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Differential release of proteoglycans during human B lymphocyte maturation

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Abstract

Proteoglycans interact with soluble proteins such as growth factors and thereby regulate extracellular signals. During B lymphocyte maturation, secretion of proteoglycans may be functionally related to the different requirements of the respective maturation stage. In order to address this question we compared structures of proteoglycans released by three B lymphocyte lines which correspond to different maturation stages. Plasma-cell type U266 cells secreted the largest proteoglycans (150 kDa), followed by mature B cells JOK-1 (130 kDa) and pre-B cells Nalm 6 (90 kDa). On average, secreted proteoglycans carried four glycosaminoglycan chains with molecular masses ranging each from 32 kDa (U266) to 23 kDa (Nalm 6). All three cell lines secreted more than 90% of their proteoglycans possessing chondroitin sulfate chains having chondroitin-4-sulfate (ΔDi-4S) as the prevalent disaccharide unit. In these proteochondroitin sulfates, unsulfated chondroitin (ΔDi-0S) was present in smaller quantities and chondroitin-6-sulfate (ΔDi-6S)-containing proteoglycan was released only by Nalm 6 and U266 cells. Cell line Nalm 6 exclusively produced proteochondroitin sulfate, whereas in culture medium of JOK-1 and U266 a small amount of proteoheparan sulfate was found also. In all three cell lines, treatment with chondroitinase ABC released a protein of 30 kDa and chemical deglycosylation resulted in a core protein of 21 kDa. In addition to pure proteochondroitin sulfate, a small portion of proteoheparan sulfate with a protein moiety of 30 kDa was detected after heparitinase treatment in supernatants of JOK-1 and U266. Thus, our results indicate that released proteoglycans may undergo modulations in their glycosaminoglycan moieties during B-cell differentiation. This may have functional consequences at the level of growth factor regulation. © 1997 Elsevier Science Ltd.

Keywords: B-cell differentiation; Proteoglycans; Secretion; Serglycin

Abbreviations: Di-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S (-6S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4 (or 6)-*O*-sulfo-D-galactose; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; *Enzymes*: Chondroitinase ABC, EC 4.2.2.4. Chondroitinase AC, EC 4.2.2.5. Heparinase, EC 4.2.2.7. Heparitinase, EC 4.2.2.8

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1. Introduction

During their differentiation, B lymphocytes are driven by many soluble factors. The coordinated recognition of these signals is regulated by the stage-specific expression of surface receptors and the secretion of autocrine factors interacting with extracellular signals [1]. For several non-hematopoietic cells, proteoglycans have been described as modulators of growth factor activities [2]. For instance, the extracellular matrix proteoglycan decorin binds to and neutralizes transforming growth factor β (TGFβ) activity [3]. TGFβ also inhibits B-cell proliferation and immunoglobulin secretion [4]. It is tempting to speculate that proteoglycans possibly released by B lymphocytes may take part in extracellular regulation of growth factors such as TGFβ. Provided proteoglycans play a regulatory role during B-cell differentiation, one would expect changes in proteoglycan release that correspond to the specific requirements of distinct B lymphocyte maturation stages.

In previous publications it has been reported that surface-expressed syndecan-1, a proteoglycan involved in adhesion to extracellular matrix components and regulation of basic fibroblast growth factor (bFGF) activity, exhibits differential gene expression during murine B-cell maturation [5]. Recently, we found that cell-surface-expressed proteochondroitin sulfate of pre-B Nalm 6 cells, in contrast to that of B-lymphoblastoid JOK-1 cells, contributes to cell adhesion to the extracellular matrix component laminin [6]. Cellular proteoglycans of these two B-cell lines are distinguished by their different sulfation patterns [7]. Changes may also occur with proteoglycans released into the extracellular environment. Proteoglycans may not only undergo changes in the level of their synthesis and secretion but also in their fine structure. These structural changes may involve both the glycosaminoglycan and the protein moiety. To pursue this question, we analyzed proteoglycans released by three human B-cell lines. These B-cell lines are equivalent to bone marrow-derived pre-B cells (Nalm 6), mature B cells of the peripheral blood (JOK-1) and immunoglobulin-secreting plasma cells (U266) as defined by their origin, morphology and cell-surface differentiation markers [8]. Differences found in the glycosylation of their secreted proteoglycans point to a regulatory role of soluble proteoglycans during B-cell maturation.

2. Results

Purification and size determination of proteoglycans.—After [^{35}S]-sulfate labeling, proteoglycans were isolated from culture supernatants of cell lines Nalm 6, JOK-1 and U266 by sequential steps of CsCl density-gradient centrifugation, anion-exchange chromatography, and gel filtration. Macromolecular material was subjected to CsCl density-gradient ultracentrifugation under dissociative conditions. Most of the [^{35}S]-label was enriched in the fractions of highest density, while the major portion of proteins was found in lower-density fractions (Fig. 1A). Fractions 1 and 2 from the bottom of the tube (indicated in Fig. 1 as preparation A) were combined and used for further purification of high-density proteoglycans. In the culture supernatant of cell line U266 the prevalent portion of [^{35}S]-label was concentrated in the first two fractions, whereas in the supernatants of JOK-1 and Nalm 6 a significant portion of radioactive label was found in fractions 3–6 (preparation B). In Nalm 6 culture supernatants, the [^{35}S]-label was also present in fractions of lower density. Macromolecules of preparations A and B were precipitated separately and subjected to anion-exchange chromatography.

Sulfated macromolecules from all three cell lines eluted on a DEAE-Sephacel column as broad peaks in the range of 0.6–0.75 M NaCl (Fig. 2). For each cell line the elution profiles of supernatant material of preparations A and B were almost identical (comparative profiles not shown). After anion-exchange chromatography the fractions containing the [^{35}S]-labeled glycoconjugates were pooled, precipitated, and chromatographed on a Sepharose CL-4B column.

Gel filtration of the supernatant molecules of the three cell lines revealed an increase in size in the direction Nalm 6 < JOK-1 < U266 (Fig. 1B). The elution profiles of the culture supernatant preparations of cell lines Nalm 6 and JOK-1 showed two peaks: a major peak I (JOK-1: $K_{av} = 0.38$) and (Nalm 6: $K_{av} = 0.32$) and a minor peak II (JOK-1: $K_{av} = 0.6$) and (Nalm 6: $K_{av} = 0.64$) (Fig. 1B). The sulfated molecules of U266 eluted as a single peak with $K_{av} = 0.28$. Extrapolating from K_{av}-values of standard glycosaminoglycans in gel filtration on Sepharose CL-4B, the hydrodynamic sizes of proteoglycan material from culture supernatants were estimated to be in the range 150–90 kDa, with U266 having the largest proteoglycans of approximately

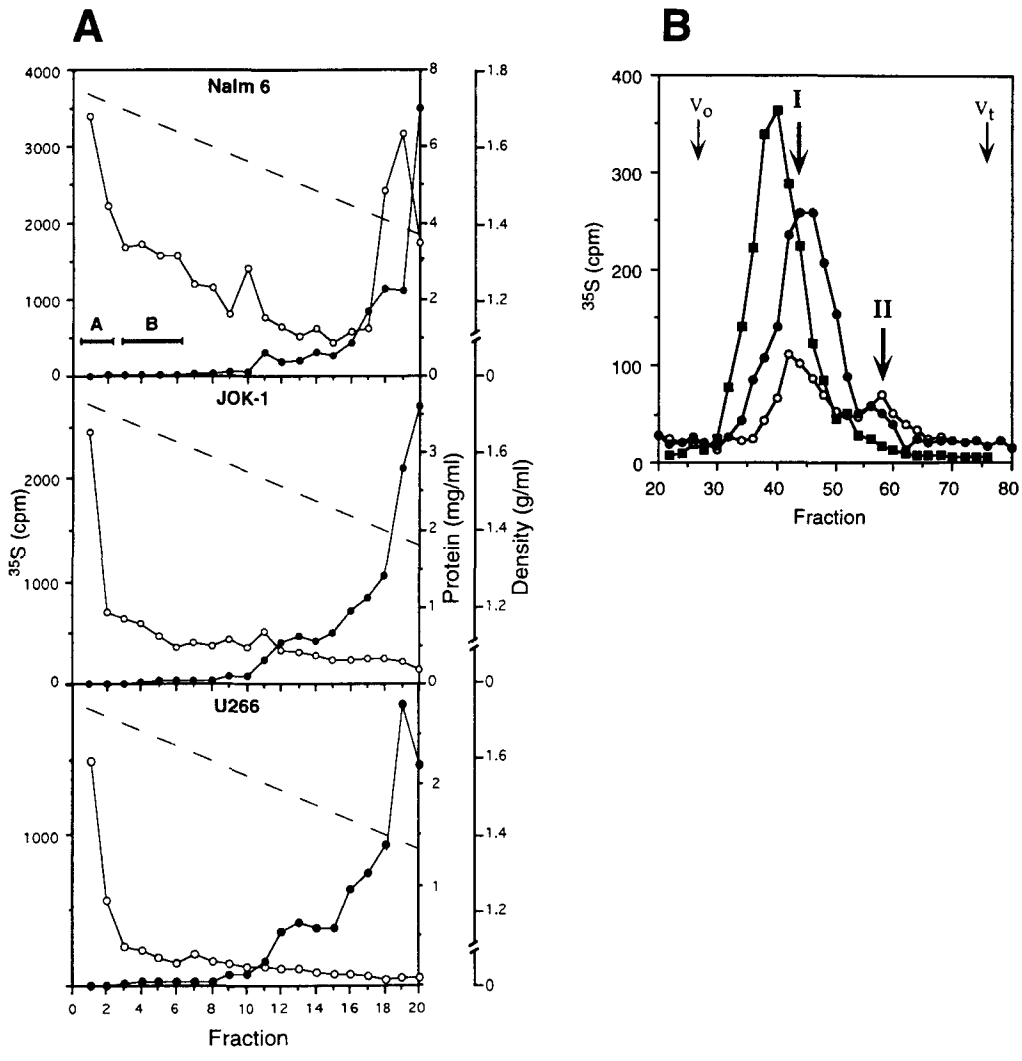

Fig. 1. (A) *CsCl density-gradient ultracentrifugation of culture medium proteoglycans.* Distribution of [^{35}S]-labeled material from culture supernatants of Nalm 6, JOK-1, and U266 cells after CsCl density-gradient ultracentrifugation was monitored by determination of [^{35}S]-radioactivity (○), protein content (●), and density (dashed line) in each fraction (2 mL each). Fractions used for further purification are indicated by bars. (B) *Sepharose CL-4B chromatography of culture medium proteoglycans.* [^{35}S]-labeled molecules obtained by sequential CsCl ultracentrifugation and DEAE-Sephacel ion-exchange chromatography were applied to a Sepharose CL-4B gel filtration column. Fractions of 1 mL were collected and [^{35}S]-radioactivity of 30 μL aliquots was determined. Nalm 6 (○), JOK-1 (●), U266 (■). V_0 and V_t are indicated by arrows.

150–120 kDa, followed by those of JOK-1 and Nalm 6 peak I material of ca. 130–90 kDa (Fig. 1B).

Since, hypothetically, proteoglycans isolated from the culture supernatant may also be derived from fetal calf serum, regularly added at 5% (v/v) to the culture medium, in some experiments we also extracted proteoglycans from serum-free PFHM medium conditioned by JOK-1 cells. When proteoglycan fractions derived from serum-free and serum-containing conditioned culture supernatant were compared, measurements both of [^{35}S]-label and hexosamine content yielded the same profile after CsCl density-gradient ultracentrifugation and ion-exchange chromatography (Fig. 2). These results indicate that supernatant-derived proteoglycan used for subsequent analysis of glycosaminoglycans and protein structure is indeed produced by the respective B-cell lines.

Size and nature of glycosaminoglycan chains.—To determine the sizes of glycosaminoglycan chains of proteoglycans and the proportion of intact proteoglycans present in the preparations, [^{35}S]-labeled material of cell-culture supernatants was chromatographed on Superose 6 with and without prior alkaline β-elimination (Fig. 3A). Supernatant preparations A of

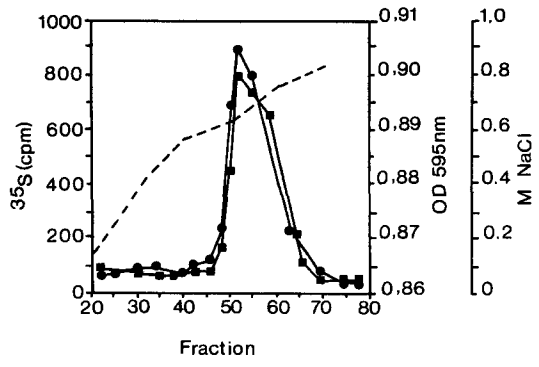

Fig. 2. DEAE-Sephacel chromatography of culture medium proteoglycan of JOK-1 cells. Preparation A of high-density proteoglycans from JOK-1 culture supernatant obtained by CsCl ultracentrifugation was subjected to anion-exchange chromatography. Fractions of 0.75 mL were harvested and elution of proteoglycans was monitored by determination of [^{35}S]-radioactivity (●) and content of hexoamines at 595 nm (■). NaCl gradient is indicated with a dashed line.

all three cell lines gave a shift to smaller molecular masses after alkaline borohydride treatment, proving that these preparations contained proteoglycans. When peak II material of Nalm 6 and JOK-1 was pooled and separately subjected to alkaline treatment, no shift to smaller molecular mass was observed (data not shown). Therefore sulfated macromolecules of peak II most likely contain free glycosaminoglycan chains. Thus, culture supernatant [^{35}S]-labeled macromolecules from Nalm 6 consisted of 30% and those from JOK-1 of 15% of free glycosaminoglycan chains (Fig. 3A). U266 Cells predominantly released intact proteoglycans into the medium. In the supernatant preparation B (proteoglycans/glycosaminoglycans in fractions 3–6 obtained after CsCl density-gradient centrifugation) of JOK-1 and Nalm 6, the ratio of free glycosaminoglycans to intact proteoglycans was about 15% higher compared to preparation A (data not shown). We assume that the wider distribution of [^{35}S]-labeled material derived from these two cell lines after CsCl density-gradient ultracentrifugation (Fig. 1A) may be caused by a higher content of free glycosaminoglycan chains. However, it cannot be excluded that there are other species of proteoglycans present in the lower-density fractions of these cell lines. The size of the glycosaminoglycan chains obtained after alkaline β-elimination was estimated on Superose 6 using glycosaminoglycan of defined molecular mass as standard. The proteoglycans found in culture supernatants of Nalm 6 had glycosaminoglycan chains with the smallest size (on the average 23 kDa), followed by JOK-1 (28 kDa) and those of U266 with the highest molecular mass (32 kDa) (Fig. 3A). Together, the sizes of the single glycosaminoglycan chains (Fig. 3A) and the size estimations of intact secreted proteoglycans (90–150 kDa) (Fig. 1B) are consistent with an average of 4 glycosaminoglycan chains being bound to the core protein of 30 kDa. The influence of a small core protein of 30 kDa, as outlined below, on the migration behaviour of complex proteoglycans is negligible in this type of gel filtration chromatography.

Aliquots of preparations obtained after CsCl density-gradient centrifugation were chromatographed on Superose 6 with and without prior digestion by chondroitinase ABC or heparitinase (Fig. 3B). As observed for all three cell lines, chondroitinase ABC treatment resulted in an almost total shift of [^{35}S]-labeled peak material to the V_t, whereas heparitinase treatment yielded only a slight reduction of peak material (Fig. 3B). The percentage of chondroitin sulfate and heparan sulfate contained in the respective preparations after specific enzymatic treatment in relation to the radioactive label of untreated peak material is shown in Table 1. Elution profiles of proteoglycans/glycosaminoglycans after chondroitinase ABC and AC treatment were identical (data for chondroitinase AC not shown). Therefore, it may be that dermatan sulfate was absent or present only in small contents which are not detectable by the method used. In culture medium of Nalm 6 only chondroitin sulfate and no heparan sulfate was observed.

The disaccharide composition of the chondroitin sulfate chains of proteoglycans was analyzed by HPLC (Table 2). ΔDi-4S was the predominant disaccharide component. ΔDi-6S was also detected in the supernatant preparations of U266 and Nalm 6. In the supernatant preparation of JOK-1 only trace amounts of ΔDi-6S were visible. ΔDi-0S was found in all three cell lines, in Nalm 6 and U266 to a higher degree than in JOK-1. Supernatant preparations of U266 and JOK-1 cells contained a small portion of heparan sulfate. With supernatant-derived proteoglycans from U266 and, to a lesser extent, with those from JOK-1, a broadening of the elution profile occurred after heparitinase treatment (Fig. 3B, arrows III and IV). A reduction in molecular mass was also observed when these enzymatically treated fractions were separated on a large-pore gradient SDS polyacrylamide gel. In this separation it was obvious that the sizes of supernatant-derived proteoglycans were slightly reduced after heparitinase treatment (Fig. 4). The slight shift in molecular mass after heparitinase

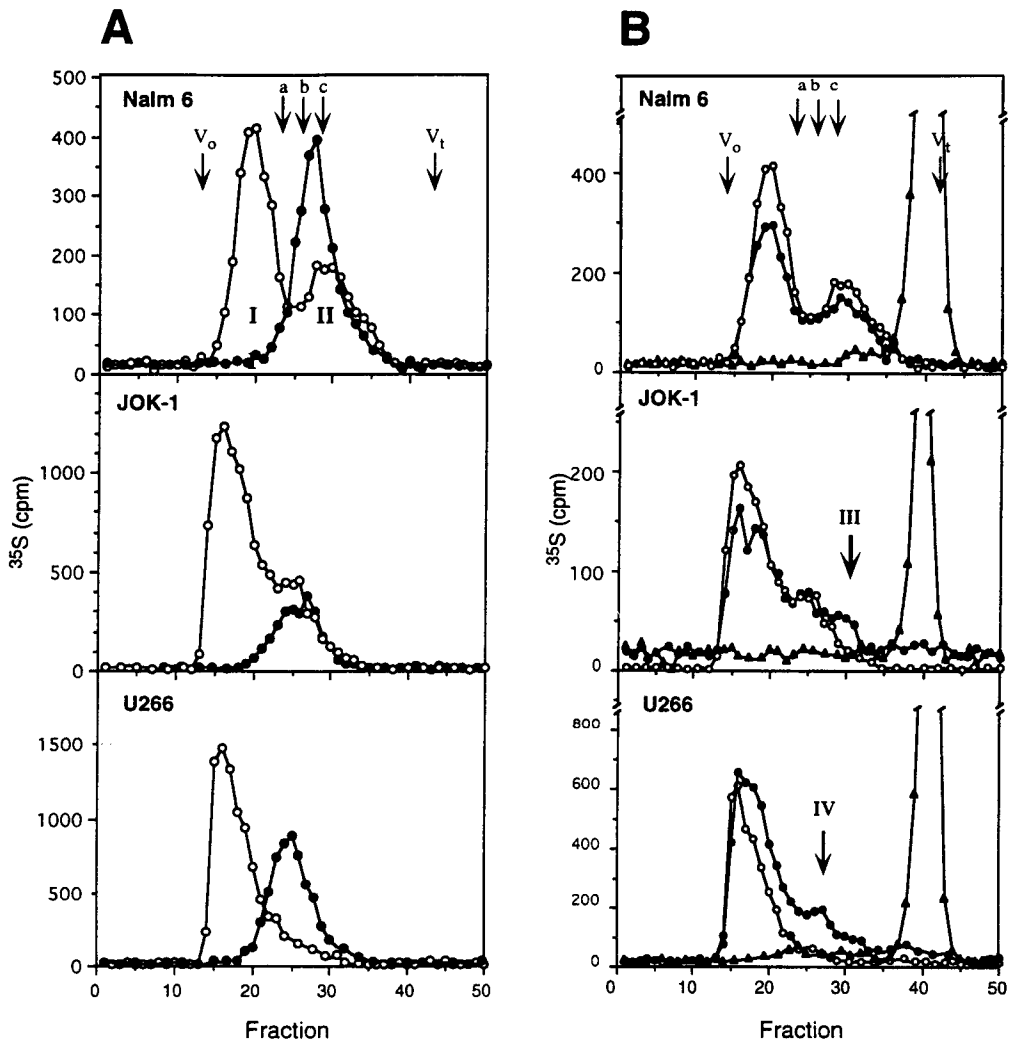

Fig. 3. (A) *Gel filtration on Superose 6 of proteoglycans with and without NaOH / $NaBH_4$-treatment.* Portions of [^{35}S]-labeled proteoglycans from culture supernatants (preparation A) were applied on a Superose 6 column after treatment with alkaline borohydride (●) or without treatment (○). The peak material of U266 and peak I material of JOK-1 partly eluted in V_0 of Superose 6 before β-elimination. (B) Gel filtration on Superose 6 of proteoglycans after enzymatic treatment. Portions of [^{35}S]-labeled proteoglycans from culture supernatants (preparation A) were applied on a Superose 6 column after treatment with chondroitinase ABC (△), heparitinase (●), or without treatment (○). Fractions of 0.5 mL were collected, and [^{35}S]-radioactivity of the fractions was determined. V_0, V_t, and the elution positions of the glycosaminoglycan standards $a = 42.5$, $b = 26.5$, and $c = 18.5$ kDa are indicated by arrows.

treatment may indicate that heparan sulfate chains are attached to the same core protein as the chondroitin sulfate chains. Due to the small shift in molecular size after heparitinase treatment it can be assumed that the portion of heparan sulfate is much smaller compared to that of the chondroitin sulfate moiety. Hence, in supernatant proteoglycans of differentiated B cells represented by JOK-1 and U266, at least part of heparan sulfate may be expressed as a hybrid proteoglycan-containing chondroitin sulfate and heparan sulfate chains.

Characterization of core proteins.—Chondroitinase ABC digestion of radioiodinated proteoglycans isolated from culture supernatants of each cell line resulted in a strong protein band of 30 kDa (Fig. 5). Consecutive treatment of the samples with chondroitinase ABC and heparitinase also showed the 30-kDa core protein (data not presented). As shown in Fig. 3B, heparitinase treatment resulted in a reduced molecular mass of U266 proteoglycans. To look for proteoheparan sulfate we separated radioiodinated proteoglycans after heparitinase treatment on SDS-PAGE. Supernatant proteoglycans derived from JOK-1 and U266 cells yielded a faint protein band of 30 kDa after heparitinase digestion when using a high amount of [^{125}I]-label (about 300,000 cpm/lane)

Table 1
Chondroitin sulfate (CS) and heparan sulfate (HS) composition of culture medium proteoglycans and glycosaminoglycans

Cell line	Composition (% of total [^{35}S]-labeled macromolecules)	
	CS	HS
Nalm6	99±2	<1
JOK-1	98±1	~2
U266	95±2	5±2

Proteoglycans and glycosaminoglycans isolated from culture medium were subjected to chondroitinase ABC and heparitinase digestion and subsequently applied to a Superose6 column as indicated in the Experimental and shown in Fig. 3B. The amount of disaccharides and oligosaccharides which eluted after enzymatic treatment in the V_t of the column was estimated by measurement of [^{35}S]-label and used to calculate the percentage of CS and HS in the respective preparation. Values are presented as mean±SD of three independent experiments.

Table 2
Composition of chondroin sulfate disaccharides

Cell line	Composition (% of total CS)		
	ΔDi-0S	ΔDi-4S	ΔDi-6S
Nalm6	15±9	73±14	12±4
JOK-1	6±2.5	91±7	<1
U266	12±7	82±8	6±4

Proteoglycans and glycosaminoglycans derived from culture medium were subjected to chondroitinase ABC digestion. The resulting unsaturated disaccharides (ΔDi-0S, ΔDi-4S, and ΔDi-6S) were identified by HPLC on a Biosil Amino5S column. Values are shown as mean±SD of 4 experiments.

(shown for JOK-1 in Fig. 5D). Similar results were obtained with U266 cells (data not shown). This indicates the existence of small amounts of pure proteoheparan sulfate in the culture supernatants of JOK-1 and U266. In the same preparation after heparitinase digestion a broader band in SDS polyacrylamide gels was located predominantly in the high molecular range (Fig. 5D). This result also indicates the existence of hybrid proteoglycans.

To further define the molecular mass of the protein moiety of secreted B-cell proteoglycans we subjected proteoglycans from U266 cells to treatment with trifluormethansulfonic acid. Chemical deglycosylation resulted in a protein band of 21 kDa (Fig. 6) which points to a carbohydrate portion of approximately 9 kDa which remains at the 30-kDa core protein obtained after chondroitinase ABC or heparitinase treatment. This residual carbohydrate moiety comprises short stubs of GlcA–Gal–Gal–Xyl representing the glycosaminoglycan–protein linkage region inert to chondrotinase ABC treatment and possibly N- or O-linked oligosaccharide chains.

3. Discussion

Our results demonstrate that human B-cell lines which represent different maturation stages of the B-cell ontogeny release proteoglycans with variations in their glycosaminoglycan moieties. The differences in size, type, and sulfation patterns of glycosaminoglycans may reflect different functions of these proteoglycans. In an earlier study, using pulse-chase experiments, we found that the B-lymphoblastoid cell line LICR-LON-HMy2 released approximately 40% of proteoglycans synthesized during a 60-min pulse into the culture supernatant within 4 h [9]. Although these data speak in favour of a directed secretory process we cannot exclude that in the present study soluble proteoglycans may have been released from the plasma membrane by shedding. All three cell lines released predominantly proteochondroitin sulfate, a feature which has previously been described for other hematopoietic cells [10–12]. In contrast to Nalm6 cells, U266 and JOK-1 also released proteoheparan sulfate. Another difference between the three cell lines was the abundant occurrence of free glyco-

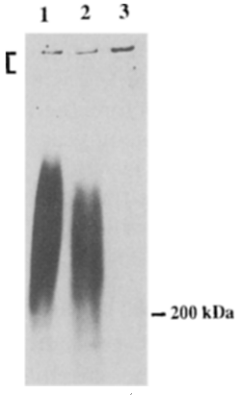

Fig. 4. Electrophoretic separation of [^{125}I]-labeled proteoglycans derived from culture supernatant of cell lines JOK-1. Proteoglycans were separated on a 1.3–10% SDS-PAGE either without enzymatic treatment (lane 1), treated with heparitinase (lane 2), or with chondroitinase ABC (lane 3). Only the high molecular range of the gel is depicted (calibration marker 200 kDa is indicated at the right margin). Chondroitinase ABC cleavage products were too small for the separation range of this gel type. The bracket at the left margin marks the region of the stacking gel.

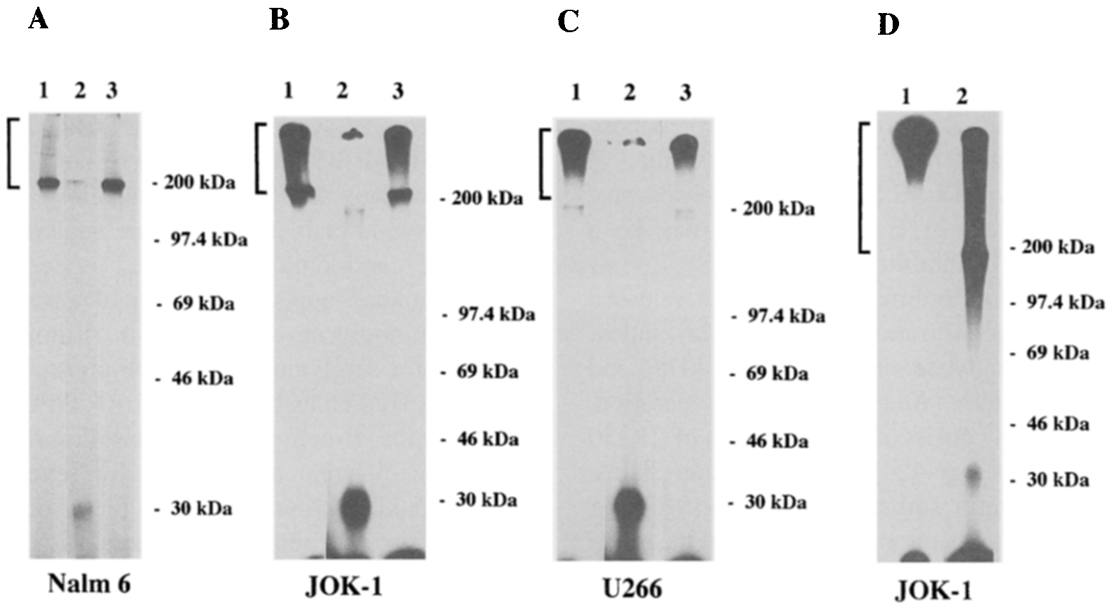

Fig. 5. Autoradiograms of radioiodinated proteoglycans and core proteins from culture supernatants of Nalm 6, JOK-1, and U266. Proteoglycans without treatment (lane 1) and treated with chondroitinase ABC (lane 2) or heparitinase (lane 3) were separated on a 10% SDS-PAGE (A–C). In D, lane 1 shows untreated material and lane 2 heparitinase-treated proteoglycans. In A, B, and C 20,000 cpm per lane, in D 300,000 cpm per lane were applied. Brackets at the left side of the autoradiograms mark the region of the 5% stacking gel.

saminoglycan chains in the culture medium of Nalm 6 (about 30% of all [^{35}S]-labeled macromolecules in the culture supernatant fraction) and to a lesser extent in that of JOK-1 cells. U266 cells exclusively released intact proteoglycans. In cell lines of T-cell and monocyte origin [10,11,13] and in a B-lymphoblastoid cell line [9], only the release of intact proteoglycans has been observed. Analyzing the proteoglycan turnover of the murine myeloid progenitor cell line FDCP-1,

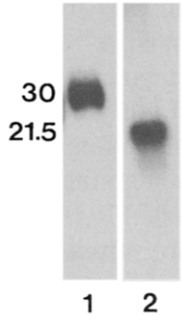

Fig. 6. Chemical deglycosylation of the secreted proteoglycan derived from the culture supernatant of cell line U266. [^{125}I]-labeled proteoglycans were treated with trifluormethansulfonic acid as described in Materials. Protein separation was done on 15% SDS-PAGE. The core protein obtained after chondroitinase ABC treatment is shown in lane 1, the chemically deglycosylated core protein in lane 2. Positions of calibration proteins (kDa) are indicated at the margin.

Minguell and Tavassoli [14] found that in the absence of stroma cells, membrane-bound proteoglycans were released into the medium where they were partially degraded. In the presence of hematopoietic stroma cells, however, the release of membrane-associated proteoglycan into the medium was almost completely suppressed. In a study of a hematopoietic stem cell line, the release of free glycosaminoglycan chains into the culture medium was reported, and it was suggested that this might be a process associated with early stages of hematopoiesis [15]. The large amount of glycosaminoglycan chains released by the pre-B cell line Nalm 6 may indicate that precursor B cells of bone marrow depend on interaction with stroma cells for stable proteoglycan expression.

In all three cell lines the major disaccharide unit of chondroitin sulfate chains from supernatant proteoglycans was identified as ΔDi-4S. Medium-derived chondroitin sulfate from Nalm 6 and U266 cells additionally contained ΔDi-6S. Differences in the content of ΔDi-0S may influence the negative charge of the corresponding proteoglycans. Also, different proportions of ΔDi-4S and ΔDi-6S may influence the secondary structure of proteoglycans. It has been shown that the different charge distribution of ΔDi-4S and ΔDi-6S in one glycosaminoglycan chain highly influences its structure and its ability to aggregate with other glycosaminoglycan chains [16]. Recently

we found that released proteochondroitin sulfate of JOK-1 and U266 was able to bind to the complement protein C1q and to inhibit complex formation of C1 [17]. In earlier studies, C1q-inhibiting proteochondroitin sulfate isolated from serum was described to contain exclusively ΔDi-4S [18]. Thus, released proteochondroitin sulfate of B lymphocytes may be a source of serum C1q inhibitor.

Taking the size determinations of intact released proteoglycans by gel filtration (90–150 kDa) and of single glycosaminoglycan chains (23–32 kDa) and those for core proteins after SDS-PAGE separation, these proteoglycans consist of a core protein of 21/30 kDa to which on average 4 glycosaminoglycan chains, usually of chondroitin sulfate, are attached. Similar size estimations have been made for proteoglycans of other hematopoietic cells: For example, human peripheral blood mononuclear cells secrete a proteochondroitin sulfate of 130 kDa, with 4 chondroitin sulfate chains of 25 kDa each, attached to a core protein of 35 kDa [19]; human platelet proteochondroitin sulfate (136 kDa) contains 4 chondroitin sulfate chains of each 28 kDa linked to a core protein of 27 kDa [20]. The sizes of proteoglycans secreted by human T cells are in the same range [11].

A difference between the cell lines was the presence of heparan sulfate found in JOK-1 and U266. The portion of heparan sulfate was low in released proteoglycans of JOK-1 and U266 cells. After heparitinase treatment of supernatant proteoglycans from JOK-1 and U266 cells a weak protein band of 30 kDa became visible which points to the presence of small amounts of proteoheparan sulfate in the culture medium in addition to proteochondroitin sulfate. The presence of hybrid proteoglycans containing both chondroitin sulfate and heparan sulfate chains attached to one core protein is mainly indicated by a shift of the molecular mass of labeled material as seen in SDS-PAGE of [^{125}I]-labeled proteoglycans after enzymatic treatment. Hybrid proteoglycans also seem to have a core protein of 30 kDa as observed after consecutive treatment with chondroitinase ABC and heparitinase. Differences between the content of heparan sulfate and chondroitin sulfate of proteoglycans during B-cell development have been reported for murine lymphocytes. The membrane-associated proteoglycan syndecan-1 is expressed on murine pre-B cells and plasma cells, but not on circulating mature B lymphocytes [5]. Syndecan-1 is a proteoheparan sulfate which may in some cells also contain a small amount of chondroitin sulfate chains attached to the same core protein [21,22]. For at least two reasons the proteoheparan sulfate we found in B-cell supernatants is unlikely to be syndecan-1: a) syndecan is not transcribed in JOK-1 cells [7] and b) the protein core of syndecan migrated to an higher position in SDS-PAGE as compared to our results [5].

A proteoglycan found in secretory vesicles of hematopoietic cells and in culture supernatant of the yolk sac carcinoma cell line L2 is serglycin [23]. Serglycin may appear with several variations in its glycosaminoglycan moiety [24]. Although mRNA specific for serglycin seems to be present in all three cell lines studied as determined by RT-PCR (data not shown), the B-cell proteoglycans described in this study are different to serglycin for several reasons. The 21.5-kDa protein core of these proteoglycans contains distinct peptide sequences without homologies to the published serglycin protein sequence [9]. Further, in our experiments, we could not clearly define a protein band of 10 kDa after chondroitinase ABC treatment which would be comparable to the 10-kDa protein of secreted serglycin [23]. Due to the high content of Ser and Gly residues in their 21.5/30-kDa core protein [9] these B-cell proteoglycans may belong to a family of Sergly proteoglycans.

4. Experimental

Materials.—$H_2[^{35}S]O_4$ was purchased from DuPont-New England Nuclear (Boston, MA, USA); Na[^{125}I] from Amersham Buchler (Braunschweig, FRG); prepacked PD10 columns, DEAE-Sephacel, Sepharose CL-4B, and Superose 6 (HR 10/30) from Pharmacia LKB Biotechnology Inc. (Freiburg, FRG); BioGel P10, BioRad assay for protein determination, and a BioSil Amino5S column from Bio-Rad (Munich, FRG); ultrapure guanidinium chloride and urea, NEM, PMSF, (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (CHAPS), chondroitinase ABC, chondroitinase AC, and iodobeads from Sigma (Munich, FRG); and the ultrafiltration equipment 'Minitan' from Millipore (Eschborn, FRG). Unsaturated chondroitin sulfate disaccharide standards for HPLC analysis, heparinase (EC 4.2.2.7), and heparitinase (EC 4.2.2.8) were purchased from Seikagaku Kogyo (Tokyo, Japan). Culture medium RPMI 1640, sulfate-free RPMI 1640 kit, and fetal calf serum were purchased from GIBCO (Eggenstein, FRG). Glycosaminoglycan standards (chondroitin sulfate/dermatan sulfate 45 kDa, keratan sulfate 25 kDa, keratan sulfate 18 kDa) were kindly provided by Dr. H.W. Stuhlsatz, University of Aachen, FRG.

Cell culture and labeling.—The human B-cell lines Nalm 6, patient-derived from an acute lymphocytic leukemia, and U266, a myeloma line, were purchased from the American Type Culture Collection (Bethesda, MA) and cell line JOK-1, from a hairy cell leukemia, was kindly provided by Dr. G. Moldenhauer, Heidelberg. The three cell lines were extensively characterized with regard to the patterns of their differentiation surface markers (CD antigens) [8]. Cell line Nalm 6 carries markers of early B-cell maturation (CD10, CD19, CD24, CD72), JOK-1 cells express markers of activated B cells (CD21, CD22, CDw75), and cell line U266 is deficient for almost all known B-cell differentiation markers except for CD44, CD73, and CDw75 which characterizes late stages of B-cell activation. For optimal production and secretion of proteoglycans, all cell lines were regularly grown in suspension culture using RPMI 1640 medium containing 10 mM HEPES, 5% heat inactivated fetal calf serum, and 2 mM L-glutamine. For some control experiments, cells were grown in "protein-free hybridoma medium" (PFHM II, Gibco, Paisley, UK) without addition of fetal calf serum. For radioactive labeling of proteoglycans, 0,37 MBq/mL $H_2[^{35}S]O_4$ was added to 1×10^6 cells/mL in sulfate-free RPMI for 24 h. After labeling, over 95% of the cells were found to be vital as assessed by the trypan blue exclusion test. Cultures were centrifuged at $400 \times g$ for 10 min and cell pellet and supernatant were collected separately.

Incorporation of $[^{35}S]$-label into macromolecules of the culture supernatant was calculated after desalting the lyophilized culture medium on a BioGel P10 column (3×70 cm) equilibrated with 0.5 M NH_4HCO_3, 1 mM NEM/PMSF/EDTA. The macromolecular fraction eluting at the void volume (V_o) was pooled and the radioactivity of an aliquot was determined.

Isolation of proteoglycans.—The non-labeled culture supernatant was concentrated 20-fold using a Minitan ultrafiltration membrane and dialyzed against 0.5 M NH_4HCO_3, 1 mM NEM/PMSF/EDTA. After lyophilization and addition of 1 part supernatant containing $[^{35}S]$-labeled material to 19 parts non-labeled supernatant, the supernatant was dissolved in buffer A (4 M guanidine/HCl, 0.1 M Tris, 1 mM EDTA, 1 mM NEM, 1 mM PMSF, pH 7.5) prior to CsCl density-gradient ultracentrifugation. The labeled macromolecular fraction of the culture supernatant was dissolved in buffer A and brought to a starting density of 1.45 g/mL by adding solid CsCl. After centrifugation at $200{,}000 \times g$ at 20 °C for 18 h (40-mL quickseal tubes, Beckman L2-centrifuge, VTI 50-rotor), fractions of 2 mL were collected from the bottom of the tube and analyzed for their density and $[^{35}S]$-sulfate incorporation. Protein content was determined by using the BioRad assay. Proteoglycan content was quantitatively assessed by a spectrophotometric assay measuring hexosamine content of glycosaminoglycan [25]. The fractions 1 and 2 with the highest density and the highest content of $[^{35}S]$-labeled material were collected as preparation A (as indicated in Fig. 1) and used for the purification of proteoglycans. In some experiments fractions 3–6 were pooled as preparation B and analyzed in identical fashion.

A column of DEAE-Sephacel (1×5 cm) was equilibrated with 7 M urea, 0.1 M Tris, 1 mM EDTA/NEM/PMSF, 0.1% CHAPS (w/v), pH 7.5 (buffer B). Macromolecular material obtained after density-gradient centrifugation was precipitated with 80% EtOH (v/v), then dissolved in buffer B and subsequently applied to anion-exchange chromatography. Column-bound proteoglycans and glycosaminoglycans were eluted with a 40-mL gradient of 0–1 M NaCl in buffer B. Fractions of 0.75 mL were collected. The peak fractions containing $[^{35}S]$-labeled macromolecules were pooled and precipitated with 80% EtOH.

The precipitate was resuspended in buffer A and then subjected to gel filtration on a column of Sepharose CL-4B (1×95 cm, flow rate 4 mL/h) equilibrated in the same buffer. Fractions of 1 mL were collected and measured for their content of $[^{35}S]$-labeled material. The appropriate fractions were pooled and brought to a concentration of 80% EtOH.

Enzymatic digestions and β-elimination of glycosaminoglycan chains.—Samples of $[^{35}S]$-labeled material (about 5,000–10,000 cpm each, purified as described above), were digested with 0.15 units of chondroitinase ABC and AC, respectively, in a total volume of 20–50 mL 0.1 M Tris, 0.1 M NaCl, pH 7.5 at 37 °C for 15 h, or with 2.5 units of heparitinase in 0.1 M Tris, 0.1 M NaCl, 10 mM $CaCl_2$, pH 7.5 at 37 °C for 15 h. The additional presence of 2.5 units of heparinase in the reaction mixture had no further degradative effect as compared to treatment with heparitinase alone. Therefore in this study, degradation of heparan sulfate moieties was performed for most experiments with heparitinase. The incubation mixtures were boiled for 3 min to terminate enzymatic degradation.

For alkaline β-elimination of glycosaminoglycans, portions of purified proteoglycans were treated with 0.5 M NaOH containing 1 M $NaBH_4$ at room tem-

perature for 16 h. The reaction was terminated by adding 33% CH_3COOH (v/v) while cooling with ice. Size determination of the glycosaminoglycan chains was performed by separation on a Superose 6 column calibrated with glycosaminoglycan standards.

Analysis of the glycosaminoglycan composition of proteoglycans.—After enzymatic treatment with chondroitinase ABC or heparitinase the reaction products were analyzed on a Superose 6 column (1 × 28 cm, flow rate 0.3 mL/min) eluted with 0.1 M Tris/HCl, 1 mM EDTA, 0.1% SDS (w/v), pH 7.5, using a FPLC-chromatography system from Pharmacia. Fractions of 0.5 mL were collected.

Analysis of the disaccharide composition.—After treatment of purified proteoglycans with chondroitinase ABC or AC, samples were boiled for 5 min and after cooling down to 4 °C were then mixed with a 4-fold excess of EtOH in order to precipitate proteins overnight. After centrifugation for 15 min at 17,000 × g, the supernatants containing the disaccharides were collected and dried in a SpeedVac (Bachofer, Reutlingen, FRG). The pellet was redissolved in water and analyzed on a BioSil Amino5S HPLC column eluted with 150 mM NaH_2PO_4, pH 4 (flow rate 1 mL/min), using the Beckmann (Munich, FRG) HPLC 'Gold'-system. Disaccharides were detected at 232 nm. Retention times were compared to those of unsaturated chondroitin sulfate disaccharide standards.

Characterization of core proteins.—Purified proteoglycans (approximately 20 μg) were radioiodinated with 18.5 MBq Na[^{125}I] using bead-coupled chloramine T (iodobeads) [26]. For separation from free [^{125}I], the reaction products were desalted on prepacked PD10 columns. Both intact and enzyme digested proteoglycans were analyzed on large-pore polyacrylamide gradient gels (1.3–10%) with agarose stacking gels as described [27]. To determine the molecular mass of the core proteins, samples were digested with chondroitinase ABC and heparitinase (incubation time 3 h at 37 °C) and separated on SDS polyacrylamide gels under reducing conditions. Preliminary kinetic experiments showed that 3-h incubation with the aforementioned enzymes was sufficient to release the protein. No further product became apparent after prolonged incubation for up to 24 h. For chemical deglycosylation of proteoglycans, radioiodinated proteoglycans were subjected to treatment with trifluoromethane sulfonic acid as previously described [28].

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References

[1] E.A. Clark and J.L. Lane, *Annu. Rev. Immunol.*, 9 (1991) 97–127.
[2] E. Ruoslahti and Y. Yamaguchi, *Cell*, 64 (1991) 867–869.
[3] Y. Yamaguchi, D.M. Mann, and E. Ruoslahti, *Nature*, 346 (1990) 281–284.
[4] J.H. Kehrl, A.B. Roberts, L.M. Wakefield, S. Jakowlew, M.B. Sporn, and A.S. Fauci, *J. Immunol.*, 137 (1986) 3855–3860.
[5] R.D. Sanderson, P. Lalor, and M. Bernfield, *Cell. Regul.*, 1 (1989) 27–35.
[6] L. Blase, A. Merling, P. Möller, and R. Schwartz-Albiez, *Leukemia*, 10 (1996) 1000–1011.
[7] S. Engelmann, O. Ebeling, and R. Schwartz-Albiez, *Biochim. Biophys. Acta*, 1267 (1995) 6–14.
[8] R. Schwartz-Albiez and G. Moldenhauer, in W. Knapp, B. Dörken, W.R. Gilks, P. Rieber, R.E. Schmidt, H. Stein, and A.E.G.Kr. von dem Borne (Eds), *Leucoyte Typing IV. White Cell Differentiation Antigens*, Oxford University Press, Oxford, 1989, pp. 142–154.
[9] H. Butz, H. Stuhlsatz, G. Maier, and R. Schwartz-Albiez, *J. Biol. Chem.*, 267 (1992) 3402–3408.
[10] D.J. McQuillan, M. Yanagishita, V.C. Hascall, and M. Bickel, *J. Biol. Chem.*, 264 (1989) 13245–13251.
[11] W.P. Steward, S.E. Christmas, M. Lyon, and J. Gallagher, *Biochim. Biophys. Acta*, 1052 (1990) 416–425.
[12] L. Uhlin-Hansen, D. Langvoll, T. Wik, and S.O. Kolset, *Blood*, 80 (1992) 1058–1065.
[13] S.O. Kolset, *Exp. Cell Res.*, 168 (1987) 318–324.
[14] J.J. Minguell and M. Tavassoli, *Blood*, 73 (1989) 1821–1827.
[15] A.J. Morris, T.M. Dexter, and J.T. Gallagher, *Biochem. J.*, 260 (1989) 479–486.
[16] E. Scott, Y. Chen, and A. Brass, *Eur. J. Biochem.*, 209 (1992) 675–680.
[17] M. Kirschfink, L. Blase, S. Engelmann, and R. Schwartz-Albiez, *J. Immunol.*, 158 (1997) 1324–1331.
[18] L. Silvestri, J.R. Baker, L. Rodin, and R.M. Stroud, *J. Immunol.*, 256 (1981) 7383–7387.
[19] D. Levitt, and P.-L. Ho, *J. Cell. Biol.*, 97 (1983) 351–358.
[20] M. Okayama, K. Oguri, Y. Fujiwara, H. Nakanishi, H. Yonekura, T. Kondo, and N. Ui, *Biochem. J.*, 233 (1986) 73–81.
[21] A. Rapraeger, *J. Cell Biol.*, 109 (1989) 2509–2518.
[22] R.D. Sanderson, T.B. Sneed, L.A. Young, G.L. Sullivan, and A.D. Lander, *J. Immunol.*, 148 (1992) 3902–3911.

[23] M.A. Bourdon, M. Shiga, and E. Ruoslahti, *J. Biol. Chem.*, 261 (1986) 12534–12537.
[24] D.E. Humphries, C.F. Nicodemus, V. Schiller, and R.L. Stevens, *J. Biol. Chem.*, 267 (1992) 13558–13563.
[25] R.W. Farndale, D.J. Buttle, and A.J. Barrett, *Biochim. Biophys. Acta*, 883 (1986) 173–177.
[26] M.A.K. Markwell, *Anal. Biochem.*, 125 (1982) 427–432.
[27] V. Vilim and J. Krajickova, *Anal. Biochem.*, 197 (1991) 34–39.
[28] A.S.B. Edge, C.R. Faltynek, L. Hof, L.E. Reichert, and P. Weber, *Anal. Biochem.*, 118 (1981) 131–137.