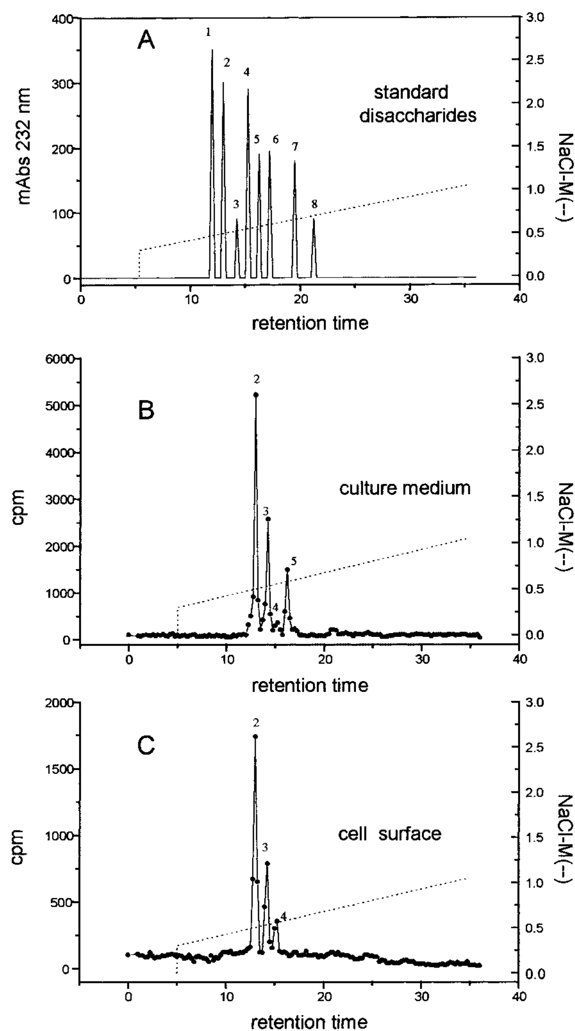

Fig. 5. Strong anion-exchange HPLC analysis of the lyase-derived disaccharides from radiolabeled HS of AFT024 cells. A mixture of disaccharide standards (**A**) and the disaccharides formed by exhaustive action of heparin + heparan sulfate lyases on radiolabeled HS from the culture medium (**B**) and 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 Figure 4.

noticed that the proportion of *N*-sulfation was nearly the same (Table V). 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 V).

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. Expression of mRNA for both syndecan4 and glypican were detected in the two stromal cell lines. Only AFT024 expressed mRNA for betaglycan. Syndecan1 and syndecan2 mRNAs were not detected in the two stromal cell lines (Fig. 6).

Cell Layer PI-PLC-Susceptible ^{35}S-GAGs

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 glycosylphosphatidylinositol (GPI)-anchored cell surface PGs. ^{35}S-radioactivity in the medium was several fold higher in PI-PLC-treated cells than in control cells (Fig. 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 HS had a stimulatory activity on FDC-P1 cells proliferation in the presence of a constant quantity of recombinant murine GM-CSF, indicating that stroma-derived GAGs could modulate the GM-CSF activity [Carvalho et al., 2000]. We have now questioned whether the HS that interacted with GM-CSF signaling pathway were associated with the GPI-anchored or transmembrane PGs. We have 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, whilst the ^{35}-S labeled GPI-anchored GAGs that could be dislodged by PI-PLC did not show a significant modulation of the GM-CSF activity (Fig. 8). Taken together, these results indicate the existence of both transmembrane HSPG and GPI-HSPG, the former ones only being involved in modulation of the GM-CSF stimulation of FDC-P1 proliferation in vitro.

DISCUSSION

The present study of GAGs synthesis translocation to the cell surface and secretion into

TABLE IV. Lyase-Derived Sulfated Disaccharide Composition of the Radiolabeled HS From the Cell Surface and Culture Medium of Stromal Cell Lines

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

n.d., not detected.
[a]The radioactivity under the peaks in Figures 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.
[b]Standard peak number in order of elution (Figs. 4 and 5).

the culture medium in two murine stromal cell lines, a bone marrow-derived cell line S17 and a fetal liver-derived cell line AFT024, has shown that they produce HS and CS with a similar distribution in the intracellular, cell surface, and extracellular compartments. No differences were observed in the relative proportions of HS and CS within each compartment. High amounts of CS were secreted into the culture medium, while HS was found to be more abundant in the cell-associated fractions. These results are in agreement with previous reports on the GAG composition of these two stromal cell lines [Siczkowski et al., 1992; Punzel et al., 1999; Carvalho et al., 2000]. We extended now this study to the fine structure of these GAGs, as well as to the identification of the core protein.

Specific sulfation patterns of HS are required for binding and modulation of the activity of cytokines that stimulate proliferation of hematopoietic progenitors [Lindahl et al., 1998; Turnbull et al., 2001]. 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 6-*O*-sulfation in the HS from the cell surface to the culture medium. 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 com-

TABLE V. Sulfation Characteristics of Disaccharides From HS Obtained From Cell Surface and Culture Medium of Stromal Cell Lines

	S17		AFT024	
Sulfation[a]	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

[a]The calculations on sulfation characteristics shown are based on the overall disaccharide composition data (Table IV).

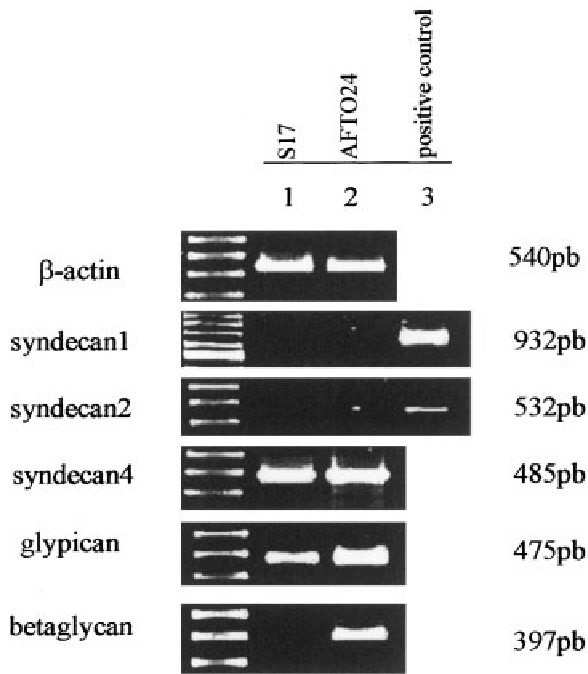

Fig. 6. RT-PCR analysis of expression of HSPGs genes in S17 and AFT024 stromas. Amplification of cDNA for β-actin, syndecan1, syndecan2, syndecan4, glypican, and betaglycan is shown in **lane 1** for S-17 and **lane 2** for AFT024. **Lane 3** shows the positive control of queratinocytes for syndecan1 and positive control of whole bone marrow for syndecan2.

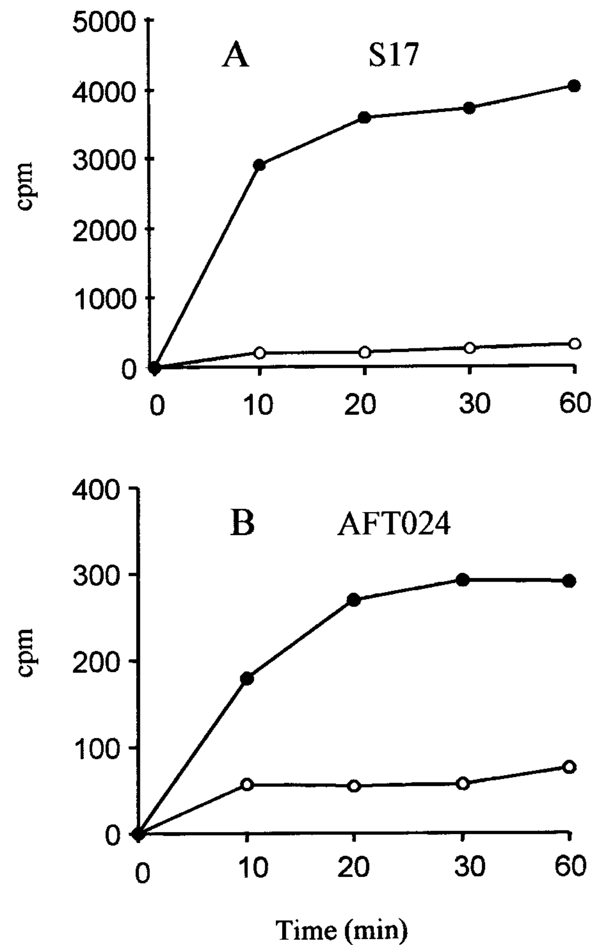

Fig. 7. Time course of labeled sulfated macromolecules release from cellular layer of S17 (**A**) and AFT024 (**B**) by PI-PLC treatment. Stromal cells were labeled with [^{35}S]sulfate. After removing the labeling medium, cells were incubated in fresh medium with 0.4 U/ml PI-PLC (●) or without (○, control) 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.

pared with the respective compartments of AFT024 cells (Tables IV and V). 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 hemopoietic stromal cells are scarce and controversial. Schofield et al. [1999] have reported that glypican1, syndecan3, and syndecan4 are the major cell-membrane HSPG species in human marrow stroma and that perlecan is the major ECM PG. Conversely, Drzeniek et al. [1997] have reported that the murine bone marrow stromal cell line MS-5 synthesizes several different HSPGs: syndecans1–4, glypican, betaglycan, and perlecan. Our results indicate that syndecan4 and glypican are apparently present in all the hematopoietic stromas, whilst syndecans1 and 2, and betaglycan are not necessarily required for sustaining of hematopoiesis.

The identification of GPI-anchored and transmembrane PGs that potentially bear the highly sulfated HS chains relevant for interaction with growth factors and determinant for sustaining proliferation of blood cell progenitors prompted us to question whether both are equally involved in these controls. We found that only the GAGs from membrane-anchored PGs were biologically active in GM-CSF signaling. Molecular aspects of interaction between the involved transmembrane receptors and the PGs are object of ongoing studies.

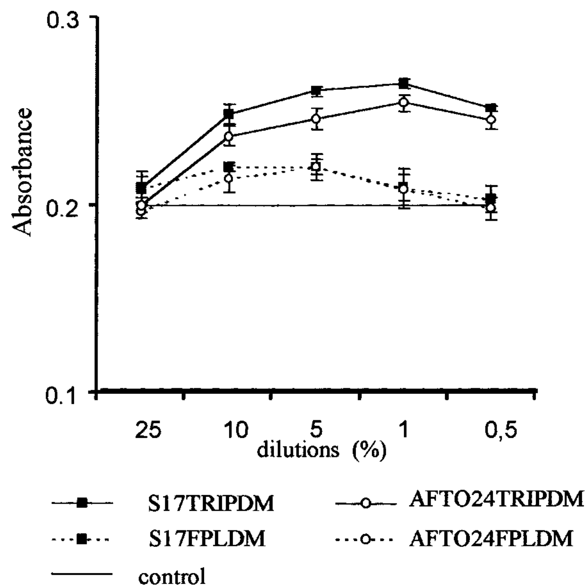

Fig. 8. Myeloid cell proliferation assay in the presence of 1 ng rGM-CSF in standard culture medium supplemented with different dilutions of S17 and AFT024 cell-derived (4×10^7) dislodged with tripsyn (TRIPDM) and phospholipase C (FPLDM). Results are expressed in absorbance mediated by rGM-CSF alone (control). Data represent mean value of two experiments done in quadruplicate.

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REFERENCES

Allen TD, Dexter TM, Simmons PJ. 1990. Marrow biology and stem cells. In: Dexter TM, Garland JM, Testa NG, editors. Colony stimulating factors. New York: Marcel Dekker. p 1–38.

Alonso S, Minty A, Bourlet Y, Buckinghan M. 1986. Comparison of three actin coding sequences in the mouse: Evolutionary relationships between the actin gene of warm blooded vertebrates. J Mol Evol 23:11–22.

Alvarez-Silva M, Borojevic R. 1996. GM-CSF and IL-3 activities in schistosomal liver granulomas are controlled by stroma-associated heparan sulfate proteoglycans. J Leukoc Biol 59:435–441.

Brito JM, Borojevic R. 1997. Liver granulomas in schistosomiasis: Mast cell-dependent induction of SCF expression in hepatic stellate cells is mediated by TNF-alpha. J Leukoc Biol 62:389–396.

Brucato S, Fagnen G, Villers C, Bonnamy PJ, Langris M, Bocquet J. 2001. Biochemical characterization of integral membrane heparan sulfate proteoglycans in Sertoli cells from immature rat testis. Biochim Biophys Acta 1510: 474–487.

Cardell S, Sander B. 1990. Interleukin 2, 4 and 5 are sequentially produced in mitogen-stimulated murine spleen cell cultures. Eur J Immunol 20:389–395.

Carvalho MA, Arcanjo K, Silva LCF, Borojevic R. 2000. The capacity of connective tissue stromas to sustain myelopoiesis depends both upon growth factors and the local intercellular environment. Biol Cell 92:605–614.

Collins LS, Dorshkind K. 1987. A stromal cell line from myeloid long-term bone marrow culture can support myelopoiesis and B lymphopoiesis. J Immunol 138:1082–1087.

Degen SJ, Stuart LA, Han S, Jamison CS. 1991. Characterization of the mouse cDNA and gene coding for a hepatocyte growth factor-like protein: Expression during development. Biochemistry 30:9781–9791.

Dexter TM. 1987. Growth factors involved in haemopoiesis. J Cell Sci 88:1–6.

Drzeniek Z, Siebertz B, Stocker G, Just U, Ostertag W, Greiling H, Haubeck HD. 1997. Proteoglycan synthesis in haematopoietic cells: Isolation and characterization of heparan sulphate proteoglycans expressed by the bone-marrow stromal cell line MS-5. Biochem J 327:473–480.

Faham S, Linhardt RJ, Rees DC. 1998. Diversity does make a difference: Fibroblast growth factor-heparin interactions. Curr Opin Struc Biol 8:5–55.

Flanagan JG, Chan DC, Leder P. 1991. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. Cell 64:1025–1035.

Gordon MY, Riley GP, Watt SM, Greaves MF. 1987. Compartmentalisation of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 326:403–405.

Gough NM, Gough J, Metcalf D, Kelso A, Grail D, Nicola NA, Burgess AW, Dunn AR. 1984. Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. Nature 309:763–767.

Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. 2000. Human LTC-IC can be maintained for at least 5 weeks in vitro when interleukin-3 and a single chemokine are combined with O-sulfated heparan sulfates: Requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins. Blood 95:147–155.

Hangoc G, Daub R, Maze RG, Falkenburg JH, Broxmeyer HE, Harrington MA. 1993. Regulation of myelopoiesis by murine fibroblastic and adipogenic cell lines. Exp Hematol 21:502–507.

Lindahl U, Kusche-Gullberg M, Kjellén L. 1998. Regulated diversity of heparan sulfate. J Biol Chem 273:24979–24982.

Martins RCL, Lima FRS, Werneck CC, Moura-Neto V, Silva LCF. 2000. Patterns of synthesis and secretion of sulfated glycosaminoglycans in primary cortical and cerebellar astrocytes in vitro. Biol Cell 92:421–427.

Metcalf D. 1993. Hematopoietic regulators: Redundancy or subtlety? Blood 82:3515–3523.

Morris AJ, Turnbull LE, Riley GP, Gordon MY, Gallagher JT. 1991. Production of heparan-sulfate proteoglycans by human bone-marrow stromal cells. J Cell Sci 99:149–156.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Punzel M, Gupta P, Roodell M, Mortari F, Verfaillie CM. 1999. Factor(s) secreted by AFT024 fetal liver cells

following stimulation with human cytokines are important for human LTC-IC growth. Leukemia 13:1079–1084.

Rajavashisth TB, Eng R, Shadduck RK, Waheed A, Ben-Avram CM, Shively JE, Lusis AJ. 1987. Cloning and tissue-specific expression of mouse macrophage colony-stimulating factor mRNA. Proc Natl Acad Sci USA 84:1157–1161.

Roberts R, Gallagher JT, Spooncer E, Allen TD, Bloomfield F, Dexter TM. 1988. Heparan sulphate bound growth factors: A mechanism for stromal cell mediated haemopoiesis. Nature 332:376–378.

Saito H, Yamagata T, Suzuki S. 1968. Enzymatic methods for determination of small quantities of isomeric chondroitin sulfates. J Biol Chem 243:1536–1542.

Sakata T, Iwagami S, Tsuruta Y, Teraoka H, Tatsumi Y, Kita Y, Nishikawa S, Takai Y, Fujiwara H. 1990. Constitutive expression of interleukin-7 mRNA and production of IL-7 by a cloned murine thymic stromal cell line. J Leukoc Biol 48:205–212.

Schofield KP, Gallagher JT, David G. 1999. Expression of proteoglycan core proteins in human bone marrow stroma. Biochem J 343:663–668.

Shively JE, Conrad HE. 1976. Formation of anhydrosugars in the chemical depolymerization of heparin. Biochemistry 15:3932–3942.

Siczkowski M, Robertson D, Gordon MY. 1992. Synthesis and deposition of glycosaminoglycans in the murine hemopoietic sromal line S17: Modulators of the hemopoietic microenvironment. Exp Hematol 20:1285–1290.

Spillmann D, Witt D, Lindhal U. 1998. Defining the interleukin-8-binding domain of heparan sulfate. J Biol Chem 273:15487–15493.

Turnbull JE, Fernig DG, Ke Y, Wilkinson MC, Gallagher JT. 1992. Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. J Biol Chem 267:10337–10341.

Turnbull J, Powell A, Guimond S. 2001. Heparan sulfate: Decoding a dynamic multifunctional cell regulator. Trends Cell Biol 11:75–82.

Werneck CC, Oliveira-dos-Santos AJ, Silva LCF, Villa-Verde DMS, Savino W, Mourão PAS. 1999. Thymic epithelial cells synthesized a heparan sulfate with highly sulfated region. J Cell Physiol 178:51–62.

Werneck CC, Cruz MS, Silva LCF, Villa-Verde DMS, Savino W, Mourão PAS. 2000. Is there a glycosaminoglycan-related heterogeneity of the thymic epithelium? J Cell Physiol 185:68–79.

Witt DP, Lander AD. 1994. Differential binding of chemokines to glycosaminoglycan subpopulations. Curr Biol 4:394–400.

Yayon A, Klagsbrun M, Esko JD, Leder P, Omitiz DM. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64:841–848.

Ymer S, Tucher WQJ, Sanderson CJ, Hapel AJ, Campbell HD, Young IG. 1985. Constitutive synthesis of interleukin-3 by leukaemia cell line WeHi-3B is due to retroviral insertion near the gene. Nature 317:255–258.

Yokota T, Lee F, Rennick D, Hall C, Arai N, Mosmann T, Nabel G, Cantor H, Arai K. 1984. Isolation and characterization of a mouse cDNA clone that expresses mast-call growth-factor in monkey cells. Proc Natl Acad Sci USA 81:1070–1074.