Expression of glypican-4 in haematopoietic-progenitor and bone-marrow-stromal cells

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Heparan sulphate proteoglycans and the extracellular matrix of bone-marrow-stromal cells are important components of the microenvironment of haematopoietic tissues and are involved in the interaction of haematopoietic stem and stromal cells. Previous studies have emphasized the role of heparan sulphate proteoglycan synthesis by bone-marrow-stromal cells. In the present study we describe the expression of glypican-4 (GPC-4), belonging to the glypican family, in bone-marrow-stromal cells and haematopoietic-progenitor cells of human and murine origin. Expression of GPC-4 was shown on the mRNA-level by reverse transcription–PCR and Northern blot analysis. Amplification products were cloned and sequenced, to confirm these results. To analyze the expression of GPC-4 on the protein level, polyclonal antibodies against selected peptides were raised in rabbits. Western blot analysis showed expression of GPC-4 as a heparan sulphate proteoglycan in the human haematopoietic-progenitor cell line TF-1 and normal human bone marrow. These results were confirmed by FACS analysis of TF-1 cells. Furthermore, GPC-4-positive progenitor cells and stromal cells were enriched from normal human bone marrow by magnetic-cell sorting and analysed by confocal laser-scanning microscopy.

Key words: haematopoiesis, heparan sulphate proteoglycans, kidney.

INTRODUCTION

Heparan sulphate proteoglycans represent a heterogeneous family of macromolecules which are involved in fundamental biological processes like cell–cell interaction and control of cell growth and differentiation [1–12]. Cell-membrane-bound heparan sulphate proteoglycans are either anchored in the cell membrane via a membrane-spanning domain, like the syndecans [for reviews see 8–10], or covalently linked to membrane lipids of the glycosylphosphatidylinositol (GPI) type [for reviews see 9,13,14]. The first heparan sulphate proteoglycan with a GPI-anchor was described in 1990 [15], and after molecular cloning it was named glypican (glypican-1, GPC-1). Recently several other heparan sulphate proteoglycans, which are also GPI-anchored, have been identified and characterized by cDNA sequence analysis: cerebroglycan (GPC-2) from embryonic-rat brain [16], OCI-5 (an older name for GPC-3) from an embryonic-rat intestinal-epithelial cell line [17], the human GPC-3 which is associated with the Simpson–Golabi–Behmel overgrowth syndrome [18], K-glypican (GPC-4) from mouse kidney [19] and GPC-5 [20]. In addition to their GPI-anchor, these heparan sulphate proteoglycans share several structural features with glypican, for example a similar core-protein size of approx. 60 kDa and the clustering of the glycosaminoglycan attachment site near the C-terminus [10]. These similarities have led to the establishment of the so called glypican-gene family.

Recently it has been shown that heparan sulphate proteoglycans might play an important role in the interaction of haematopoietic-stem cells and stromal cells [1,21–26]. In this context we have analyzed the expression of heparan sulphate proteoglycans in several haematopoietic-progenitor cell lines and bone-marrow-stromal cell lines. Using reverse transcription (RT)-PCR and Northern blot analysis we have observed the expression of GPC-4 in several haematopoietic-progenitor cell lines and bone-marrow-stromal cell lines. Here we report on the analysis of GPC-4 expression in haematopoietic cell lines and normal human bone marrow.

MATERIALS AND METHODS

Cell culture and cell lines

The human haematopoietic-progenitor cell line TF-1 was established from a patient with an erythroleukaemia [27]. TF-1 cells can be induced to differentiate into more mature erythroid cells, but also into macrophage-like cells, and therefore might be regarded as a haematopoietic-progenitor cell line with bipotent differentiation potential. The HEL cell line was obtained from the A.T.C.C. (Rockville, MD, USA). HEL cells are similar to TF-1 cells in their differentiation potential [28]. The murine multipotent-haematopoietic cell line FDCP-Mix-A4 was isolated from long-term bone marrow cultures [29]. The murine committed erythroid progenitor cell line ELM-D has been established from an erythroblastic leukaemia and was described by Itoh et al. [30]. ELM-D cells grow strictly dependent on stromal cells, like the MS-5 stromal cell line [31]. The murine haematopoietic multilineage cell line Myl-D7 has been established recently [32]. Myl-D7 cells have the potential to differentiate into the myeloid-macrophage, erythroid or lymphoid pathway. The murine

Abbreviations used: FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPC, glypican; GPI, glycosylphosphatidylinositol; MACS, magnetic cell sorting; RT, reverse transcription.

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haematopoietic cell line MS-5 has been established from long-term bone marrow cultures [31]. MS-5 cells have the capacity to support the growth of murine and human haematopoietic stem and progenitor cells *in vitro*. Normal human kidney, bone marrow and cervix tissues were obtained during surgery.

RNA isolation and cDNA synthesis

Approx. 10^7 cells were collected by centrifugation and total RNA was isolated according to the method of Chomczynski and Sacchi [33]. 2 μg of total RNA were used to synthesize cDNA by RT (cDNA Preamplification kit, Gibco BRL, Eggenstein, Germany) starting with oligo dT. cDNA was synthesized in 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 500 μM each dNTP, 500 nM oligo dT and 200 units of reverse transcriptase (Superscript II) at 42 °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.

RT-PCR

2 μl of synthesized cDNA were used for PCR reactions. Primers were synthesized according to the previously published sequence for murine GPC-4 [19] or according to the respective sequence of human GPC-4 of sequence-analysed clones. PCR reactions were performed in 10 mM KCl, 20 mM Tris/HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X-100, 250 μM dNTP with 0.5 units of vent Polymerase (New England Biolabs) or in 67 mM Tris/HCl (pH 8.8), 6.7 mM $MgCl_2$, BSA at 170 μg/ml, 16.6 mM $(NH_4)_2SO_4$, 250 μM each dNTP, 2.5 units of *Taq* polymerase (Ampli-*Taq*, Perkin-Elmer Cetus, Überlingen, Germany) and 30 pmoles of the respective 5′-primers and 3′-primers. Oligonucleotide primers were synthesized by MWG Biotech (Ebersberg, Germany). PCR was performed in a thermal cycler (Trioblock, Biometra, Göttingen, Germany). Denaturation was at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec. The number of cycles was 25 or 50 (if necessary, nested RT-PCR was performed). PCR amplification products were analysed on a 1.2 % (w/v) agarose gel (Seakem LE, FMC, Biozym, Hameln, Germany) and visualized by ethidium bromide staining.

Primers used for testing the expression in different cell lines and tissues were, 5′-GGTCCGCACCATGGCACGCTT-3′ (459–470), 5′-CTCACACATTCCAAGTACTCA-3′ (1021–1040) and the nested primer 5′-TAAATAGCTCTGAATTTT-GCATG-3′ (865–885). The position in brackets indicates the relative position of the primers from the murine cDNA [19]. In addition, the following glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-primers were used: 5′-primer, 5′-GGTGAA-AGTCGGAGTCAACGGA-3′ and 3′-primer, 5′-CTGGGGTC-ACGCTCCTGGAAGA-3′.

Cloning of PCR-amplified DNA fragments

DNA-fragments were extracted from agarose gels (Quiex, Qiagen, Hilden, Germany), ligated into pCR TM II (Invitrogen, Leek, Netherlands) for 16 hours at 14 °C and transformed into competent XL1-Blue cells (Stratagene, Heidelberg, Germany). Plasmid DNA from positive clones was checked for the right insert by sequence analysis [34].

Northern blot analysis

1 μg of poly-A^+-RNA was sized fractionated on a formaldehyde-containing 1 % agarose gel. Gels were blotted on to nylon membranes (Boehringer, Mannheim, Germany) and hybridized against digoxygenin-labelled probes (1 μl/5 ml) at 50 °C for 16 h. Labelling of the probes was performed by PCR (Boehringer, Mannheim, Germany). Filters were washed twice for 5 min at room temperature in 2 × SSC, 0.1 % SDS and twice at 50 °C in 0.2 × SSC, 0.1 % SDS for 15 minutes (where SSC is 0.5 M NaCl/0.015 M sodium citrate). Detection of the chemiluminescent signal was performed following the manufacturer's instructions (Boehringer, Mannheim, Germany). Films were exposed overnight.

Polyclonal antisera against human GPC-4

Comparison of the human and mouse amino-acid sequence allowed us to select 5 peptides for the generation of polyclonal antisera: Peptide 1, amino acids 88–101; peptide 2, amino acids 54–66; peptide 3, amino acids 367–382; peptide 4, amino acids 509–520; peptide 5, amino acids 220–233 (Figure 1). Peptides were synthesized by Eurogentec (Seraing, Belgium). Immunization of rabbits was performed according to standard procedures using about 250 μg of the respective antigen, coupled to keyhole-limpet haemocyanin (KLH) as carrier, in complete Freund's adjuvant. Antibody titres were analysed by a semi-quantitative enzyme immunoassay and compared to preimmune sera.

Analysis of GPC-4 expression in haematopoietic cell lines, normal human bone marrow and kidney

GPC-4 expression in haematopoietic-progenitor and bone-marrow-stromal cell lines, normal human bone marrow and kidney was analyzed by dot blot and Western blot as described previously [35]. Briefly, TF-1 cells were washed once with RPMI 1640 medium and lysed in lysis buffer [50 mM Tris/HCl, 150 mM NaCl, pH 7.4, 1 % Nonidet P40, 1 % sodium deoxycholate, 5 mM EDTA, 0.4 mM Pefablock SC PLUS (Boehringer Mannheim, Germany)] for 30 min on ice. Debris was removed by a short centrifugation and the cell lysate was stored at −20 °C. Human bone marrow and kidney tissue, frozen in liquid nitrogen, was homogenized and proteins were extracted in RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 % Nonidet P40, 5 mM EDTA, 1 % sodium deoxycholate and 1 mM PMSF) on ice for 60 min. Insoluble fractions were pelleted and removed by centrifugation. The supernatant was stored at −20 °C until further analysis. Proteoglycans were isolated by anion-exchange chromatography (Sepharose Q), treated with heparinase/heparitinase, subjected to SDS/PAGE (4–15 %, w/v) under reducing conditions and blotted on to Biodyne B membrane as described previously [35]. Detection of GPC-4 was performed using the polyclonal anti-(GPC-4 peptide) rabbit antiserum against peptide 4 and affinity-purified peroxidase-labelled donkey anti-rabbit antiserum as a second antibody.

Analysis of GPC-4 expression by immunohistochemistry, confocal laser-scanning microscopy and FACS

Immunohistochemistry

Sections of human kidney were fixed in 4 % (v/v) formaldehyde and stained using the polyclonal rabbit antiserum against peptide 4 and a second peroxidase-conjugated goat anti-rabbit antibody according to standard procedures.

FACS analysis

The human haematopoietic-progenitor cell line TF-1 was stained with the polyclonal rabbit antiserum against peptide 4 and a second fluorescein isothiocyanate (FITC)-conjugated goat anti-

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Peptide 1 (aa 88-101)
                          *
GPC-4      hK-Gly     SEQCNHLQAVFASR
GPC-4      mK-Gly     SEQCNHLQAIFASR
GPC-1      h-Gly      RDSSRVLQAMLATQ
GPC-2      r-Cbr      EDSGSFLIHTLAAR
GPC-3      r-Oci      QSASMELKFLIIQN
GPC-5      -          QTSSSTLKFLISRN
GPC-6      -          EETSHFVRTTFVSR

Peptide 2 (aa 54-66)
                        **
GPC-4      hK-Gly     HLKIC-PQGSTCCS
GPC-4      mK-Gly     HLKIC-PQDYTCCS
GPC-1      h-Gly      HLRIC-PQGYTCCT
GPC-2      r-Cbr      HLQIC-PQEYTCCS
GPC-3      r-Oci      DLQVCLPKGPTCCS
GPC-5      -          DLQVCISKKPTCCT
GPC-6      -          HLRICP-QEYTCCT

Peptide 3 (aa 367-382)
                      *   *
GPC-4      hK-Gly     RPHHPEERPTTAAGTSL
GPC-4      mK-Gly     RPYHPEQRPTTAAGTSL
GPC-1      h-Gly      GKLAPRERPPSGT---L
GPC-2      r-Cbr      RSSAEEERPTTAAGTNL
GPC-3      r-Oci      IDKKVLKVARVEHEETL
GPC-5      -          KEKHGMKTTTRNSEETL
GPC-6      -          RPYNPEERPTTAAGTSL

Peptide 4 (aa 509-520)

GPC-4      hK-Gly     SE---FDYNAT---------DHAG
GPC-4      mK-Gly     SEFEYNATDH--------SGKSAN
GPC-1      h-Gly      RK---VSRKSSSS--RTPLTHALP
GPC-2      r-Cbr      GA---PVVPPARPPRPPRPPRRDG
GPC-3      r-Oci      GGSGDGMIKVKNQLRFLAELAYDL
GPC-5      -          GGSGSGE--VKRTLKITDWMPDDM
GPC-6      -          TE---FEFVTT---------EAPA

                      *     *
GPC-4      hK-Gly     SEFDYNATDHAG
GPC-4      mK-Gly     SEFEYNATDHSG

Peptide 5 (aa 220-233)
                          *
GPC-4      hK-Gly     TFAQGLAVAGDVV
GPC-4      mK-Gly     TFAQGLAVARDVV
GPC-1      h-Gly      SFVQGLGVASDVV
GPC-2      r-Cbr      ALVQGLETGRNVV
GPC-3      r-Oci      IFLQALNLGIEVI
GPC-5      -          TFLQALNLGIEVI
GPC-6      -          TFVQGLTVGREVA
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Figure 1 Peptides used for the generation of GPC-4-specific antisera

The figure shows a comparison of amino acid sequences derived from different members of the GPC family. The first column provides the current nomenclature for members of the GPC family and the second column gives the older nomenclature for the same proteins along with an indication of the species from which the sequence was determined. Peptide sequences of human GPC-4 were selected according to differences between murine and human GPC-4 [differences are indicated by (*)]. Whereas murine and human GPC-4 sequences show a high sequence similarity, comparison with the deduced protein sequences of other members of the glypican gene family show that the sequence similarity is lower and for peptide 4 there is no sequence similarity. For peptide 4, two different alignments are shown that were generated by comparison of either the whole sequence or the peptide. Abbreviations used: aa, amino acids; h, human; m, murine; r, rat.

rabbit antibody at 4 °C without fixation and subjected immediately to FACS analysis using a FACS-Calibur cell sorter (Beckton-Dickinson, Heidelberg, Germany).

Enrichment of GPC-4 positive cells and confocal laser-scanning microscopy

Normal human bone marrow was obtained during surgery. Haematopoietic cells were isolated after mechanical disruption from the spongiosa by washing with PBS. GPC-4-expressing cells were enriched by magnetic cell sorting (MACS) using an AS column (Miltenyi, Bergisch Gladbach, Germany). Bone marrow cells were incubated without fixation with the polyclonal antiserum against peptide 4 for 1 h at 4 °C. After washing with PBS, cells were incubated for 1 h at 4 °C with magnetic microbeads (Miltenyi) conjugated with a polyclonal goat anti-(rabbit IgG) antiserum. After washing, cells were separated by MACS according to the manufacturers instructions. Eluted cells were then stained by a second FITC-conjugated goat anti-(rabbit IgG) antiserum for 1 h at 4 °C. After washing of the cells, cytospins were prepared and analysed by confocal laser-scanning microscopy using a LSM 410 (Zeiss, Cologne, Germany). Confocal scanning was performed using a Plan Neofluar objective (40×) with a numerical aperture of 0.75 and an argon laser (15 mW). Fluorescence detection was performed at the wavelength of 488 nm.

RESULTS

Expression of GPC-4 by haematopoietic-progenitor and bone-marrow-stromal cell lines

GPC-4, isolated from murine kidney cells was recently described as a heparan sulphate proteoglycan with a restricted expression pattern [19]. However, analysis of the expression of heparan sulphate proteoglycans in haematopoietic-progenitor and bone-marrow-stromal cells clearly indicates GPC-4 expression in these haematopoietic cells (Figure 2). RT-PCR analysis was performed with oligonucleotides derived from the known murine cDNA sequence [19] in a nested PCR reaction. Several murine and human haematopoietic-progenitor and bone-marrow-stromal cell lines were analysed: the murine stromal cell line, MS-5; the murine haematopoietic-progenitor cell lines, Myl-D7, FDCP-mix

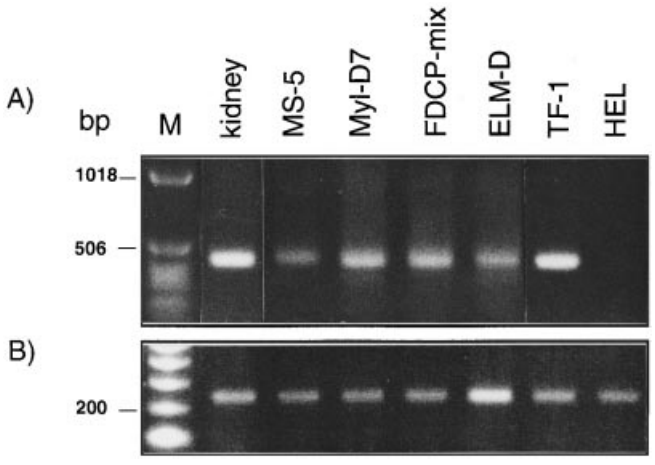

Figure 2 RT-PCR analysis of GPC-4 in haematopoietic-stem and -progenitor cell lines and the bone-marrow-stromal cell line MS-5

(**A**) RT-PCR analysis was performed using specific primer pairs for GPC-4. mRNA of human kidney was used as a positive control. The cell lines MS-5, Myl-D-7, FDCP-Mix-A4 and ELM-D are of murine origin, and TF-1 and HEL are of human origin. M, molecular size markers. (**B**) As a positive control for expression of a house-keeping gene RT-PCR analysis was performed using specific primers for GAPDH.

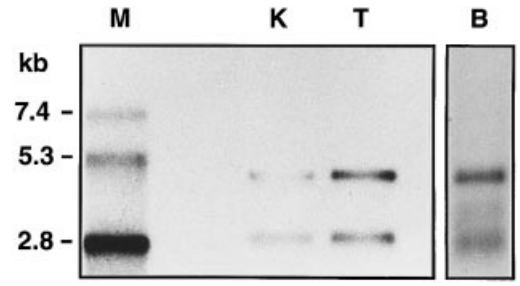

Figure 3 Northern blot analysis of GPC-4 from human kidney, the human haematopoietic-progenitor cell line TF-1 and normal human bone marrow

(K) Kidney (0.7 μg), (B) bone marrow (1.0 μg), and (T) TF-1 (1.0 μg) poly A (+) RNA was run on an 1% agarose gel, blotted and hybridized against a random digoxygenin-labelled PCR fragment of GPC-4.

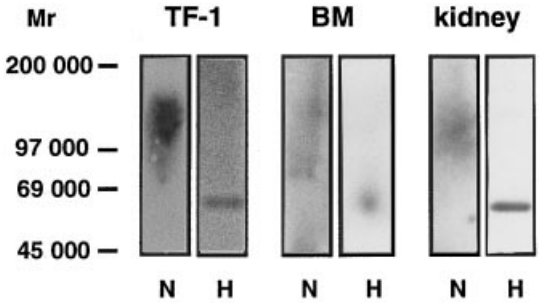

Figure 4 Western blot analysis of GPC-4 expression in human kidney, normal bone marrow and the haematopoietic-progenitor cell line TF-1

GPC-4 expression in human kidney, normal human bone marrow (BM) and the human haematopoietic-progenitor cell line TF-1 was analyzed by SDS/PAGE and Western blotting using the polyclonal rabbit antiserum against peptide 4. Proteoglycans from cell or tissue extract were precipitated with ethanol and submitted to SDS/PAGE [4–15% (w/v) gel] either prior to (N) or after (H) digestion with heparinase/heparitinase. The positions of the molecular mass markers (Mr in Da) are shown on the left.

A4, and ELM-D; the human progenitor cell lines, TF-1 and HEL. A specific amplification product of the expected size (426 bp) was obtained for all of these haematopoietic cell lines, with the exception of the HEL cell line and the cervix-fibroblast cell line (Figure 2). GAPDH primers were used in a control RT-PCR to ensure the quality of cDNA synthesis. As the cDNA sequence of human GPC-4 had not been described when this study was performed, we determined the complete cDNA sequence of human GPC-4 from one of these cell lines, TF-1. However, while this manuscript was under review, the cDNA sequence of human GPC-4, derived from a human foetal-brain library, was reported by Veugelers et al. [36] and confirmed our results.

Northern blot analysis

To determine the cellular expression pattern of human GPC-4, poly-A$^+$RNA from human kidney cells and TF-1 cells was compared by northern blot analysis using a non-radioactive digoxygenin-labelled probe. The chemiluminescent signals showed 2 bands of approx. 4.6 and 2.9 kb for kidney, bone marrow and TF-1 cells (Figure 3). Both bands seem to be specific for GPC-4 since the use of three different probes of the cDNA coding region, from the N-terminal, C-terminal and mid-region, all gave rise to the same two bands (results not shown).

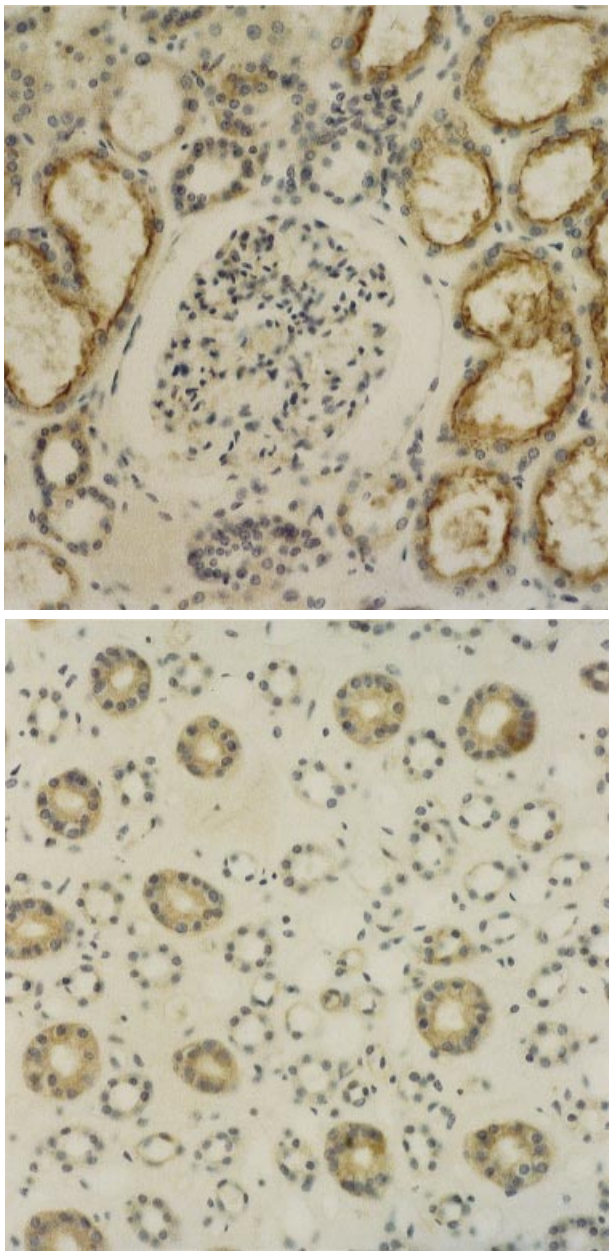

Figure 5 Immunohistochemical analysis of GPC-4 expression in human kidney

Sections of human kidney (cortex, upper panel; medulla, lower panel) were formaldehyde fixed and stained using the polyclonal rabbit antiserum against peptide 4 and a second peroxidase-conjugated goat anti-rabbit antibody. The brown-coloured staining indicates the presence of GPC-4. Magnification: upper panel, 1280 $\times$; lower panel, 640 $\times$.

Analysis of GPC-4 expression in haematopoietic cell lines, human bone marrow and kidney

GPC-4 expression in haematopoietic-progenitor and bone-marrow-stromal cell lines and human kidney was investigated on the protein level by dot blot and Western blot analysis. Antisera raised against the different peptides (Figure 1) revealed a high antibody titre against their respective peptides, when tested by

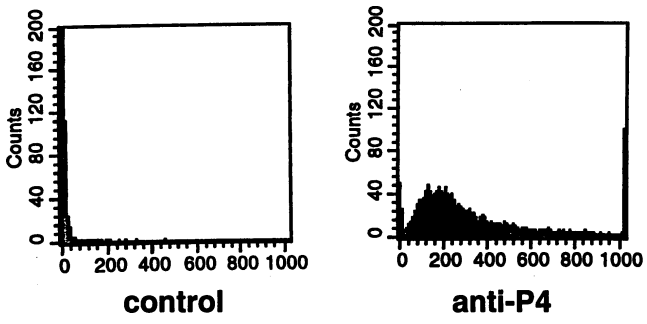

Figure 6 FACS analysis of GPC-4 expression in the human haematopoietic-progenitor cell line TF-1

TF-1 cells were stained with the polyclonal rabbit antiserum against peptide 4 (anti-P4) and a second FITC-conjugated goat anti-(rabbit IgG) antibody without fixation and used immediately for FACS-analysis. Control: TF-1 cells were stained with pre-immune serum and the FITC-conjugated second antibody.

enzyme immunoassay. However, all the antisera were not suitable for immunoprecipitation of ^{35}S-labelled proteoglycans from different cell lines. Therefore GPC-4 expression was studied by dot blot and Western blot analysis. Dot blot analysis showed a positive reaction with human kidney and TF-1 cell extracts for all anti-peptide antisera (results not shown). In contrast, no specific staining was observed for the human haematopoietic-progenitor cell line HEL, the murine haematopoietic-progenitor cell line FDCP-Mix-A4 and the murine bone-marrow-stromal cell line MS-5. For the HEL cell line this result is in agreement with the lack of a signal in RT-PCR and Northern blot analysis (Figure 2 and results not shown). For the murine cell lines FDCP-Mix-A4 and MS-5, this might reflect the specificity of the anti-peptide antisera for human GPC-4 and indicates that the antisera do not crossreact with mouse GPC-4. In the following experiments, the antiserum against peptide 4, which is highly specific for GPC-4 and according to the amino acid sequences (Figure 1) should not react with other members of the glypican gene family, was used for Western blot and immunohistochemical analysis. Proteoglycans from the cell or tissue extract were subjected to SDS/PAGE prior to and after digestion with heparinase/heparitinase. These experiments show that GPC-4 is expressed as a heparan-sulphate proteoglycan in human kidney, bone marrow and the haematopoietic-progenitor cell line TF-1 (Figure 4). The molecular mass distribution of the native heparan sulphate proteoglycan was in the range of 90 to $>$ 200 kDa and the size of the core protein was approx. 57 kDa.

Analysis of GPC-4 expression by immunohistochemistry, confocal laser-scanning microscopy and FACS

Formaldehyde-fixed sections of human kidney was stained using the polyclonal rabbit antiserum against peptide 4. In the cortex only a weak (brown-coloured) staining was observed for glomeruli, while a strong staining of the luminal surface of epithelial cells in the proximal tubules showed up (Figure 5, upper panel). In the medulla, a distinct (brown-coloured) staining of collecting ducts was visible, whereas distal tubules showed only a faint, if any, staining (Figure 5, lower panel).

For the human haematopoietic-progenitor cell line TF-1, FACS analysis of unfixed cells using the polyclonal rabbit antiserum against peptide 4 showed substantial cell labelling (Figure 6, right side) compared with the control (Figure 6, left side) in which the cells were treated with pre-immune serum.

GPC-4 expression was also analysed in normal human bone marrow. GPC-4 positive cells (below 1 % of nucleated cells) were

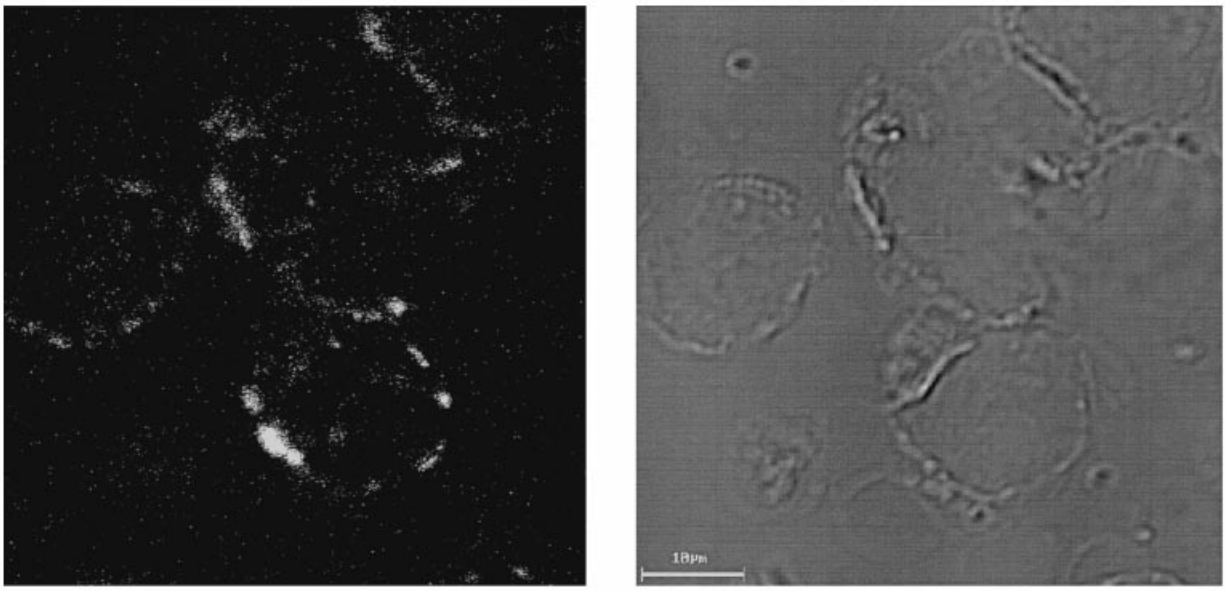

Figure 7 Analysis of GPC-4 expression in normal human bone marrow by confocal laser-scanning microscopy after enrichment of GPC-4-expressing cells by MACS

GPC-4-expressing cells from normal human bone marrow were enriched by MACS using the polyclonal rabbit antiserum against peptide 4 and magnetic beads conjugated with anti-(rabbit IgG) antibodies. Enriched cells were then stained with a second FITC-conjugated goat anti-(rabbit IgG) antiserum. Cytospin preparations of these cells were analysed by confocal laser-scanning microscopy and show the expression of GPC-4 by normal human bone marrow cells and its localization at the cell membrane. Size and shape of stained cells indicate staining of two progenitor cells and one stromal cell (right panel). Fluorescence microscopy (left panel) and phase-contrast microscopy (right panel) pictures are shown. (Magnification: 2500 $\times$; bar, 10 μm).

enriched by MACS using the antiserum against peptide 4 and analysed by confocal laser-scanning microscopy. Figure 7 (left panel) clearly shows the expression of GPC-4 in normal human bone marrow cells and its localization at the cell membrane. The morphology of positive cells, as analysed by phase-contrast microscopy, is compatible in size and shape with progenitor cells (Figure 7, right panel). In addition, some cells with a morphology of stromal cells were detected, even though part of the stromal cells might have been lost during the initial isolation of cells from the bone-marrow spongiosa. This is in agreement with our findings that GPC-4 is expressed by the bone-marrow-stromal cell line MS-5 and several haematopoietic-progenitor cell lines but not by mature leukocytes (Figure 2).

DISCUSSION

Recently it has been shown that heparan sulphate proteoglycans might play an important role in the interaction of haematopoietic-stem and stromal cells [1,21–26]. Binding and presentation of haematopoietic-growth factors by heparan sulphate proteoglycans has been shown to be an important mechanism for this interaction [21,22]. However, our knowledge on the heparan sulphate proteoglycans, involved in the interaction of haematopoietic-progenitor and stromal cell lines, is still limited. In fact, there are only few reports on proteoglycans from haematopoietic-bone-marrow cells [24,37–44] pointing to stromal cells, rather than haematopoietic-stem cells or progenitor cells, as the source of heparan sulphate proteoglycans [24,37–40,42–44]. Recently, we have analysed the expression of heparan sulphate proteoglycans in several murine and human haematopoietic-progenitor and stromal cell lines [43]. In contrast with the bone-marrow-stromal cell line MS-5, which expressed at least seven different heparan sulphate proteoglycans, the expression of heparan sulphate proteoglycans in haematopoietic-progenitor cell lines is limited [35,43].

Further characterization of the heparan sulphate proteoglycan found in TF-1 cells [35] has shown that this heparan sulphate proteoglycan is anchored in the cell membrane by a GPI anchor, indicating that this heparan sulphate proteoglycan belongs to the glypican-gene family (unpublished work, Z. Drzeniek, G. Stöcker, B. Siebertz, U. Just and H.-D. Haubeck). Therefore, we have analysed the expression of known members of the glypican-gene family in TF-1 cells. Whereas for GPC-1, GPC-2 and GPC-3 no expression was observed on the mRNA level, GPC-4 was expressed in this cell line.

In the present study we have analyzed the expression of GPC-4 in several haematopoietic-progenitor and bone-marrow-stromal cell lines of human and murine origin. The cDNA sequence of mouse GPC-4 was recently described by Watanabe et al. [19]. In their study it was shown that the expression of GPC-4 is restricted, with a high level of expression in kidney and embryonic brain, moderate expression in liver and lung and low expression in adult brain and spleen. Our results clearly show that the expression of GPC-4 is not restricted to these organs. GPC-4 is also expressed in a number of haematopoietic-progenitor and bone-marrow-stromal cells (Figure 2). As the cDNA sequence of human GPC-4 had not been described when this study was performed, we have determined the complete cDNA sequence of human GPC-4 from one of these cell lines, TF-1. However, while this manuscript was under review, the cDNA sequence of human GPC-4, derived from a human foetal brain library, was reported by Veugelers et al. [36] and confirmed our sequence (results not shown). Northern blot analysis revealed two mRNA signals with sizes of 2.9 kb and 4.6 kb (Figure 3). The use of three different cDNA probes (N-terminal, C-terminal and mid-regions) led to the same pattern in the Northern blot analysis, indicating that both mRNA species are specific for GPC-4. They most probably result from the use of different polyadenylation signals or different splicing in the 5′- or 3′-regions of a pre-mRNA. In contrast, in the mouse only one mRNA species of 3.4 kb was observed [19].

In the present study we have shown the expression of GPC-4 at the mRNA level in a number of bone-marrow-stromal and haematopoietic-progenitor cells of human and murine origin and in normal human bone marrow. To confirm that GPC-4 is expressed on the protein level, specific polyclonal antisera against selected peptides designed from the deduced human amino acid sequence were raised in rabbits. Immunochemical analysis showed that K-glypican is expressed as a heparan sulphate proteoglycan in normal human bone marrow, human kidney and the haematopoietic-progenitor cell line TF-1. For the TF-1 cell line this was confirmed by FACS analysis (Figure 6). For the analysis of human bone-marrow cells, enrichment of GPC-4 positive cells (calculated to $< 1\,\%$ of nucleated cells) by MACS was necessary. The morphology of positive cells as analysed by phase-contrast microscopy is compatible, in size and shape, with progenitor cells (Figure 7). In addition, some cells with a morphology of stromal cells were detected. However, part of the stromal cells might have been lost during the initial isolation of cells from the bone-marrow spongiosa. These results are in agreement with our findings that GPC-4 is expressed by the bone-marrow-stromal cell line MS-5 and several haematopoietic-Progenitor cell lines but not by mature leukocytes (Figure 2) [36].

Furthermore, immunohistochemical analysis of GPC-4 expression in human kidney, which had not been analysed until now, revealed an interesting distribution that was in accordance with *in situ* hybridization signals in the developing mouse kidney [19]. Whereas glomeruli in the cortex showed weak if any staining, a strong staining of the luminal surface of epithelial cells in proximal tubules was observed. In contrast, in the medulla a clear but diffuse staining of epithelial cells in the collecting ducts was visible, whereas distal tubules showed only a faint (if any) staining. This distinct distribution might be of relevance for the function of GPC-4 in kidney.

We have shown previously the expression of several different heparan sulphate proteoglycans in the stromal cell line MS-5 and of at least one heparan-sulphate proteoglycan with a core protein size of 59 kDa in the TF-1 cell line [35,43]. However, for the haematopoietic-progenitor cell lines FDCP-Mix-A4 and Myl-D-7, we and others have shown by metabolic labelling that they do not express a native heparan sulphate proteoglycan ([35,37] and unpublished results, Z. Drzeniek, G. Stöcker, B. Siebertz, U. Just, K. Itoh and H.-D. Haubeck). Therefore, expression of GPC-4 on the mRNA level raises the question of whether GPC-4 is expressed in these cell lines as a free core protein that might be lost during standard proteoglycan purification protocols. In fact, the existence of some free core protein has been observed when crude extracts were analysed (results not shown). However, we have shown recently that the induction of erythroid differentiation in the FDCP-Mix A4 cell line induces the expression of heparan sulfate proteoglycans [45]; therefore an alternative explanation would be that GPC-4 is expressed only during specific differentiation stages of haematopoietic-progenitor cells. Since the antiserum against peptide 4 is specific for human GPC-4, the question of whether GPC-4 is expressed in the murine haematopoietic-progenitor and stromal cells as a free core protein cannot be answered at present. GPC-4 might be expressed as a free core protein in these cell lines, and it might also be involved in the regulation of proliferation and differentiation of haematopoietic-stem and -progenitor cells. This is an interesting possi-

bility, as it has been shown recently that the free core protein of syndecan-4 is part of focal adhesions and plays an important role in the cell adhesion process and might also be involved in protein kinase C-mediated signalling [46,47].

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