Proteoglycan synthesis in human and murine haematopoietic progenitor cell lines: isolation and characterization of a heparan sulphate proteoglycan as a major proteoglycan from the human haematopoietic cell line TF-1

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Proteoglycans of bone-marrow stromal cells and their extracellular matrix are important components of the microenvironment of haematopoietic tissues. Proteoglycans might also be involved in the interaction of haematopoietic stem and stromal cells. Recently, several studies have been reported on the proteoglycan synthesis of stromal cells, but little is known about the proteoglycan synthesis of haematopoietic stem or progenitor cells. Here we report on the isolation and characterization of proteoglycans from two haematopoietic progenitor cell lines, the murine FDCP-Mix A4 and the human TF-1 cell line. Proteoglycans were isolated from metabolically labelled cells and purified by several chromatographic steps, including anion-exchange and size-exclusion chromatography. Biochemical

characterization was performed by electrophoresis or gel-filtration chromatography before and after digestion with glycosaminoglycan-specific enzymes or HNO₂ treatment. Whereas FDCP-Mix A4 cells synthesize a homogeneous chondroitin 4-sulphate proteoglycan, isolation and characterization of proteoglycans from the human cell line TF-1 revealed, that TF-1 cells synthesize, in addition to a chondroitin sulphate proteoglycan, a heparan sulphate proteoglycan as major proteoglycan. For this heparan sulphate proteoglycan a core protein size of approx. 59 kDa was determined. Immunochemical analysis of this heparan sulphate proteoglycan revealed that it is not related to the syndecan family nor to glypican.

INTRODUCTION

The control of growth and differentiation in the haematopoietic system is mediated by complex interactions of haematopoietic cells, growth factors and the bone-marrow microenvironment [1]. Whereas the importance of cytokines, growth factors and colony-stimulating factors (CSFs) has been shown by numerous studies (for reviews, see [2,3]), the essential role of the haematopoietic microenvironment, e.g. the bone-marrow stromal cells and their extracellular matrix, was realized only during the last few years [4–10]. The importance of the haematopoietic microenvironment 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* [1,6]. In these cultures, haematopoietic stem-cell self renewal, differentiation and development into mature cells does not depend on added haematopoietic growth factors, but requires direct physical contact between the stromal cells and the haematopoietic cells. However, the molecular mechanisms of the interaction of stromal cells and haematopoietic cells are not fully understood. Whereas stromal cells have been shown to produce a variety of growth factors, either constitutively or after activation, 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 of primitive haematopoietic progenitor cells and stromal cells [7,8,11,12]. Roberts et al. [8] have shown that interleukin-3 (IL-3) and granulocyte–macrophage (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 also in other systems [13–17]. Recent data indicate that proteoglycans might be involved also in cell adhesion and homing of haematopoietic stem and progenitor cells [12,18–20].

Whereas these studies have shown that proteoglycans might play a major role in the interaction of haematopoietic stem and stromal cells, a detailed analysis of these proteoglycans is, due to the limited availability of primitive haematopoietic progenitor and stromal cell lines, still lacking. In fact, there are only a few reports on proteoglycans from haematopoietic cell lines [18,21–26]. In most of the earlier studies, either whole bone-marrow or long-term bone-marrow cultures have been used [5,27]. However, this approach does not allow either the direct identification of the cells that synthesize the proteoglycans or a localization of the proteoglycans in the microenvironment. Most studies have pointed to stromal cells rather than haematopoietic stem cells or progenitor cells as the source of heparan sulphate proteoglycans [18,21–25].

Unlike most other haematopoietic cell lines, the murine FDCP-Mix cell line and the human TF-1 cell line are dependent for their survival and growth on either soluble haematopoietic growth factors or on a close contact with stromal cells or cell lines in the absence of added growth factors. These characteristics make TF-1 and FDCP-mixA4 cells particularly useful for studying the

Abbreviations used: IL-3, interleukin 3; GM-CSF, granulocyte–macrophage colony-stimulating factor; IMDM, Iscove's modified Dulbecco's medium; HS, horse serum; FCS, fetal-calf serum; SN, supernatant; Ex, extract.

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molecular mechanisms underlying the interactions of haematopoietic progenitor and stromal cells. In the present study we have compared the proteoglycan synthesis of the murine multipotent haematopoietic cell line FDCP-Mix A4 and the human bipotent haematopoietic cell line TF-1. Whereas FDCP-Mix A4 cells synthesize a homogeneous chondroitin 4-sulphate proteoglycan [22], isolation and characterization of proteoglycans from the human haematopoietic cell line TF-1 revealed that TF-1 cells synthesize, in addition to a chondroitin sulphate proteoglycan, a heparan sulphate proteoglycan as major proteoglycan.

MATERIALS AND METHODS

Materials

Carrier-free $\text{H}_2^{35}\text{SO}_4$ was obtained from New England Nuclear (Dreieich, Germany) and $[\text{H}]$ serine from Amersham Buchler (Braunschweig, 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 obtained from Pharmacia/LKB (Freiburg, Germany). The Biosil Amino 5S column was purchased from Bio-Rad (Munich, Germany).

^{14}C -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). Hydroxyapatite was purchased from Bio-Rad, (Munich, Germany) and guanidinium chloride was from Pierce (Cologne, Germany).

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)], and heparitinase [heparan sulphate lyase (EC 4.2.2.8)] were purchased from Seikagaku (Tokyo, Japan). Iscove's modified Dulbecco's medium (IMDM), RPMI 1640, horse serum (HS) and fetal-calf serum (FCS) were obtained from GIBCO (Eggenstein, Germany). Spectra Por 6 dialysis tubes with a cut-off value of 1 kDa were obtained from Spectrum (Houston, TX, U.S.A.).

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

Radiolabelling of cell lines

The human bipotent haematopoietic progenitor cell line TF-1 was established from a patient with an erythroleukaemia. TF-1 cells can be induced to differentiate into the erythroid, but also the monocyte-macrophage lineage, and therefore are at least bipotent [28]. These cells are dependent upon IL-3, GM-CSF or erythropoietin for their survival and proliferation. Alternatively, TF-1 cells can be maintained in close contact with stromal cells in the absence of added growth factors (W. Ostertag and U. Just, unpublished work). TF-1 cells were routinely kept in RPMI 1640 medium supplemented with 10% FCS and recombinant human GM-CSF (5×10^3 units/ml).

The murine multipotent haematopoietic cell line FDCP-Mix clone A4 line was isolated from long-term bone-marrow cultures [6]. These cells are not leukaemic. FDCP-Mix A4 cells are dependent on IL-3 for their survival and self-renewal. Cultured with stromal layers, FDCP-Mix A4 cells can be induced to proliferate and differentiate without added growth factors [6]. FDCP-Mix A4 cells were routinely grown in IMDM supplemented with 20% HS and WEHI-3BD-conditioned medium as a source of IL-3 at a concentration that stimulated optimal cell growth.

The fibroblast cell line was isolated from a human cervix sample excised during delivery for medical reasons, by the outgrowth method as described elsewhere [29] and was kept in RPMI-1640 supplemented with 10% FCS.

The cells were metabolically radiolabelled with $40 \mu\text{Ci/ml}$ $[\text{H}]$ serine and $80 \mu\text{Ci/ml}$ $[\text{S}]$ sulphate in serine- and sulphate-reduced medium (TF-1: serine- and sulphate-free RPMI 1640 with 10% FCS and 5×10^3 units/ml recombinant human GM-CSF; FDCP-Mix A4: serine- and sulphate-free IMDM with 10% HS and 10% WEHI-3BD-conditioned medium at a cell density of $3 \times 10^5/\text{ml}$ for 36 h; and cervix fibroblasts in serine- and sulphate-free RPMI 1640 for 24 h). To obtain a more efficient core-protein labelling in some of the experiments, FCS was dialysed against serine- and sulphate-depleted RPMI 1640 using Spectra Por 6 dialysis tubes.

Isolation of proteoglycans

Proteoglycans were isolated as described elsewhere [30,31]. Briefly, proteoglycans were extracted from metabolically labelled cells (cell layer, Ex) with 4 M guanidinium chloride, pH 5.8, containing 50 mM sodium acetate, 50 mM EDTA, 0.1 M 6-aminohexanoic acid, 50 mM benzamidine hydrochloride, 5 mM PMSF, 10 mM *N*-ethylmaleimide and 0.5% (w/v) CHAPS. Proteoglycans were also isolated from the culture medium (supernatant, SN). Extracts and supernatants were cleared by centrifugation at 2000 *g* for 20 min.

The total incorporation of $[\text{S}]$ sulphate in proteoglycans was calculated from size-exclusion chromatography on TSK 3000 column for supernatants and extracts. Elution was performed by 20 mM sodium phosphate containing 4 M guanidinium chloride and 0.05% (w/v) CHAPS, pH 6.0. The elution profile was monitored by liquid-scintillation counting.

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% 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 a 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 and 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 'high salt fraction' was eluted with 4 M guanidinium chloride in buffer A. 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.

Further purification of guanidinium chloride-containing samples was performed by hydroxyapatite chromatography. Briefly, samples were diluted with water to a final concentration of 5 mM sodium phosphate and applied to a 0.5 ml hydroxyapatite column equilibrated with 5 mM sodium phosphate buffer, pH 6.8. Washing was performed by 6 vol. of 5 mM sodium phosphate buffer. Proteoglycans were eluted with 0.3 M sodium phosphate buffer, pH 6.8.

For further purification of heparan sulphate proteoglycans, samples from the supernatants of TF-1 cells were diluted with digestion buffer [32] and digested with chondroitinase AC/ABC (2 h, 37 °C). Digested samples were diluted to approx. 0.15 M NaCl with 50 mM Tris buffer, pH 7.4, and applied to a DEAE-

Sephacel column (2 ml) equilibrated with 50 ml of Tris/HCl/2 M urea/0.1 % Triton X-100/0.15 M NaCl/1 mM PMSF, pH 7.4. Elution was performed with 6 ml of 50 mM Tris/HCl/2 M urea/0.1 % Triton X-100/1 M NaCl/1 mM PMSF, pH 7.4. Heparan sulphate proteoglycans were precipitated with 90 % ethanol in the presence of 50 μ g of BSA at -20°C [33].

For analysis of the hydrophobicity of isolated proteoglycans, the chromatographic separation of liposome-incorporated proteoglycans was performed on a Sepharose CL 4B column as described by Lories et al. [34]. As a positive control, proteoglycans of human cervix fibroblasts were used.

Immunochemical analysis of proteoglycans

Immunochemical analysis of proteoglycans was performed either by dot-blot and Western-blot analyses. The following monoclonal antibodies against cell-surface heparan sulphate proteoglycans were used: S1 (anti-glypican), 2E9 [anti-(syndecan 1 + 3)], 1C7 [anti-(syndecan 3)], and 8G3 [anti-(syndecan 4)]. In addition, the monoclonal antibody 3 G 10 against an epitope generated in heparitinase-digested heparan sulphate proteoglycans was used (monoclonal antibodies were kindly provided by G. David, University of Leuven, Leuven, Belgium). For dot-blot analysis, samples were immobilized on Biotodyne B membrane (Pall, Dreieich, Germany). Blocking was performed with 5 % BSA/1 % dry milk in 0.02 M Tris/0.3 M NaCl, pH 7.5, for 1 h at room temperature. Incubation with specific monoclonal antibodies (1–10 μ g/ml) was performed overnight at 4°C . Detection of bound antibodies was carried out according to standard procedures using either peroxidase-conjugated second antibodies and diaminobenzidine or a chemiluminescence detection system (ECL; Amersham, Braunschweig, Germany). Western-blot analysis was performed according to standard procedures. Detection was done by measuring chemiluminescence (ECL).

Characterization of proteoglycans

Biochemical characterization of the isolated proteoglycans was done by electrophoresis before and after digestion with glycan-specific enzymes using fluorography. Enzymic digestion with heparinase (heparin lyase; EC 4.2.2.7) and heparitinase (heparan sulphate lyase; EC 4.2.2.8), chondroitin sulphate lyase AC (EC 4.2.2.5) and chondroitin sulphate lyase ABC (EC 4.2.2.4) was performed in the presence of proteinase inhibitors as described in [32,35]. For enzymic digestion of proteoglycans from the 'high-salt fraction', proteoglycans were precipitated three times at -20°C with 4 vol. of 95 % ethanol. Electrophoresis was performed as described by Laemmli using gradient gels (8–25 % or 4.5–22 % acrylamide and 3 % bisacrylamide cross-linker). After electrophoresis, gels were treated with En³Hance (NEN) according to the manufacturer's instructions. Kodak XAR-5 film was used for fluorographic detection. Exposure times ranged from 1 to 60 days.

In addition, molecular-mass distributions of native and HNO_2 -treated proteoglycans were estimated by size-exclusion chromatography on TSK 4000.

β -Elimination and HNO_2 degradation

The size of the glycosaminoglycan chains was determined after alkaline β -elimination [36] by gel-permeation chromatography on a TSK 4000 column.

HNO_2 degradation of heparan sulphate was performed as described by Shively and Conrad [37]. Briefly, proteoglycans were treated with 0.6 M HNO_2 , pH 1.5, for 10 min at room

temperature. Thereafter samples were neutralized by the addition of 1 M Na_2CO_3 , pH 8.0.

Disaccharide analysis

Disaccharide analysis was performed as described elsewhere [31]. Briefly, proteoglycans were digested with either chondroitinase AC or chondroitinases AC/ABC. Digested samples were submitted to anion-exchange chromatography on a Biosil Amino 5S column (Bio-Rad). The elution profile was monitored by liquid-scintillation counting.

RESULTS

Isolation of proteoglycans

Proteoglycans were isolated from metabolically labelled murine and human haematopoietic cell lines and purified by several chromatographic steps. The content of metabolically labelled proteoglycans (^{35}S incorporation) of supernatants and extracts was calculated from size-exclusion chromatography on a TSK 3000 column. Whereas about 80 % of the proteoglycans (^{35}S -labelled material) were found in the cell extract of the murine multipotential cell line FDCP-Mix A4 (20 % SN, 80 % Ex), for the human haematopoietic progenitor cell line TF-1 the majority of proteoglycans was found in the supernatant (70–90 % SN, 10–30 % Ex).

Figure 1 Anion-exchange chromatography on MEM-SEP QMA of the extract from the human haematopoietic progenitor cell line TF-1

Proteoglycans (^{35}S -labelled material) from the extract (Ex) of the human haematopoietic stem-cell line TF-1 were submitted to anion-exchange chromatography. The column was equilibrated with 20 mM sodium phosphate/0.1 M NaCl/0.1 LiCl/0.05 % CHAPS, pH 4.5. Elution was performed with a two-step linear NaCl gradient (0.2–0.75 M and 0.75–3.0 M). High-salt fractions were eluted by the use of 4 M guanidine hydrochloride (Gua/HCl). The elution profile was monitored by liquid-scintillation counting (^3H , ^{35}S).

Figure 2 Size-exclusion chromatography on TSK 4000 column of proteoglycans from the cell extract of TF-1 cells

Proteoglycans eluted in the salt gradient of the anion-exchange chromatography (Figure 1) were submitted to size-exclusion chromatography on a TSK 4000 column. Elution profiles for the two partially separated peaks (TF-1 Ex A and B) are shown for the native (—) and HNO_2 -treated (---) samples. Elution profiles were monitored by liquid-scintillation counting.

Purification and characterization of proteoglycans from the human haematopoietic cell line TF-1

Purification of proteoglycans was performed by an initial anion-exchange chromatography of supernatants and cell extracts from the human haematopoietic cell line TF-1.

Proteoglycans (^{35}S -labelled material) was eluted in the salt

Figure 3 SDS/PAGE on a 4.5–20% gradient gel of proteoglycans from the salt gradient of the anion-exchange chromatography of TF-1 cell extract

Proteoglycans from fractions of pool A (a) and pool B (b) of the anion-exchange chromatography (Figure 1) were treated with chondroitinase AC/ABC (lane A), heparinase/heparitinase (lane H) or both (lane A/H). Untreated (lane N) and treated samples were submitted to SDS/PAGE under reducing conditions. Detection was performed by fluorography.

gradient as well as in the 'high-salt fraction' (eluted by 4 M guanidinium chloride). For the TF-1 cell line, relative amounts of proteoglycans, eluted in the salt gradient and in the high-salt fraction respectively differed markedly. Whereas from the extract comparable amounts of proteoglycans were found in the gradient and in the 'high-salt fraction' (Figure 1), at least 90 % of the proteoglycans from the supernatant of TF-1 (which represented about 80 % of the total proteoglycans) eluted in the salt gradient (see Figure 5 below). Proteoglycans from the high-salt fraction were further characterized by gel-permeation chromatography in the presence of 4 M guanidinium chloride, ethanol precipitation of ^{35}S -containing material and subsequent anion-exchange chromatography. By this procedure, for the cell extract, approx. 50 % of the ^{35}S -containing material could be eluted in the salt gradient. The molecular-mass distribution of these proteoglycans was comparable with those which eluted in the salt gradient of the initial anion-exchange chromatography.

The molecular-mass distribution of proteoglycans, purified by anion-exchange chromatography, was analysed by size-exclusion chromatography on TSK 4000 column and SDS/PAGE. The relative proportion of heparan sulphate proteoglycans was calculated by size-exclusion chromatography of native and HNO_2 -treated samples. By this procedure, 39 % of the total proteoglycans (9 % in the cell extract and 30 % in the supernatant) could be identified as heparan sulphate proteoglycans.

For the cell extract of TF-1, the two partially separated peaks from anion-exchange chromatography (Figure 1, salt gradient) were analysed separately (Figures 2A and 2B). In both samples, two heparan sulphate populations with K_{av} values of 0.10–0.50 and 0.65–0.90 respectively were observed. However, the relative content of the heparan sulphate proteoglycan (K_{av} 0.10–0.50) was higher in sample B than in sample A. Analysis of the HNO_2 -treated samples revealed an additional chondroitin sulphate proteoglycan that was eluted in the K_{av} range of 0.05–0.55. These results have been confirmed by electrophoresis of the samples before and after digestion with glycosaminoglycan-specific enzymes using fluorographic detection (Figures 3a and 3b). From SDS/PAGE the molecular-mass distributions of proteoglycans

Figure 4 Size-exclusion chromatography on a TSK 4000 column of proteoglycans from the high-salt fraction of the anion-exchange chromatography of a TF-1 cell extract

Proteoglycans eluted in the high-salt fraction of the anion-exchange chromatography (Figure 1) were submitted to size-exclusion chromatography on a TSK 4000 column. Elution profiles are shown for the native (—) and HNO_2 -treated (---) samples. Elution profiles were monitored by liquid-scintillation counting.

Figure 5 Anion-exchange chromatography on MEM-SEP QMA of the supernatant from the human haematopoietic cell line TF-1

Proteoglycans from the supernatant (SN) of the human haematopoietic cell line TF-1 were submitted to anion-exchange chromatography. Elution conditions are the same as in Figure 1. Fractions were pooled as indicated by the bars. Gua/HCl, guanidinium chloride.

were estimated as about > 200–130 kDa for the heparan sulphate proteoglycan and 60–20 kDa for the second heparan sulphate population, calculated from the electrophoretic mobility compared with ^{14}C -labelled standard proteins. For the small heparan sulphate β -elimination did not change the elution profile (results

Figure 6 Size-exclusion chromatography on TSK 4000 column of proteoglycans from the supernatant of TF-1 cells eluted in the salt gradient of the anion-exchange chromatography

Proteoglycans from the supernatant (SN) of TF-1 cells eluted in the salt gradient of the anion-exchange chromatography (Figure 5) were submitted to size-exclusion chromatography on a TSK 4000 column. Elution profiles are shown for the native ('nativ'; top panel) and HNO_2 -treated (' HNO_2 ', middle panel) and chondroitinase AC/ABC-treated ('AC/ABC', bottom panel) samples. Elution profiles were monitored by liquid-scintillation counting.

not shown), indicating that this heparan sulphate represents free glycosaminoglycan chains rather than a distinct proteoglycan. In addition, the molecular-mass distribution of the chondroitin sulphate proteoglycan was estimated to be about > 200–100 kDa. Size-exclusion chromatography of proteoglycans from the high-salt fraction of the cell extract of TF-1 cells is shown in Figure 4. For the native sample proteoglycans in the K_{av} range 0–0.50 were detected. HNO_2 treatment of this sample revealed that it contained a chondroitin sulphate proteoglycan and a heparan sulphate proteoglycan with comparable molecular-mass distributions. SDS/PAGE analysis showed a molecular-mass distribution of about > 200–100 kDa for both proteoglycans (results not shown). The molecular-mass distribution of these proteoglycans were comparable with those which were eluted in the salt gradient of the initial anion-exchange chromatography.

Anion-exchange chromatography of the supernatant of TF-1 cells (Figure 5) showed that a major fraction (about 80%) of biosynthetically labelled proteoglycans was eluted in the salt gradient, whereas a minor fraction was eluted in the high-salt fraction. The elution profile obtained by size-exclusion chromatography of the proteoglycans from the salt gradient is shown in Figure 6 (top panel). In this sample, the content of heparan sulphate proteoglycans was determined to be approx. 30% as demonstrated by HNO_2 treatment (Figure 6, middle panel). The relative content of chondroitin sulphate proteoglycans was approx. 70% (Figure 6, bottom panel). The molecular-mass distribution is comparable with that obtained for the respective proteoglycans from cell extract with a K_{av} of 0.15–0.55 for the heparan sulphate proteoglycan and of 0.05–0.55 for the chondroitin sulphate proteoglycan.

Figure 7 Size-exclusion chromatography on a TSK 4000 column of glycosaminoglycan chains obtained after β -elimination of proteoglycans from the supernatant of TF-1 cells eluted in the salt gradient of the anion-exchange chromatography

Proteoglycans from the supernatant of TF-1 cells eluted in the salt gradient of the anion-exchange chromatography (Figure 5) were treated first either by heparinase/heparitinase ('Hep/Hept beta', top panel) or chondroitinase AC/ABC ('AC/ABC beta', bottom panel) and thereafter submitted to β -elimination. Size-exclusion chromatography was performed on a TSK 4000 column. Elution profiles were monitored by liquid-scintillation counting.

In addition to the analysis of the native proteoglycans, the molecular-mass distribution of their glycosaminoglycan moieties was analysed after β -elimination (Figure 7). For heparan sulphate relatively small glycosaminoglycan chains with a K_{av} of 0.55–0.85 were observed, corresponding to an molecular mass of approx. 20–6 kDa when compared with glycosaminoglycan chains of known molecular-mass distribution [38] (Figure 7, bottom panel),

Figure 8 SDS/PAGE of proteoglycans eluting in the salt gradient of the anion-exchange chromatography of TF-1 supernatant

(a) Proteoglycans from the salt gradient of the anion exchange chromatography (Figure 5) were treated with chondroitinase AC/ABC (lane A), heparinase/heparitinase (lane H) or both (lane A/H). Untreated (lane N), and treated samples were submitted to SDS/PAGE on a 4.5–22% gradient gel under reducing conditions. Detection was performed by fluorography. (b) Proteoglycans from the salt gradient of the anion-exchange chromatography (Figure 5) were concentrated by ethanol precipitation and submitted to SDS/PAGE on a 8–25% gradient gel under reducing conditions before (lane 1) and after (lane 2) chondroitinase AC/ABC treatment. (c) Proteoglycans from the salt gradient of the anion-exchange chromatography (Figure 5) were treated with chondroitinase AC/ABC and thereafter submitted to anion-exchange chromatography on DEAE-Sephacel. The eluted heparan sulphate proteoglycans were concentrated by ethanol precipitation and submitted to SDS/PAGE on an 8–25% gradient gel under reducing conditions before (lane 1) and after (lane 2) heparinase/heparitinase treatment.

whereas the size of the chondroitin sulphate chains was larger, with a K_{av} of 0.2–0.65 (Figure 7, top panel). Comparable data were obtained for the glycosaminoglycan chains of proteoglycans isolated from the cell extract (results not shown).

Analysis of the high-salt fraction from the supernatant, representing a minor portion of the proteoglycans, revealed a heparan sulphate proteoglycan representing about 50% of proteoglycans in this sample (results not shown). In addition, a chondroitin sulphate proteoglycan with a comparable size distribution was detected. The molecular-mass distribution of both proteoglycans was estimated to be in the K_{av} range 0–0.55.

Analysis of proteoglycan core proteins

Identification of core proteins of the proteoglycans from the TF-1 cell line was attempted for the major proteoglycans and was successful for those eluted in the salt gradient of the supernatant, which contained at least 70% of the total proteoglycans. Analysis of core proteins was done by electrophoresis before and after digestion with glycan-specific enzymes using fluorography. For the heparan sulphate proteoglycan, additional purification steps were applied (see above under 'Purification of proteoglycans'). Figure 8(a) shows the molecular-mass distribution of the native proteoglycans from the supernatant of TF-1 cells with a molecular mass range of > 300–100 kDa for the chondroitin sulphate proteoglycan and of > 200–100 kDa for the heparan sulphate proteoglycan (4.5–22% gradient gel). Identification of the core protein of the chondroitin sulphate proteoglycan by enzymic digestion required concentration of the sample by ethanol precipitation. After chondroitinase AC/ABC digestion (Figure 8b, lane 2), two core proteins in the range of 20–24 kDa could be detected by SDS/PAGE (8–25% gradient gel). In addition, a band of approx. 90 kDa showed up in the smear of the remaining heparan sulphate proteoglycan.

Analysis of the heparan sulphate proteoglycan was performed after further purification. The chondroitinase AC/ABC-digested sample was applied to ion-exchange chromatography and concentrated by ethanol precipitation. SDS/PAGE revealed a heparan sulphate proteoglycan core protein of approx. 59 kDa (Figure 8c, lane 2), calculated from the electrophoretic-mobility comparison with ^{14}C -labelled standard proteins.

The hydrophobicity of proteoglycans from the supernatant and cell extract of the TF-1 cell line was analysed by liposome incorporation. Chromatographic analysis on Sepharose CL-4B revealed about 15% hydrophobic proteoglycans in the cell-extract fraction. For the proteoglycans in the supernatant, less than 2% incorporation into lipid vesicles was observed (results not shown).

Immunochemical analysis of proteoglycans from the haematopoietic progenitor cell line TF-1

To answer the question as to whether the heparan sulphate proteoglycan isolated from the haematopoietic progenitor cell line TF-1 is related to known cell-surface-associated heparan sulphate proteoglycans, immunochemical analysis of proteoglycans was performed by dot-blot and Western-blot analysis. The following monoclonal antibodies against cell-surface heparan sulphate proteoglycans were used: S1 (anti-glypican), 2E9 [anti-(syndecan 1 + 3)], 1C7 and 8G3 (anti-syndecan 4). In addition the monoclonal antibody 3G10 against an epitope generated in heparitinase-digested heparan sulphate proteoglycans was used (monoclonal antibodies were kindly provided by G. David, University of Leuven, Leuven, Belgium). As a positive control, heparan sulphate proteoglycans isolated from a human cervix fibroblast cell line (cell extract) were used. The

Table 1 Reactivity of the proteoglycans from the supernatant and cell extract of TF-1 cells and the cervix fibroblasts with the different monoclonal antibodies in the semiquantitative dot-blot analysis

Portions (5 µl) of proteoglycan-containing samples were applied to a Biotodyne B membrane; subsequently, membranes were blocked by 5% BSA/1% dry milk as described in the Materials and methods section. Detection was performed by the use of the indicated specific monoclonal antibodies against the different heparan sulphate proteoglycans and the ECL system. Samples were analysed at different dilutions. Staining intensities were scored: —, no staining; +/—, faint staining, if any; +, positive staining of the undiluted sample; ++, positive staining of 1:5-diluted sample; +++/++++, positive staining of 1:10-diluted sample; +++++, positive staining of 1:100-diluted samples

mAb against:	TF-1 supernatant	TF-1 cell extract	Cervix fibroblast cell extract
Syndecan 1 + 3 (2E9)	—	—	+++
Syndecan 3 (1C7)	—	—	—
Syndecan 4 (8G3)	+	+/—	+++
Glypican (S1)	—	—	+++
Heparan sulphate proteoglycans (3G 10)	++++	++++	+++

Figure 9 Immunochemical detection of proteoglycans isolated from human cervix fibroblasts and human haematopoietic cell line TF-1

Proteoglycans of human cervix fibroblasts (cell extract, lane 1) and the haematopoietic cell line TF-1 (lane 2) were treated with heparitinase and submitted to SDS/PAGE on a 4.5–15% gradient gel and subsequently blotted on to a Biotodyne B membrane. Detection was performed using monoclonal antibody 3 G 10 (left panel) and anti-glypican monoclonal antibody S1 (right panel) and the ECL (enhanced chemiluminescence) detection system.

reactivity of the proteoglycans from the supernatant and cell extract of TF-1 cells and the cervix fibroblasts with the different monoclonal antibodies in the semiquantitative dot-blot analysis is summarized in Table 1. Using the monoclonal antibody 3 G 10 against a common neoepitope generated after heparitinase digestion of heparan sulphate proteoglycans [39], a strong staining was observed for proteoglycans from the human cervix fibroblast cell line and in the supernatant and cell extract of the haematopoietic cell line TF-1. Whereas for the cervix fibroblasts this staining could be attributed by semiquantitative dot-blot analysis to syndecan 1, syndecan 4 and glypican, for the TF-1 cell line only a weak staining for syndecan 4 was observed. These findings were confirmed by Western-blot analysis (Figure 9). Whereas for cervix fibroblasts heparan sulphate proteoglycan core-protein signals for the major known heparan sulphate proteoglycans in the range of 34–125 kDa could be detected, for the haematopoietic cell line TF-1, only one strong signal of the order of

Figure 10 Anion-exchange chromatography on MEM-SEP QMA of the cell extract of the murine haematopoietic cell line FDCP-Mix A4

Proteoglycans from the cell extract of the murine haematopoietic cell line FDCP-Mix A4 were submitted to anion-exchange chromatography. Elution conditions were the same as in Figure 1. Fractions were pooled as indicated by the bar. Gua/HCl, guanidium chloride.

approx. 60 kDa could be demonstrated with monoclonal antibody 3 G 10 (Figure 9, left). This band corresponds to the signal observed for the metabolically labelled core protein of the heparan sulphate proteoglycan from the supernatant of TF-1 cells (Figure 8c). To confirm the results of the semi-quantitative dot-blot analysis, Western blot analysis was performed also using the specific monoclonal antibodies against syndecans and glypican. Figure 9 (right-hand panel) shows the immunochemical reactivity with the monoclonal antibody S1 (anti-glypican). It should be noted, that comparable amounts of proteoglycans from TF-1 and cervix fibroblasts have been applied in both Western blots. Whereas for cervix fibroblasts a clear signal for glypican is obvious, for TF-1 cells no glypican could be detected. The heparan sulphate proteoglycan from the TF-1 cell line reacted only with the monoclonal antibody 3 G 10 after heparit-

Figure 11 SDS/PAGE on a 4.5–20% gradient gel of proteoglycans from the anion-exchange chromatography of cell extract of the murine haematopoietic cell line FDCP-Mix A4

Proteoglycans eluted in the salt gradient of the anion-exchange chromatography (fractions 17–21, as indicated by the bar in Figure 9) were treated with chondroitinase AC/ABC (A), heparinase/heparitinase (H) or both (A/H). Untreated (lane N) and treated samples were submitted to SDS/PAGE under reducing conditions. Detection was performed by fluorography.

inase digestion in dot-blot, but also in Western-blot, analysis. In addition, a core protein of comparable size was detected in the cell extract of TF-1 cells by the monoclonal antibody 3 G 10 (results not shown).

The possible relationship of the 90 kDa core protein to a CD 44 proteoglycan was tested by dot-blot analysis using the specific monoclonal antibody 7D8 (kindly provided by G. David) and the monoclonal antibody F10-44-2 (Boehringer, Mannheim, Germany), which should react with all known CD 44 variants [40]. A relationship of the 90 kDa core protein to a CD 44 proteoglycan variant could not be confirmed.

Characterization of proteoglycans from the murine haematopoietic progenitor cell line FDCP-Mix A4

The murine multipotential haematopoietic cell line FDCP-Mix A4 was analysed by the procedure described above. Anion-exchange chromatography of the cell extract is shown in Figure 10. Whereas a homogeneous peak (^{35}S -labelled material) was eluted in the salt gradient, no ^{35}S -labelled proteoglycans could be observed in the high-salt fraction.

Analysis of proteoglycans from the homogeneous peak in the salt gradient by SDS/PAGE, before and after treatment with different glycosaminoglycan-specific enzymes, revealed a chondroitin sulphate proteoglycan with a molecular-mass distribution of 200–70 kDa as the only proteoglycan (Figure 11). These results were confirmed by size-exclusion chromatography on a TSK 4000 column and by disaccharide analysis (results not shown).

DISCUSSION

Heparan sulphate proteoglycans represent a heterogeneous family of macromolecules which are involved in fundamental biological processes such as 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 [11,41–48]. Recently it has been shown that proteoglycans might play a major role in the

interaction of haematopoietic stem and stromal cells [7,8,11,12,25,49]. Most of these studies have pointed to stromal cells rather than haematopoietic stem cells or progenitor cells as the source of heparan sulphate proteoglycans [18,21–23,25,49]. Whereas analysis of the proteoglycans from stem cells was precluded previously by their low frequency in the bone marrow, the recent establishment of factor-dependent multipotent progenitor cell lines that are able to adhere and grow on stromal cells in the absence of added growth factors [6,28] has made a detailed analysis of their proteoglycans possible. Studies on the murine haematopoietic cell lines FDCP-Mix [22] FDCP-1 and FDCP-2 [23,26] have shown a chondroitin 4-sulphate proteoglycan to be the only proteoglycan in these murine haematopoietic cell lines. In the present study we have confirmed these results for the FDCP-Mix A4 cell line and have found a chondroitin 4-sulphate proteoglycan with a molecular-mass distribution of 200–70 kDa. However, the question is whether these results are representative for all haematopoietic progenitor cells and especially also for human progenitor cells.

Our results for the human bipotent haematopoietic progenitor cell line TF-1 clearly show that this is not the case. Analysis of proteoglycans from the TF-1 cell line has shown a heparan sulphate proteoglycan as a major proteoglycan with a molecular mass in the range of > 200–100 kDa in the cell extract and in the supernatant which has been calculated to represent about 40% of the total proteoglycans (30% supernatant, 9% cell extract). In addition, a chondroitin sulphate proteoglycan was found with a molecular mass of > 300–100 kDa in the supernatant and of about > 200–100 kDa in the cell extract. The comparable molecular-mass distribution of the respective proteoglycans in the supernatant and cell extract would suggest, that they represent the same or closely related proteoglycans. This is supported not only by a core protein of a similar size of approx. 59 kDa for the heparan sulphate proteoglycan in the supernatant and cell extract, but also by the analysis of the glycosaminoglycan chains, showing a comparable molecular-mass distribution of heparan sulphate chains (K_{av} 0.55–0.85, corresponding to a size of approx. 20–6 kDa). From the size of the native heparan sulphate proteoglycan (molecular mass > 200–100 kDa), the size of the core protein and the calculated size of the glycosaminoglycan chains an average number of three to six heparan sulphate chains per core protein can be estimated.

The hydrophobicity of the proteoglycans from the supernatant and cell extract was analysed by incorporation into lipid vesicles. For the proteoglycans from the supernatant, no incorporation into lipid vesicles was found. The relative content of proteoglycans with a hydrophobic domain in the cell extract could be estimated to be of the order of about 15% of proteoglycans in the cell extract.

In the ion-exchange chromatography, some of the proteoglycans could be eluted only by 4 M guanidinium chloride (high-salt fraction). By re-chromatography we have shown that at least part of these proteoglycans are similar to, or identical with, those eluted by the salt gradient. These data indicate that proteoglycans from the high-salt fraction may have formed aggregates, presumably with other components of the extracellular matrix, and therefore showed stronger interactions with the resin of the anion-exchange column.

Analysis of the core proteins was rather difficult, as in several cell-culture experiments the incorporation of [^3H]serine into proteoglycan core proteins was very low. This problem could be partially overcome by dialysis of the serum component of the culture medium against serine-free medium before culture. By this approach, characterization of the core proteins was possible for the major proteoglycans. Proteoglycans from the supernatant

represent about 80% of the total proteoglycans (^{35}S -labelled material) and were used successfully for the determination of core-protein sizes. For the chondroitin sulphate proteoglycan, two core proteins in the range of 20–24 kDa were observed. In addition, a band in the range of about 90 kDa was detectable. The size of the smaller core proteins accords excellently with that of the well known proteoglycan serglycin, which has been described as a major proteoglycan in haematopoietic cells [11,49,50]. The possible relationship of the 90 kDa core protein to a CD 44 proteoglycan could not be confirmed by immunochemical analysis using the specific monoclonal antibody 7D8 and monoclonal antibody F10-44-2, which should react with all known CD 44 variants [40]. For the heparan sulphate proteoglycan from the supernatant of TF-1 cells a core protein of approx. 59 kDa was found. To answer the question of whether the heparan sulphate proteoglycan isolated from the haematopoietic progenitor cell line TF-1 is related to known cell-surface-associated heparan sulphate proteoglycans, immunochemical analysis of proteoglycans was performed by dot-blot and Western-blot analysis. Monoclonal antibodies against the different syndecan isoforms and glypican were used. The heparan sulphate proteoglycan from the TF-1 cell line did not react with any of these monoclonal antibodies. This was in contrast with heparan sulphate proteoglycans that have been isolated from a human cervix fibroblast cell line and which were used as a control. The molecular-mass distribution of the native proteoglycan, the core protein size and the lack of immunochemical reactivity with monoclonal antibodies against known heparan sulphate proteoglycans might indicate a novel heparan sulphate proteoglycan. However, this has to be confirmed by sequence analysis. For the second heparan sulphate, which was eluted in the salt gradient with a molecular-mass distribution of about 60–20 kDa, it has been shown by β -elimination that it did not represent a small heparan sulphate proteoglycan, but rather free heparan sulphate glycosaminoglycan chains. A similar finding was reported by Yeaman and Rapraeger [52], who described free heparan sulphate chains accumulating intracellularly in the macrophage-like cell line P388D1.

Thus, in contrast with the results for the murine haematopoietic progenitor cell lines of the FDCP series, where only a homogeneous chondroitin sulphate proteoglycan is found, the human bipotent haematopoietic cell line TF-1 synthesizes different proteoglycans. The distribution of the proteoglycans differs greatly between FDCP-Mix and TF-1 cells. Whereas the majority of the proteoglycans in the FDCP-Mix cell line is found to be cell-associated, for the TF-1 cell line most of the proteoglycans were found in the supernatant. Detailed analysis of these proteoglycans revealed a heparan sulphate proteoglycan as a major proteoglycan. Our results were supported in part by an analysis of proteoglycans from the human HEL cell line [53]. The HEL cell line, similarly to the TF-1 cell line, can be induced to differentiate into erythrocytes and macrophage-like cells and therefore might also be regarded as a multipotential or at least bipotential haematopoietic cell line. Even though Schick and Senkowski-Richardson have focused on the analysis of the chondroitin sulphate proteoglycans and did not study the heparan sulphate proteoglycans, it can be concluded from their data that HEL cells synthesize, in addition to two or three different chondroitin sulphate proteoglycans, also heparan sulphate proteoglycans. Whether the differences between the murine and human haematopoietic cell lines are due to the different species, e.g. human and mouse, or reflect the state of differentiation of these haematopoietic cell lines, requires investigation.

The different functions of the individual proteoglycans synthesized by the human haematopoietic cell line TF-1 have not

been studied until now. However, Gordon et al. [7] and Roberts et al. [8] have shown, that heparan sulphate proteoglycans expressed on stromal cells can bind growth factors and present them in a biologically active form to haematopoietic stem and progenitor cells. Such a binding of growth factors by heparan sulphate proteoglycans has also been shown in other cell systems [16,54,55]. The heparan sulphate proteoglycan synthesized and secreted by TF-1 cells found in the supernatant might have a similar function in the extracellular matrix and bind growth factors. The function of cell-associated or membrane-bound heparan sulphate proteoglycans on haematopoietic progenitor cells may be different. These 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 [17]. Membrane-associated proteoglycans may be also involved in the adhesion and homing of haematopoietic cells either to stromal cells or to the extracellular matrix [12,18,20,49,51]. Chondroitin sulphate proteoglycans, like serglycin, are found also in secretory granules of several haematopoietic cells and might be involved in the storage of proteolytic enzymes [11].

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