Proteoglycan synthesis in haematopoietic cells: isolation and characterization of heparan sulphate proteoglycans expressed by the bone-marrow stromal cell line MS-5

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Proteoglycans of bone-marrow stromal cells and their extracellular matrix are important components of the haematopoietic microenvironment. Recently, several studies have indicated that they are involved in the interaction of haematopoietic stem and stromal cells. However, a detailed characterization of the heparan sulphate proteoglycans synthesized by bone-marrow stromal cells is still lacking. Here we report on the isolation and characterization of proteoglycans from the haematopoietic stromal cell line MS-5, that efficiently supports the growth and differentiation of human and murine haematopoietic progenitor cells. Biochemical characterization of purified proteoglycans

revealed that the haematopoietic stromal cell line MS-5 synthesizes, in addition to chondroitin sulphate proteoglycans, several different heparan sulphate proteoglycans. Immunochemical analysis, using specific antibodies against the different members of the syndecan family, glypican, betaglycan and perlecan, showed that MS-5 cells synthesize all these different heparan sulphate proteoglycans. These data were further supported by reverse-transcriptase PCR and confirmed by sequence and Northern blot analysis. The relative abundance of the different heparan sulphate proteoglycans was estimated on the protein and mRNA levels.

INTRODUCTION

The importance of the haematopoietic microenvironment for the control of growth and differentiation in the haematopoietic system has been shown in long-term bone-marrow cultures, where stromal cells are necessary to support the proliferation and differentiation of haematopoietic stem and precursor cells *in vitro* [1,2]. In these cultures self-renewal, differentiation and development of haematopoietic stem and precursor cells into mature cells not only depends on added haematopoietic growth factors but also requires direct physical contact between stromal and haematopoietic cells [1–6]. However, the molecular mechanisms of the interaction between stromal cells and haematopoietic cells are not fully understood. It has been shown that stromal cells produce a variety of growth factors, either constitutively or after activation. However, the requirement of a direct cell–cell contact between stromal cells and haematopoietic cells indicates that additional signals are needed. Recently it has been shown that proteoglycans might be involved in the interaction between primitive haematopoietic progenitor cells and stromal cells [7–11]. In these studies it was shown that interleukin-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF) can be bound by heparan sulphate proteoglycans from bone-marrow stromal cells or their extracellular matrix and can be presented in a biologically active form to haematopoietic cells. These data indicate that binding of growth factors by heparan sulphate proteoglycans might be an important mechanism for the interaction of haematopoietic stem and stromal cells. Such a binding of growth factors to heparin and heparan sulphate proteoglycans has been observed in other systems as well [12–17].

These studies have shown that proteoglycans might play an important role in the interaction of haematopoietic stem and stromal cells. Until the present study, the limited availability of

bone-marrow stromal cell lines has precluded a detailed analysis of the proteoglycans from stromal cells [11,18]. In fact, there are only a few reports on proteoglycans from haematopoietic cell lines [11,18–23]. In most of the earlier studies, either whole bone marrow or long-term bone-marrow cultures have been used [4,18,24]. However, by this approach it was impossible to identify the cells that synthesize these proteoglycans. The establishment of bone-marrow stromal cell lines [11,25] has made a detailed analysis of their proteoglycans possible. In the present study we have analysed the proteoglycans synthesized by the murine bone-marrow stromal cell line MS-5. This murine stromal cell line efficiently supports the growth and differentiation of human and murine haematopoietic progenitor cells [25] and therefore might be extremely useful for studying the molecular mechanisms underlying the interactions of haematopoietic progenitor and stromal cells.

MATERIALS AND METHODS

Materials

Carrier-free H₂³⁵SO₄ was obtained from New England Nuclear (Dreieich, Germany). MEM-SEP Q 10/10 cartridges were obtained from Millipore (Eschborn, Germany) and TSK G 3000 SW, TSK G 4000 SW columns, equipped with guard columns, and DEAE-Sephacel were from Pharmacia (Freiburg, Germany).

¹⁴C-labelled molecular mass standards for SDS/PAGE were obtained from Amersham Buchler. CHAPS, benzamidine hydrochloride, 6-aminohexanoic acid, PMSF and *N*-ethylmaleimide were purchased from Sigma (Deisenhofen, Germany). Guanidinium chloride was purchased from Pierce (Cologne, Germany).

Abbreviations used: RT, reverse transcriptase; mAb, monoclonal antibody; DIG, digoxigenin.

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Chondroitin sulphate lyase AC (EC 4.2.2.5), chondroitin sulphate lyase ABC (EC 4.2.2.4), heparinase (heparin lyase, EC 4.2.2.7), heparitinase (heparan sulphate lyase, EC 4.2.2.8) and monoclonal antibody (mAb) 3G10 were purchased from Seikagaku (Tokyo, Japan). RPMI 1640 and horse serum were obtained from Gibco (Eggenstein, Germany) and the polyclonal antiserum (no. 823) against betaglycan from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The following blotting membranes were used for Western blot analysis: Biotrans B (Pall, Dreieich, Germany) and PVDF-Immobilon N⁺ (Millipore, Eschborn, Germany).

Reverse transcriptase (RT; Superscript II) was obtained from Gibco, and *Taq* polymerase (Ampli-Taq) from Perkin-Elmer Cetus (Überlingen, Germany). Oligo(dT), the digoxigenin (DIG)-RNA labelling kit and the DIG-Easy Hyb solution were purchased from Boehringer (Mannheim, Germany). The Quiaex gel extraction kit was obtained from Qiagen (Hilden, Germany). The pCR TM II plasmid cloning kit was purchased from Invitrogen (Leek, Netherlands). The Optiprime kit and XL1-Blue cells were obtained from Stratagene (Heidelberg, Germany). FMC-Seakem LE agarose and NuSieve agarose were obtained from Biozym (Hameln, Germany). Positively charged nylon membranes were from Boehringer. Oligonucleotide primers were synthesized by MWG Biotech (Ebersberg, Germany) and Dynabeads Oligo(dT)25 were obtained from Dynal (Hamburg, Germany).

All remaining reagents were of analytical grade (Merck, Darmstadt, Germany).

Cell culture and radiolabelling of cell lines

The murine haematopoietic stromal cell line MS-5 has been established from long-term bone-marrow cultures [25]. MS-5 cells have the capacity to support the growth of murine haematopoietic stem and progenitor cells *in vitro* [25]. In addition, the human bipotent progenitor cell line TF-1 can be maintained in close contact with MS-5 stromal cells in the absence of added growth factors. MS-5 cells were metabolically radiolabelled by 50 μ Ci/ml [³⁵S]sulphate in sulphate-reduced RPMI 1640 with 20% (v/v) horse serum at a cell density of 2.5×10^5 /ml for 24 h.

Isolation of proteoglycans

Proteoglycans were isolated as described elsewhere [26,27]. Briefly, proteoglycans were extracted from metabolically labelled cells using 4 M guanidinium chloride (pH 5.8) containing 50 mM sodium acetate, 50 mM EDTA, 0.1 M 6-aminohexanoic acid, 50 mM benzamidinium hydrochloride, 5 mM PMSF, 10 mM *N*-ethylmaleimide and 0.5% (w/v) CHAPS. Proteoglycans were also isolated from the culture medium (supernatant). Extracts and supernatants were cleared by centrifugation at 2000 *g* for 20 min. The total incorporation of [³⁵S]sulphate into proteoglycans was calculated from size-exclusion chromatography on a TSK 3000 column for supernatants and extracts.

Purification of proteoglycans was carried out by anion-exchange chromatography and size-exclusion chromatography. Briefly, to remove the guanidinium chloride from the cell extracts, extracts were precipitated with 9 vol. of 95% (v/v) ethanol. Precipitate was reconstituted in 7 M urea buffer containing 0.1 M LiCl/0.1 M NaCl/0.05% CHAPS and protease inhibitors as described above. Ion-exchange chromatography was performed on an MEM-SEP Q 10/10 cartridge (Millipore). The cartridge was equilibrated with 20 mM sodium phosphate containing 0.1 M LiCl/0.1 M NaCl/0.05% CHAPS (pH 4.5) (buffer A). Gradient elution was performed by a two-step linear NaCl

gradient from 0.2 to 0.75 M and from 0.75 to 3.0 M (buffer B = buffer A + 3 M NaCl). The elution profile was monitored by liquid-scintillation counting.

Size-exclusion chromatography was performed on a TSK 4000 column using 20 mM sodium phosphate containing 4 M guanidinium chloride and 0.05% CHAPS (pH 6.0) as eluent. The elution profile was monitored by liquid-scintillation counting.

Immunochemical analysis of proteoglycans

Immunochemical analysis of proteoglycans was performed using the following mAbs against cell-surface heparan sulphate proteoglycans: S1 (anti-glypican), 2E9 (anti-syndecan-1 + -3), 6G12 (anti-syndecan-2) and 8G3 (anti-syndecan-4) (mAbs were kindly provided by G. David, University of Leuven, Belgium). In addition, the mAb 3G10 (Seikagaku), recognizing an epitope generated in heparitinase-digested heparan sulphate proteoglycans, the mAbs MM-1 (mixture of four mAbs) against perlecan (kindly provided by G. David), and a polyclonal antiserum against betaglycan (Upstate Biotechnology) were used. Western blot analysis was performed as described [27] or alternatively after electrophoretic separation of proteoglycans in 4% (w/v) NuSieve (FMC) agarose gels in the presence of SDS. Blocking of Immobilon N⁺ or Biotrans B membranes was performed using 5% (w/v) BSA/5% (w/v) dry milk in 0.02 M Tris/0.3 M NaCl (pH 7.5) or alternatively 3% (w/v) casein in 0.02 M Tris/0.15 M NaCl and 0.02% (v/v) Tween 20 (pH 7.4) for 1 h at room temperature. Incubation with specific mAbs (1 μ g/ml) was performed overnight at 4 °C or alternatively for 2 h at room temperature. Detection of bound antibodies was carried out according to standard procedures using peroxidase-conjugated second antibodies and a chemiluminescence detection system (ECL, Amersham, Braunschweig, Germany).

Characterization of proteoglycans

Isolated proteoglycans were biochemically characterized by electrophoresis before and after digestion with glycan-specific enzymes using fluorography. Enzymic digestion with heparinase, heparitinase, chondroitin sulphate lyase AC and chondroitin sulphate lyase ABC was performed in the presence of proteinase inhibitors as described [28,29]. For enzymic digestion, proteoglycans were precipitated up to three times at -20 °C with 4 vol. of ethanol (95%). Electrophoresis was performed using gradient gels (8–25% or 4.5–22% T, 3% C). After electrophoresis gels were treated with En[³H]ance (New England Nuclear) according to the manufacturer's instructions. Kodak XAR-5 film was used for fluorographic detection. In addition, *M_r* distributions of native and HNO₂-treated proteoglycans were estimated by size-exclusion chromatography on TSK 4000.

RNA isolation and cDNA synthesis

Total RNA was isolated according to the method of Chomczynski and Sacchi [30]. Total RNA (2 μ l; 1 μ g/ μ l) was used to synthesize cDNA by RT (Superscript II, Gibco) starting with oligo(dT) (Boehringer). cDNA was synthesized in 20 mM Tris/HCl (pH 8.4)/50 mM KCl/2.5 mM MgCl₂/10 mM DTT/500 μ M each dNTP/500 nM oligo(dT)/RT (200 units; Superscript II) at 50 °C for 50 min. The reaction was terminated by incubation at 70 °C for 15 min and degradation of RNA by RNase H at 37 °C for 20 min. Poly(A)⁺ RNA was isolated by the use of Dynabeads Oligo(dT)25 (Dyna).

Northern blot

Poly(A)⁺ RNA (1 µg) was run on a 1.0% (w/v) denaturing formaldehyde gel and transferred on to positively charged nylon membranes (Boehringer). RNA blots were hybridized against DIG-labelled (Boehringer) antisense RNA probes in DIG-Easy Hyb at 68 °C for 14 h. Washing was done twice in 2 × SSC (0.3 M NaCl/0.03 M sodium citrate)/0.1% (w/v) SDS at room temperature for 5 min and twice in 0.1 × SSC/0.1% SDS at 68 °C for 15 min. Chemiluminescence detection of RNA was performed according to the manufacturer's instructions (Boehringer). Antisense probes were generated after cloning of PCR-amplification products (see below) into pCRTM 2.1 cloning vector (Invitrogen). The perlecan probe was isolated from a human kidney cDNA library (Stratagene) and corresponds to the published perlecan cDNA sequence from 12082 to 14071 bp [31]. The amount of poly(A)⁺ RNA necessary for perlecan detection was approx. 4 µg. For radioactive detection the perlecan probe was randomly labelled with [³²P]α-dCTP (Amersham).

RT PCR

PCR conditions for amplification of the different members of the syndecan family, betaglycan and glypican, were optimized using the Optiprime kit (Stratagene). Synthesized cDNA (2 µl) was used for PCR reactions. The reactions were performed in 67 mM Tris/HCl (pH 8.8)/6.7 mM MgCl₂/BSA (170 µg/ml)/16.6 mM (NH₄)₂SO₄/100 µM each dNTP/2.5 units of *Taq* polymerase (Ampli-Taq, Perkin-Elmer Cetus) and 50 pmol of the respective 5'-primers and 3'-primers for syndecan-1, -2 and -4. Oligonucleotides for syndecan 1 were: 5'-ATGAGACGCGCGGCGCTCTGGC-3' as 5'-primer and 5'-GGCGTAGAACTCCTCC-TGCTTGGT-3' as 3'-primer; for syndecan-2 5'-GCAGCCA-AGCCACCAGTCTAG-3', 5'-TGTAGAGTGCTGTACTCC-AG-3' as 5'-primers (nested PCR) and 5'-TCCTTATCGGAT-GTCAGCTCT-3' as 3'-primer; for syndecan-4 5'-CGAGAGA-CTGAGGTCATCGAC-3' as 5'-primer and 5'-CGCGTAGA-ACTCATTGGTGG-3' as 3'-primer. Primers for betaglycan were: 5'-GAGCTGTATAACACAGACCTC-3' as 5'-primer and 5'-CGTCGTCAGGAGTCACACAC-3' as 3'-primer. Amplification of betaglycan was performed in 10 mM Tris/HCl (pH 9.2)/3.5 mM MgCl₂/75 mM KCl. The primer pair for glypican was 5'-CGCCAGATCTACGGAGCCAAG-3' as 5'-primer and 5'-GAACTTGTCGGTGATGAGCAC-3' as 3'-primer. Amplification of glypican was performed in 10 mM Tris/HCl (pH 8.3)/3.5 mM MgCl₂/75 mM KCl and 4% (v/v) DMSO (Optiprime, Stratagene). Primers for syndecan-3 were 5'-CACGGCTGACATAAGGACC-3' as 5'-primer and 5'-CTC-TAGTATGCTCTTCTGAG-3' as 3'-primer. Amplification of syndecan-3 was performed in 10 mM Tris/HCl (pH 8.3)/1.5 mM MgCl₂/25 mM KCl. Oligonucleotide primers were synthesized by MWG Biotech. PCR was performed in a thermal cycler (trioblock, Biometra, Göttingen, Germany). Denaturation was at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s. Number of cycles was 40. PCR amplification products were analysed on a 1.2% (w/v) agarose gel (Seakem LE, FMC/Biozym, Hameln, Germany), visualized by ethidium bromide staining.

Cloning of PCR products

PCR-amplified DNA fragments were extracted from agarose gels (Quiex, Qiagen), ligated in pCR TM II or 2.1 (Invitrogen) for 16 h at 14 °C and transformed into competent XL1-Blue cells

(Stratagene). Positive clones were checked for the right insert by sequence analysis [32].

RESULTS

Isolation and characterization of proteoglycans from the murine haematopoietic stromal cell line MS-5

Proteoglycans were isolated from the metabolically labelled murine bone-marrow stromal cell line MS-5 and purified by several chromatographic steps. The content of metabolically labelled proteoglycans (³⁵S incorporation) of supernatants and extracts was calculated from size-exclusion chromatography on TSK 3000 columns. About 40–50% of the proteoglycans (³⁵S-labelled material) were found in the cell extract of the murine haematopoietic stromal cell line MS-5. Purification of proteoglycans was performed by an initial anion-exchange chromatography of supernatants and cell extracts. The *M_r* distribution of proteoglycans was analysed by size-exclusion chromatography on a TSK 4000 column and SDS/PAGE. The relative proportion of heparan sulphate proteoglycans was approx. 20% of the total proteoglycans, as calculated by size-exclusion chromatography of native and HNO₃-treated samples (results not shown). Comparable amounts of heparan sulphate proteoglycans were found in the supernatant and in the cell extract. Results obtained by size-exclusion chromatography were confirmed by SDS/PAGE of samples before and after digestion with glycosaminoglycan-specific enzymes using fluorographic detection. The *M_r* distributions of the heparan sulphate proteoglycans from the supernatant were estimated to be *M_r* approx. > 200 000–90 000 (results not shown). For the cell extract, three heparan sulphate proteoglycan populations with *M_r* distributions of > 200 000, *M_r* > 200 000–100 000, and *M_r* 90 000–30 000 were found (results not shown).

Figure 1 Immunoblotting detection of proteoglycans isolated from the cell extract of the bone-marrow stromal cell line MS-5

Proteoglycans from the cell extract of the murine bone-marrow stromal cell line MS-5 were treated with heparitinase and submitted to SDS/PAGE on a 4.5–15% (w/v) gradient gel and subsequently blotted to Biodyne B membrane. In (A), detection of heparan sulphate proteoglycan (HS-PG) core proteins was performed using the mAb 3G10. The mAb 3G10 recognizes a common neo-epitope, generated after heparitinase digestion of heparan sulphate proteoglycans. This mAb has been shown to detect most, if not all, heparan sulphate proteoglycan core proteins after heparitinase digestion [33]. In (B), detection of heparan sulphate proteoglycan core proteins was performed using the specific mAbs against the individual proteoglycans: lane 1, anti-syndecan-1; lane 2, anti-syndecan-2; lane 3, anti-syndecan-4; lane 4, anti-glypican; lane 5, anti-betaglycan. Detection was performed by the enhanced chemiluminescence system. Arrows indicate the specific core-protein bands of the expected size.

Figure 2 Immunoblot detection of proteoglycans isolated from the supernatant of the bone-marrow stromal cell line MS-5

Proteoglycans from the murine bone-marrow stromal cell line MS-5 were treated by heparitinase and submitted to SDS/PAGE on a 4.5–15% gradient gel and subsequently blotted to Biodyne B membrane. Proteoglycans from the supernatant of MS-5 cells were applied: (A) lane 1, in a concentrated sample, to detect also the core protein with an M_r of approx. 35 000, and (A) lane 2, in a diluted sample. In (A), detection of heparan sulphate proteoglycan core proteins was performed using the mAb 3G10. In (B), heparan sulphate proteoglycan core proteins were stained using the specific mAbs against the individual proteoglycans: lane 1, anti-syndecan-1; lane 2, anti-syndecan-4; lane 3, anti-glypican; lane 4, anti-beta-glycan. Detection was performed by the enhanced chemiluminescence system. Arrows indicate the specific core-protein bands of the expected size.

Immunoblot analysis of proteoglycans from the bone-marrow stromal cell line MS-5

For a detailed characterization of heparan sulphate proteoglycans, isolated from the bone-marrow stromal cell line MS-5, immunoblot analysis of proteoglycans was performed by Western blot analysis and confirmed by RT-PCR and Northern blot analysis. mAbs against cell-surface heparan sulphate proteoglycans of the syndecan family and glypican, and the mAb 3G10 against a common epitope generated in heparitinase-digested heparan sulphate proteoglycans [33] were used (mAbs were kindly provided by G. David). The mAb 3G10 has a higher

Table 1 Relative content of heparan sulphate proteoglycans from the bone-marrow stromal cell line MS-5 on the mRNA and protein levels

The relative content of the different heparan sulphate proteoglycans in the cell extract of MS-5 cells was estimated from the scanned Western blot, using the mAb 3G10 (shown in Figures 1 and 5). The mAb 3G10 recognizes a common neo-epitope, generated after heparitinase digestion of heparan sulphate proteoglycans. This mAb has been shown to detect most, if not all, heparan sulphate proteoglycan core proteins after heparitinase digestion [33]. The relative content of the different heparan sulphate proteoglycan mRNAs was calculated from scanned Northern blots using DIG-labelled heparan-sulphate-proteoglycan-specific probes (shown in Figures 4 and 5). There is no value in the Protein column for glypican because calculation was not possible, as glypican could not be separated as a single band in the Western blot.

	$10^3 \times$ Core protein size	mRNA (Northern blot; relative content) (%)	Protein (Western blot, mAb 3G10; relative content) (%)
Syndecan-1	69/82	3	31
Syndecan-2	48	65	24
Syndecan-3	125	25	19
Syndecan-4	35	2	19
Glypican	64	4	—
Betaglycan	120–140	< 1	1
Perlecan	~ 400	< 1	1

affinity than the specific ones and is therefore especially suitable for a more sensitive detection of heparan sulphate proteoglycan core proteins. Furthermore, the mAb 3G10 allows an estimation of the relative abundance of the different heparan sulphate proteoglycans. In addition, a polyclonal antiserum against betaglycan and the mAbs MM-1 (a mixture of four mAbs) against perlecan (kindly provided by G. David) were used.

Using the mAb 3G10, a strong staining was observed for proteoglycans from the supernatant and cell extract of the bone-marrow stromal cell line MS-5 (Figures 1 and 2). Using the specific mAbs for Western blot analysis of proteoglycans from the cell extract, this staining could be attributed to syndecan-1 (M_r approx. 69 000), syndecan-2 (48 000), syndecan-4 (35 000), glypican (approx. 69 000) and betaglycan (approx. 140 000) (Figure 1). Syndecan-3 (M_r 125 000) was deduced from the staining pattern of the mAb 2E9 against syndecan-1, which cross-reacts with syndecan-3. This was supported also by RT-PCR and Northern blot analysis (see below). In the supernatant of MS-5 cells, signals in the region of M_r approx. 35 000 and approx. 110 000–145 000 were detected (Figure 2) and could be assigned to syndecan-1 (approx. 69 000), syndecan-4 (approx. 35 000), glypican (approx. 69 000) and betaglycan (110 000–145 000) respectively. In addition, the expression of perlecan was shown immunochemically in the cell extract, using the mAbs MM-1, and by Northern blot analysis (see Figure 5). Slight differences between the expected and observed electrophoretic mobility may be explained by co-migration effects of the different proteoglycan core proteins, e.g. glypican is observed at M_r 69 000 instead of 64 000.

Relative amounts of the different heparan sulphate proteoglycans were calculated from scanned films of Western blots, using the mAb 3G10 (Figures 1 and 2). The calculation is based on the assumption that for syndecans and glypican the average number of heparan sulphate chains per core protein is approximately the same (Table 1). In the cell extract, syndecan-1 and syndecan-2 are the most abundant heparan sulphate proteoglycans, followed by syndecan-3 and syndecan-4, whereas betaglycan and perlecan were minor components. For glypican no clear-cut estimation was possible (Table 1). For the supernatant only a rough estimation could be obtained by scanning Western

Figure 3 PCR analysis of heparan sulphate proteoglycans from the bone-marrow stromal cell line MS-5

RT PCR analysis was performed using specific primer pairs for the different syndecans (1–4), glypican and betaglycan. Amplification products for syndecan-1, syndecan-2, syndecan-3, syndecan-4, betaglycan and glypican could be detected in MS-5 cells. Amplification products had the expected size and sequence, as predicted from known cDNA sequences. Numbers on the left indicate the size in bp.

blots, even after different exposure times. Whereas syndecan-2 and perlecan could not be detected, glypican and syndecan-4 were minor components (< 10 %). Syndecan-1, syndecan-3 and betaglycan appear to be the major heparan sulphate proteoglycans in the supernatant.

RT PCR analysis of proteoglycans from the bone-marrow stromal cell line MS-5

To confirm the results of the Western blot analysis, RT PCR was performed using specific primer pairs for the different syndecans (1–4), glypican, perlecan and betaglycan. The specificity of

Figure 4 Northern blot analysis of heparan sulphate proteoglycans from the bone-marrow stromal cell line MS-5

Poly (A)⁺ RNA (1 µg per lane) was run on an agarose gel and blotted, and membrane strips were hybridized against digoxigenin-labelled antisense RNA probes for syndecan-1, syndecan-2, syndecan-3, syndecan-4, glypican and betaglycan. Exposure times for RNA detection were: 30 s for syndecan-1, syndecan-2 and syndecan-3, 1 min for glypican, 5 min for syndecan-4 and 15 min for betaglycan. Numbers on the right indicate the size in kbp. The relative position of 18 S and 28 S ribosomal RNA is also given.

Figure 5 Northern (A) and Western (B) blot analysis of perlecan from MS-5 cells

(A) Poly(A)⁺ RNA from kidney and MS-5 cells were size-fractionated on an agarose gel, blotted and hybridized against a perlecan probe (perlecan cDNA fragment: 12 082 to 14 071 bp). Hybridization was performed with a radioactive-labelled probe for the kidney RNA blot (exposure time: 12 days at -80°C) and a DIG-labelled probe for the MS-5 RNA blot (exposure time: 20 min at room temperature). (B) Isolated heparan sulphate proteoglycans from the cell extract of MS-5 cells were submitted to SDS electrophoresis on a 4% agarose gel and subsequently blotted onto Immobilon N⁺ membrane. Detection was done by using perlecan-specific mAbs MM-1 and the enhanced chemiluminescence detection system. Perlecan bands are indicated by arrows in (A) and (B). For an explanation of the numbers on the left and right, see legend to Figure 4.

PCR-amplification products was confirmed by sequencing of cloned amplification products. Figure 3 shows that syndecan-1, syndecan-2, syndecan-3, syndecan-4, betaglycan, and glypican could be detected in MS-5 cells by this technique. Perlecan was not identified by RT PCR but only by Northern and Western blot analysis (see Figure 5). Unsuccessful amplification was probably due to the selected primers.

Northern blot analysis of proteoglycans from the bone-marrow stromal cell line MS-5

Expression of the different heparan sulphate proteoglycans has also been analysed at the mRNA level. Poly(A)⁺ RNA from MS-5 cells was analysed using DIG-labelled antisense RNA probes against syndecans 1–4, glypican, betaglycan and perlecan (Figures 4 and 5). The probe for syndecan-1 detected a major band at approx. 2.5 kb and a minor band at approx. 3 kb, which was only visible after longer exposure (results not shown). The probe for syndecan-2 detected two bands at 2 kb and at approx. 1.0 kb. Syndecan-3 mRNA appeared with a size of approx. 5.2 kb and syndecan-4 was detected at approx. 2.8 kb. The probe for glypican also detected two bands, a smaller one at approx. 3.9 kb and a larger one at approx. 5.0 kb. The message for betaglycan showed up with a size of approx. 6 kb and perlecan with a size of approx. 14 kb. In addition to the band of the expected size of 14 kb, extensive cross-hybridization was observed, which is most probably due to the epidermal-growth-factor-like domains of the probe, which also contains a domain with homology to domain 4A of laminin.

Comparison of the relative amounts of mRNA for the different heparan sulphate proteoglycans was calculated from scanned films of the Northern blots, corrected for exposure times and the

specific DIG activity of the different probes (Table 1). These results clearly show that on the mRNA level syndecan-2 and syndecan-3 are the most abundant species in MS-5 cells, followed by syndecan-1, glypican and syndecan-4. Betaglycan and perlecan showed the lowest amount of mRNA.

DISCUSSION

Heparan sulphate proteoglycans represent a heterogeneous family of macromolecules that are involved in fundamental biological processes like cell-cell interaction and control of cell growth and differentiation. Different types of heparan sulphate proteoglycans were found on cell surfaces, in basement membranes and in the extracellular matrix [9,34–42]. It was shown that heparan sulphate proteoglycans might also play an important role in the interaction of haematopoietic stem and stromal cells [7–11,43]. Gordon et al. [7] and Roberts et al. [8] showed that interleukin-3 and granulocyte/macrophage colony-stimulating factor can be bound by heparan sulphate proteoglycans from bone-marrow stromal cells or their extracellular matrix and can be presented in a biologically active form to haematopoietic precursor cells. Most of these studies indicated that heparan sulphate proteoglycans were synthesized by bone-marrow stromal cells rather than haematopoietic stem or progenitor cells [11,19–22,43]. However, we have shown that the human haematopoietic progenitor cell line TF-1, which can be induced to differentiate into the erythroid and also the monocyte/macrophage lineage, and is therefore considered to be at least bipotent [44], synthesizes a heparan sulphate proteoglycan, which is not related to the syndecan family [27].

Until the present study, the limited availability of bone-marrow stromal cell lines has precluded a detailed analysis of the proteoglycans from stromal cells [11,18]. However, the establishment of bone-marrow stromal cell lines [25] has made a detailed analysis of their proteoglycans possible. In the present study we have analysed the proteoglycans synthesized by the murine bone-marrow stromal cell line MS-5. This murine stromal cell line efficiently supports the growth and differentiation of human and murine haematopoietic progenitor cells [25] and therefore might be extremely useful for studying the molecular mechanisms underlying the interactions of haematopoietic progenitor and stromal cells.

Isolation and biochemical characterization of proteoglycans from the murine bone-marrow stromal cell line MS-5, revealed that MS-5 cells synthesize several different heparan sulphate proteoglycans. In the cell extract, biochemical analysis by fluorography and gel-permeation chromatography allowed the identification of three heparan sulphate proteoglycan populations with M_r values in the range of $> 200\,000$, $> 200\,000$ – $100\,000$ and $90\,000$ – $30\,000$. In the supernatant of MS-5 cells, a heparan sulphate proteoglycan population with an M_r distribution of $M_r > 200\,000$ – $90\,000$ was found (results not shown).

To answer the question of whether the heparan sulphate proteoglycans isolated from the bone-marrow stromal cell line MS-5 are related to known cell-surface-associated heparan sulphate proteoglycans, analysis of proteoglycans was performed by Western blot analysis using mAbs against the different heparan sulphate proteoglycans, as well as by RT-PCR and Northern blot analysis. In addition, the mAb 3G10 against a common neo-epitope, generated after heparitinase-digestion of heparan sulphate proteoglycans, has been used, which has been shown to detect most, if not all, heparan sulphate proteoglycan core proteins after heparitinase digestion [33]. The use of this antibody allows an estimation of the relative abundance of the different

heparan sulphate proteoglycans, independent of the relative affinities of different specific mAbs.

Using the mAb 3G10 and the specific antibodies against the different heparan sulphate proteoglycans, syndecan-1, syndecan-2, syndecan-3, syndecan-4, glypican, betaglycan and perlecan were identified in the cell extract of MS-5 cells. These data were also confirmed by RT-PCR and Northern blot analysis (Figures 3–5). In the supernatant of the bone-marrow stromal cell line MS-5, syndecan-1, syndecan-3, syndecan-4, glypican and betaglycan were detected. The presence of syndecan-3 was deduced from the staining pattern of the mAb 2E9 against syndecan-1, which has been shown to cross-react with syndecan-3. To confirm the expression of syndecan-3 and to exclude the possibility that the two bands with an M_r of approx. $110\,000$ – $140\,000$ represent a hybrid type of syndecan-1 with additional chondroitin sulphate chains, differential digestion with heparinase/heparitinase and/or chondroitinase AC/ABC was performed. Western blot analysis, using the specific mAb 2E9, showed sharp core protein bands after heparinase/heparitinase treatment, which did not change after combined heparinase/heparitinase and chondroitinase AC/ABC treatment (results not shown). Similar results have been obtained by comparison of the fluorographic patterns, as well as immunochemical staining patterns using the mAb 3G10 (results not shown). From these data it can be concluded that these bands most probably correspond to syndecan-3, and that MS-5 cells do not express hybrid forms of proteoglycans.

The expression of the different heparan sulphate proteoglycans was also studied by Northern blot analysis (Figures 3–5). RT-PCR and Northern blot analysis both demonstrate the presence of transcripts for the different heparan sulphate proteoglycans in MS-5 cells. Some mRNA signals of the heparan sulphate proteoglycans showed up with more than one signal. This has been previously observed for syndecan-1 [45,46], syndecan-2 [47–49] and glypican [50]. However, in the case of glypican we observed an additional signal for a mRNA with a size of approx. 5.0 kb. Whether this signal is specific for glypican or is due to cross-hybridization, has not been analysed further. The different transcript sizes may correspond to different genes, alternatively spliced gene products or the use of different polyadenylation sites. Such a mechanism has been proposed recently for syndecan-2, where three potential polyadenylation signal sequences have been observed [47].

Quantitative analysis of the expression of the different heparan sulphate proteoglycans was performed at the protein and mRNA levels. Comparison of the relative amounts of core proteins, calculated from Western blots using the mAb 3G10 (Figures 1 and 2), revealed that in the cell extract, syndecan-1 and syndecan-2 are the most abundant heparan sulphate proteoglycans, followed by syndecan-3 and syndecan-4, whereas betaglycan and perlecan were minor components. For glypican no estimation was possible, as no clear-cut separation of the core protein band was possible. The calculation is based on the assumption, that for syndecans and glypican the average number of heparan sulphate chains per core protein is approximately the same. In the supernatant only a rough estimation could be obtained by scanning of Western blots, even after different exposure times. Whereas syndecan-2 and perlecan could not be detected, glypican and syndecan-4 were relative minor components ($\leq 10\%$). Syndecan-1, syndecan-3 and betaglycan appear to be the major heparan sulphate proteoglycans in the supernatant. Comparison of the relative amounts of mRNA for the different heparan sulphate proteoglycans, calculated from the Northern blots (Table 1) clearly shows that on the mRNA level, syndecan-2 and syndecan-3 are the most abundant species in MS-5 cells, followed by syndecan-1, glypican and syndecan-4. Betaglycan and perlecan

showed the lowest amount of mRNA. Comparison of the relative abundance of the different heparan sulphate proteoglycans at the protein and mRNA levels showed a good correlation for syndecan-2, syndecan-3, betaglycan and perlecan. However, for syndecan-1 and syndecan-4 small amounts of mRNA are in contrast with relatively high protein levels. This might be explained in part by differences in the stability and translational efficiency of their mRNAs. A post-translational regulation of syndecan expression has been described recently for syndecan-1 [51–53], where an up-regulation on the protein level was observed without changes to the mRNA level. Therefore, post-translational regulation might be one of the mechanisms by which syndecan expression is regulated in different cell types, activation states or differentiation stages.

Whereas approx. 50% of the proteoglycans in the MS-5 cell line are found to be cell-associated, Western blot analysis revealed that syndecan-1, syndecan-3, syndecan-4, betaglycan and glypican were also found in the supernatant of MS-5 cells. This could be due either to proteolytic cleavage of the syndecans and betaglycan or to phospholipase-mediated release of the glycosylphosphatidylinositol-anchored glypican. Such a proteolytic cleavage of betaglycan by plasmin has been reported recently by Lamarre et al. [54]. For glypican a rapid release from the cell membrane into the pericellular matrix has also been shown [50]. In addition, a protease-sensitive site at the border of the transmembrane domain and ectodomain is a common feature of all members of the syndecan family [55]. Therefore release of proteoglycans from the cell surface might be an important step during activation of bone-marrow stromal cells and/or differentiation of haematopoietic precursor cells.

In the present study we focused on the analysis of the heparan sulphate proteoglycans, which appear to play a crucial role in the interaction of haematopoietic stem and stromal cells. The different functions of the individual proteoglycans, synthesized by the bone-marrow stromal cell line MS-5, have not been studied until now. Analysis of the murine bone-marrow stromal cell line MS-5 revealed, that MS-5 cells synthesize at least seven different heparan sulphate proteoglycans. These proteoglycans may be involved in several different functions, like binding of distinct growth factors by bone-marrow stromal cells, which support growth and differentiation of haematopoietic progenitor cells into the different lineages (see the Introduction). Such a binding of growth factors by heparan sulphate proteoglycans has also been shown in other cell systems [14,15,55–57]. The heparan sulphate proteoglycans synthesized and secreted by MS-5 cells might have a similar function in the extracellular matrix. The function of cell-associated or membrane-bound heparan sulphate proteoglycans may be different. Cell-associated heparan sulphate proteoglycans might function as co-receptors for growth factors, a mechanism which has been described recently for the membrane-bound betaglycan in the high-affinity binding of transforming growth factor- β to its signal-transducing receptor [16,54]. Membrane-associated heparan-sulphate and chondroitin-sulphate proteoglycans may also be involved in adhesion and homing of haematopoietic cells either to stromal cells or to the extracellular matrix [10,22,43,58–60].

This work was supported by grants Ha 1565/2-2 and Ju 197/3-2 from the Deutsche Forschungsgemeinschaft and grant W24/92/OS-4 by the Deutsche Krebshilfe.

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Received 13 March 1997/4 June 1997; accepted 19 June 1997