Glycoconjugate J (1986) 3: 287-298

Effect of *In Vitro* Differentiation on Proteoglycan Structure in Cultured Human Monocytes

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Received April 2, 1986.

Key words: monocyte in vitro differentiation, chondroitin sulfate proteoglycan, fibronectin-chondroitin sulfate interaction

Parellel to *in vitro* differentiation of human monocytes into macrophage-like cells, the cells change their synthesis of glycosaminoglycans from chondroitin 4-sulfate to highly sulfated chondroitin sulfate, containing 4,6-disulfated *N*-acetylgalactosamine units [Kolset *et al.* (1983) Biochem J 210:661-67]. After exposure of monocyte cultures to [^{35}S]sulfate for 24 h either from the onset of cultivation, prior to differentiation, or from day 4, after differentiation, ^{35}S-macromolecules from medium and cell-layer were isolated and characterized. The cell-layer of day 5 cultures contained both proteoglycans and free polysaccharide chains, while the ^{35}S-macromolecules present in the cell-layer of day 1 cultures and in medium of both monocytes and macrophage-like cells were almost exclusively of proteoglycan nature. Proteoglycans produced by macrophage-like cells were of larger size than the monocyte proteoglycans, most likely due to an increased polysaccharide chain length. These proteoglycans, in contrast to the monocyte-derived species, also showed affinity for fibronectin at physiological ionic strength.

Chondroitin sulfate proteoglycans present in cartilage have been extensively studied (see ref. 1 for review). The large proteoglycans form, together with hyaluronic acid and link proteins, aggregates which are important for the mechanical properties of cartilage. Chondroitin sulfate proteoglycans with structural features different from the large, aggregating species have also been identified in cartilage; among them a small proteoglycan (M_r ~76 000) [2]. Similar proteoglycans carrying chondroitin sulfate or dermatan sulfate have been isolated from aorta, bone, cornea, sclera and tendon [3]. Recently, the complete amino acid sequence of a small chondroitin sulfate proteoglycan has been deduced from the nucleotide sequence of the core protein cDNA [4]. The core protein contains a large region composed of alternating serine and glycine residues, relating this proteoglycan to the mast cell heparin proteoglycan [5]. Many different cell types synthesize large chondroitin sulfate or dermatan sulfate proteoglycans

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in vitro, including fibroblasts [6], smooth muscle cells [7] and glial cells [8]. The genetic relationship between these different proteoglycans has not been established.

Chondroitin sulfate proteoglycans are also present in plasma [9, 10] and as a complex with platelet factor 4 in platelets [11]. The inhibitor of complement factor C1q has recently been purified and identified as a chondroitin sulfate proteoglycan [12]. Furthermore, leukocytes [13], thymus-derived lymphocytes [14] as well as purified basophilic leukocytes [15], B- and T-cells [16] and monocytes [16, 17] synthesize chondroitin sulfate when cultured *in vitro*. Presently it is not known whether all blood cells produce the same type of proteoglycan and whether there exists a "blood-specific" proteoglycan, genetically distinct from chondroitin sulfate proteoglycans synthesized by non-circulating cells.

We have previously shown that human monocytes cultured *in vitro* produce chondroitin sulfate [17]. Parallel to *in vitro* differentiation into macrophage-like cells, which occurs within four to five days of cultivation [18, 19], the cells change their synthesis of chondroitin 4-sulfate to highly sulfated chondroitin sulfate containing 4,6-disulfated *N*-acetylgalactosamine units.

It has also been shown that *in vitro* differentiated, bone marrow derived mast cells [20]; isolated rat glomeruli [21]; and cultured chick embryo chondrocytes [22] have the ability to synthesize chondroitin sulfate containing disulfated *N*-acetylgalactosamine units. In the latter communication the expression of the disulfated sugar units could be related to the differentiation state of the cells.

In the present study, we report changes in chondroitin sulfate proteoglycan structure correlated to the transition *in vitro* of monocytes into macrophage-like cells.

Materials and Methods

Chondroitin sulfate and chondroitin sulfate fractions from bovine nasal septa with known molecular weights were gifts from Dr. Å. Wasteson, University of Uppsala, Sweden. Mono- and di-*O*-sulfated hexuronosyl-2,5-anhydro[1-^{3}H]mannitol disaccharides were prepared from heparin as described, and separated into mono- and di-*O*-sulfated species by preparative paper electrophoresis [23]. Inorganic [^{35}S]sulfate and ^3H_2O were purchased from The Radiochemical Centre, Amersham, England. Bacterial chondroitinase ABC (EC 4.2.2.4) was from Seikagaku Fine Chemicals, Tokyo, Japan. DNP-alanine, papain and bovine serum albumin were all from Sigma Chemical Co., St. Louis, MO, USA. Percoll, DEAE-Sephacel, Sepharose 4B-CL, Sepharose 6B-CL, Sephadex G-200, Sephadex G-50 and dextran blue were from Pharmacia Fine Chemicals, Uppsala, Sweden. Penicillin and streptomycin were from Gibco Bio-Cult, Paisley, Scotland. Human fibronectin coupled to cyanogen bromide activated Sepharose 4B (3 mg fibronectin/ml gel) was a gift from Dr. S. Johansson, University of Uppsala, Sweden.

Cell Cultures

Monocytes were isolated from human blood by centrifugation of defibrinated blood on Percoll (diluted to 54%) for 20 min at 1 000 × *g*. Cells at the interphase were collected,

washed (200 × *g* for 10 min) and seeded in 16 mm Costar wells (Costar, Broadway, Cambridge, MA, USA) at a density of 1 × 10^6 cells/well in 1 ml of F-10 medium (Flow laboratories, Irvine, Scotland) supplemented with 20% autologous serum and 100 U/ml of penicillin and streptomycin [17, 24]. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in air. After 2 h non-adherent cells were removed by washing three times with phosphate-buffered saline (PBS; 0.13 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4). Adherent monocytes were reincubated in F-10 medium with antibiotics and 20% serum as described above. During extended culture periods the medium was changed every second day.

Biosynthetic labeling of proteoglycans and glycosaminoglycans was performed by washing the cells with sulfate-depleted F-10 medium ($MgCl_2$ substituted for $MgSO_4$) followed by incubation in this medium supplemented with 50 μCi $^{35}SO_4$/ml and well, antibiotics as described above and 20% autologous serum. Two different labeling periods were used. Fifty per cent of the cells (usually 8 to 12 wells) were labeled for 24 h from the establishing of the cultures, while the remaining 50% was labeled from day 4 to day 5 *in vitro*. After the respective labeling periods, medium (and sometimes cell fractions) were harvested and subjected to different isolation procedures (see below).

Isolation of ^{35}S-Glycosaminoglycans

Medium and cell-layers from cultures labeled with [^{35}S]sulfate from either day 0-1 or day 4-5 were digested with papain and labeled polysaccharides were isolated as described [17].

Isolation of [^{35}S]-Macromolecules

Medium was collected from day 1 and day 5 cultures incubated in the presence of [^{35}S]sulfate for 24 h. Detached cells were removed by centrifugation for 10 min at 200 × *g*. a) For size determination by analytical gel filtration, sodium dodecyl sulfate (SDS) was added to the medium to give a final concentration of 1%. The cell-layers were solubilized by incubation with 1% SDS (1 ml/well) for 20 min at room temperature. The medium and cell fractions were heated to 100°C for 3 min and subsequently dialyzed against 0.1% SDS, 0.15 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0, to remove unincorporated [^{35}S]sulfate. b) For affinity chromatography on immobilized fibronectin, radioactive medium proteoglycans were purified by DEAE-ion exchange chromatography. Culture medium (10 ml) from day 1 and day 5 cell cultures exposed to [^{35}S]sulfate were applied to 2 ml columns of DEAE-Sephacel. After washing with 40 ml of 0.2 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0, and 0.2 M NaCl in 0.05 M sodium acetate buffer, pH 4.0, respectively, the columns were eluted with a linear 100 ml gradient ranging from 0.2 M to 1.5 M NaCl in 0.05 M sodium acetate buffer, pH 4.0. Fractions containing ^{35}S-radioactivity were pooled and concentrated.

Concentration of ^{35}S-Labeled Medium Proteoglycans

^{35}S-Labeled medium proteoglycans pooled after DEAE-ion exchange chromatography were diluted 1:4 with 0.05 M Tris-HCl buffer, pH 7.4, and applied to a 0.2 ml column of DEAE-Sephacel equilibrated with 0.2 M NaCl, 0.05 M Tris-HCl buffer, pH 7.4. The columns were subsequently washed with 5 ml equilibration buffer and eluted with 10 × 100 μl of

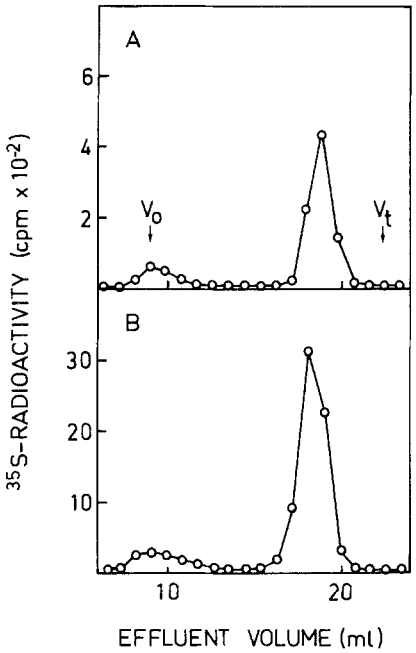

Figure 1. Chondroitinase ABC digestion of ^{35}S-labeled cell-layer glycosaminoglycans. ^{35}S-Polysaccharides obtained after papain digestion of cell-layers from day 1 (A) and day 5 (B) cultures were digested with chondroitinase ABC as described in Materials and Methods. The digested samples were applied to a column (0.6 × 90 cm) of Sephadex G-50, and eluted with 1 M NaCl at a flow rate of 5.4 ml/h. Fractions of 0.9 ml were collected and analyzed for ^{35}S-radioactivity. Dextran blue and DNP-alanine were used as markers for void (V_o) and total (V_t) volume, respectively.

0.05 M Tris-HCl buffer, pH 7.4, containing 1 M NaCl. Chondroitinase ABC digestion, alkali treatment and paper electrophoresis were performed as previously described [17].

^{35}S-Radioactivity was determined in a Packard model 2405 liquid scintillation spectrophotometer.

Results

Cultured human monocytes were incubated in [^{35}S]sulfate-containing medium for 24 h, either from day 0 to 1 or from day 4 to 5. At the start of the second labeling period the cells displayed morphological changes, previously attributed to differentiation into macrophage-like cells [18, 19]. Chondroitinase ABC treatment of day 1 and day 5 [^{35}S]-polysaccharides, isolated after papain digestion and gel chromatography, resulted in depolymerization of 90% of the [^{35}S]-macromolecules in the medium, as previously shown [17], and of 85-90% of the corresponding cell-layer polysaccharides (Fig. 1). Hence, the major sulfated polysaccharide present in medium and cell-layer of both monocytes and macrophage-like cells is of chondroitin sulfate nature. Moreover, high

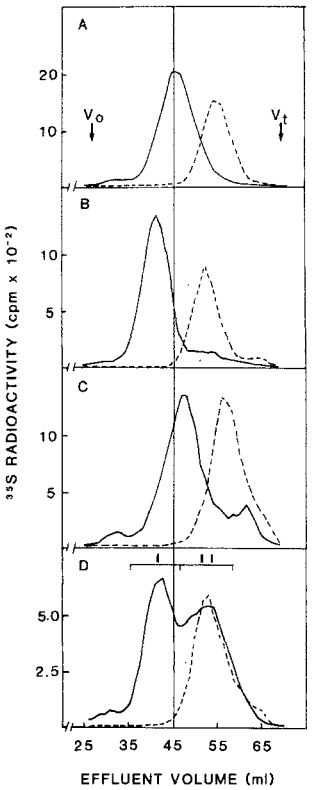

Figure 2. Gel chromatography on Sepharose CL-4B of SDS-solubilized medium and cell-layer ^{35}S-macromolecules. SDS-solubilized ^{35}S-macromolecules from medium (A,B) and cell-layer (C,D) of day 1 (A,C) and day 5 cell cultures (B,D) were applied to a column (1 × 90 cm) of Sepharose CL-4B prior to (—) and after (---) alkali treatment. The samples, containing 5 000-15 000 cpm in 0.5 ml were boiled in 1% SDS for 2 min prior to analysis. The column was eluted with 0.1% SDS, 0.15 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0, at a flow rate of 5.6 ml/h. Fractions of 1.4 ml were collected and analyzed for radioactivity. Dextran blue and DNP-alanine were used as markers for void (V_o) and total (V_t) volume, respectively.

voltage paper electrophoresis of ^{35}S-labeled disaccharides obtained after chondroitinase ABC digestion of cell-layer (day 5) polysaccharides, revealed the same characteristic appearance of disulfated disaccharides (data not shown), as previously reported for medium polysaccharides [17].

Macromolecular Properties of Chondroitin Sulfate

The macromolecular properties of the chondroitin sulfate synthesized by monocytes and macrophage-like cells were compared by gel chromatography on Sepharose CL-4B after solubilisation of medium and cell layer ^{35}S-macromolecules in SDS.

As shown in Fig. 2A and 2B the ^{35}S-labeled chondroitin sulfate from medium of both day 1 and day 5 cultures is of proteoglycan nature; alkali treatment results in a shift in elution

Table 1. K_{av}-values on Sepharose CL-4B of ^{35}S-proteoglycans from day 1 and day 5 cultures. SDS-solubilized ^{35}S-macromolecules from medium and cell-layer of day 1 and day 5 cell cultures were chromatographed on Sepharose CL-4B as described in the legend to Fig. 2. The K_{av}-values given correspond to the peak elution positions of the proteoglycans.

	Day 1 cultures		Day 5 cultures	
Blood donor	medium	cell-layer	medium	cell-layer
1	0.41	0.48	0.35	0.38
2	0.38	0.48	0.36	0.36
3	0.32	n.d.[a]	0.28	n.d.
4	0.33	n.d.	0.32	n.d.
5	0.34	n.d.	0.31	n.d.
6	0.45	n.d.	0.37	n.d.
7	0.40	0.42	n.d.	n.d.

[a] n.d. = not determined

position, reflecting the release of polysaccharide chains from the protein cores. Proteoglycans are present also in the cell layer (Fig. 2C and 2D). However, macrophage-like cells contain additional ^{35}S-labeled material with the approximate size of that of free polysaccharide chains (for further characterization, see below).

A considerable difference in size was observed when proteoglycans obtained from monocytes of different blood donors were chromatographed on Sepharose CL-4B. The calculated K_{av} peak-values for medium proteoglycans obtained from day 1 cultures varied between 0.32 and 0.45 when cell cultures from seven different blood donors were analyzed (Table 1). However, the proteoglycans recovered from day 5 cultures were found to be of larger size than the proteoglycans obtained from day 1 corresponding fractions (evident also in Fig. 2).

Polysaccharide Chain Length

To investigate whether the larger size of the proteoglycans synthesized by macrophage-like cells could be attributed to an increased polysaccharide chain length, ^{35}S-labeled proteoglycans from day 1 and day 5 cultures were subjected to alkali treatment followed by gel chromatography on Sephadex G-200. The elution profiles of ^{35}S-polysaccharides from blood donor 1 cultures are shown in Fig. 3. Polysaccharide chains from both medium and cell-layer of day 5 cultures are larger in size than their day 1 counterparts; an increase in average molecular weight of 6 500 and 10 200 for medium and cell-layer polysaccharides, respectively, could be calculated relating their elution positions to those of chondroitin sulfate standards of known molecular weight (Table 2).

When the two peaks obtained after Sepharose CL-4B chromatography of the day 5 cell fraction (see Fig. 2D) were separately pooled, treated with alkali and analyzed on the Sephadex G-200 column, polysaccharide chains from the second peak were shown to

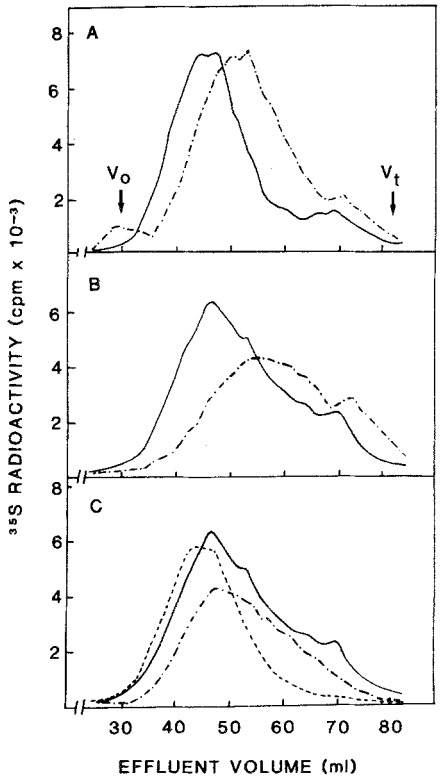

Figure 3. Gel chromatography on Sephadex G-200 of medium and cell-layer ^{35}S-labeled polysaccharide chains of blood donor 1. SDS-solubilized ^{35}S-macromolecules from medium (A) and cell-layer (B) of day 1 (-·-) and day 5 (—) cell cultures were treated with alkali, dialyzed against water and applied to a column (1 × 100 cm) of Sephadex G-200 eluted with 1 M NaCl. Cell-layer ^{35}S-macromolecules from day 5 cultures (—), and Sepharose CL-4B separated peak I (---) and peak II (--) (see Fig. 2D), were similarly treated with alkali and applied to the column (C), which was eluted at a flow rate of 3 ml/h. Fractions of 1 ml were collected and analyzed for radioactivity. Dextran blue and ^3H_2O were used as markers for void (V_o) and total (V_t) volume, respectively.

be smaller in size and more heterogenous than polysaccharides from the first peak (Fig. 3C). Furthermore, identical elution profiles were obtained for retarded peak material prior to and after alkali treatment, indicating that little or no protein was attached to the polysaccharide (data not shown). Recent studies indicate that such free chains are located intracellularly and hence, not associated with the cell surface (S.O. Kolset, unpublished data).

Paper electrophoresis of disaccharides obtained after chondroitinase ABC digestion of proteoglycan-associated (peak I) and free polysaccharides (peak II) showed a content of approximately 20% disulfated disaccharides in both preparations (data not shown).

The polysaccharide chains of day 5 proteoglycans from blood donors 2 and 3 also had a higher average molecular weight than the corresponding day 1 polysaccharides (Table 2). In contrast, similar molecular weights were obtained for day 1 and day 5 medium ^{35}S-polysaccharides from blood donor 4. Furthermore, the intact medium proteoglycans

Table 2. Average molecular weight of polysaccharide chains from ^{35}S-proteoglycans isolated from day 1 and day 5 cell cultures. Alkali-treated ^{35}S-macromolecules from day 1 and day 5 cell cultures were chromatographed on Sephadex G-200 as described in the legend to Fig. 3. The K_{av} peak values were used to calculate an approximate M_r from a standard curve of log M_r vs. K_{av}, constructed by use of well-characterized chondroitin 4-sulfate fractions [26].

	Medium		Cell-layer	
Blood donor	K_{av}	M_r	K_{av}	M_r
		Day 1 cultures		
1	0.45	14000	0.55	9500
2	0.39	17200	0.44	14400
3	0.39	17200	n.d.[a]	n.d.
4	0.30	23600	n.d.	n.d.
		Day 5 cultures		
1	0.34	20500	0.35	19700
2	0.31	22800	0.31	22800
3	0.32	22000	n.d.	n.d.
4	0.30	23600	n.d.	n.d.

[a] n.d. = not determined

obtained from this blood donor showed no significant increase in size after *in vitro* differentiation of the cells (Table 1). Taken together, these results indicate that the increased size of proteoglycans from macrophage-like cells as compared to those of monocytes is due to the synthesis of longer polysaccharide chains.

Interaction of Purified Medium Proteoglycans with Fibronectin

Medium proteoglycans from day 1 and day 5 cultures were purified by DEAE-ion exchange chromatography. As shown in Fig. 4, the ^{35}S-labeled day 5 proteoglycans were eluted at a higher ionic strength than day 1 ^{35}S-macromolecules. The retarded elution position of the ^{35}S-proteoglycans produced by macrophage-like cells is probably due to the higher sulfate content of the polysaccharide chains as previously stated [17] and the larger size of the proteoglycan molecules.

The increase in molecular weight and sulfate content of the polysaccharide chains of the proteoglycans produced by macrophage-like cells would increase its potential to interact with other macromolecules [25]. Therefore the ability of proteoglycans from day 1 and day 5 cultures to interact with fibronectin was investigated. The purified day 1 and 5 ^{35}S-proteoglycans were applied to a column of fibronectin-Sepharose equilibrated in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.05 M NaCl. The column was then eluted with a linear NaCl gradient. As shown in Fig. 5A, more than 50% of the day 1 ^{35}S-proteoglycans were washed through the column and the retained ^{35}S-proteoglycans were eluted early in the gradient. In contrast, the day 5 proteoglycans were quantitatively bound to the gel and eluted at an NaCl concentration of 0.13 M, corresponding to an ionic strength of 0.18.

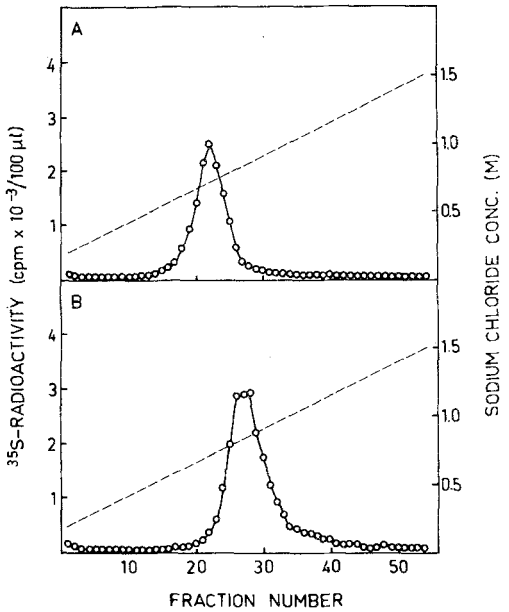

Figure 4. Preparative DEAE-ion exchange chromatography of ^{35}S-labeled medium proteoglycans. Culture medium (10 ml) from day 1 (A) and day 5 cell cultures (B), exposed to ^{35}S-sulfate for 24 h were applied to 2 ml columns of DEAE-Sephacel. After washing with 40 ml of 0.2 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0, and 0.2 M NaCl in 0.05 M sodium acetate buffer, pH 4.0, respectively, the columns were eluted with a linear 100 ml gradient ranging from 0.2 M to 1.5 M NaCl in 0.05 M sodium acetate buffer, pH 4.0. Fractions of 1.9 ml were collected and 100 µl of each fraction was analyzed for ^{35}S-radioactivity.

Discussion

We have previously shown that cultured human monocytes during the first two days *in vitro* produce chondroitin sulfate that is exclusively 4-sulfated [17]. However, after prolonged culture periods, the chondroitin sulfate synthesized becomes highly sulfated due to the appearance of 4,6-di-sulfated *N*-acetylgalactosamine residues. Recent results indicate that the formation of "oversulfated" chondroitin sulfate is related to the differentiation state of the cells [27]; monocytes cultured on fibronectin substrates for five days did not display the marked morphological changes attributed to the development of macrophage-like cells, and synthesized negligible amounts of polysaccharide containing disulfated *N*-acetylgalactosamine units.

Interestingly, the content of 4,6-di-*O*-sulfated *N*-acetylgalactosamine in chondroitin sulfate synthesized by cultured chick embryo chondrocytes has been shown to correlate with the degree of differentiation of the cells [22]. The differentiated cells expressing these specific sugar units were also shown to increase their synthesis of cartilage-specific proteoglycan. Hence a correlation may be noted between discrete changes in polysaccharide structure and altered proteoglycan synthesis related to the cellular state of differentiation.

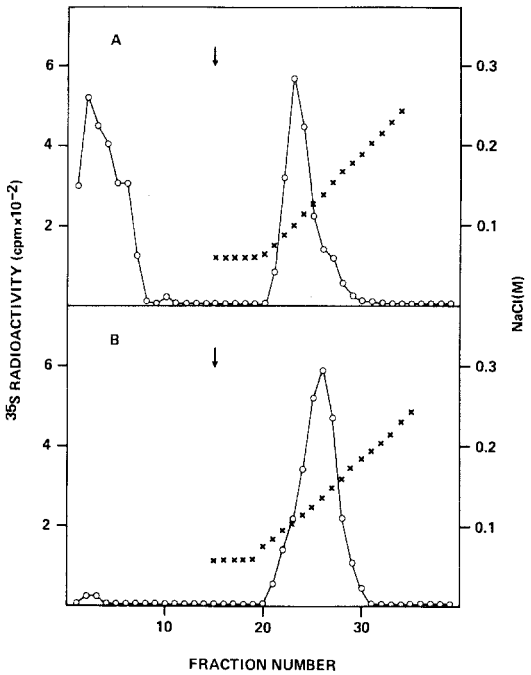

Figure 5. Affinity chromatography on fibronectin-Sepharose. ^{35}S-Proteoglycans from day 1 (A) and day 5 (B) cell culture medium were applied to a 2 ml column of fibronectin-Sepharose equilibrated in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.05 M NaCl. After washing of the column with the equilibration buffer, a salt gradient extending from 0.05 M to 0.5 M NaCl in 0.05 M Tris-HCl buffer, pH 7.4, was applied (start of gradients indicated with arrows). Fractions of 1.5 ml were collected and analyzed for ^{35}S-radioactivity.

The relationship between the monocyte proteoglycan and proteoglycans synthesized by other types of blood cells is not clear. The elution position on Sepharose CL-4B of the chondroitin sulfate proteoglycan identified as the inhibitor of complement factor C1q in plasma [12] (K_{av} = 0.35) resembles that of monocyte medium proteoglycans. The C1q inhibitor contains exclusively chondroitin 4-sulfate, relating it to the day 1 proteoglycan but not to the proteoglycan produced by macrophage-like cells. The proteoglycan present in platelets, bound to platelet factor 4, is also of chondroitin 4-sulfate nature [11]. This proteoglycan is much smaller and its amino acid composition is different from that of the C1q inhibitor, indicating that they might be distinct proteoglycan molecules. The chondroitin sulfate proteoglycan synthesized by lymphocytes [16], finally, has a molecular size similar to the monocyte proteoglycan and contains polysaccharide chains with an approximate M_r of 25 000. Hence, the proteoglycans synthesized by monocytes and lymphocytes are closely similar. As their structure resembles that of the C1q inhibitor, these cells may be responsible for the production of this proteoglycan.

Studies on the protein core of the proteoglycans synthesized by monocytes have been hampered by the low level of proteoglycan synthesis in these cells. However, pilot studies, where purified medium proteoglycans have been labeled with ^{125}Iodine and

subjected to SDS gel electrophoresis after chondroitinase ABC treatment, indicate a molecular weight of the core protein of about 11 000 (L. Kjellén and S.O. Kolset, unpublished data). Interestingly, rat basophil leukemic cells also synthesize a chondroitin sulfate proteoglycan with a core protein of similar size [28].

The results presented in this study suggest that the occurrence of disulfated disaccharides in cultured monocytes is accompanied by an increased molecular weight of the proteoglycan. Since polysaccharide chains, released by alkali treatment from the proteoglycans of macrophage-like cells, generally were of larger size than the monocyte counterparts, the increased size of the proteoglycan could simply be due to the larger size of the polysaccharide chains; the size estimates of the proteoglycan from blood donor 4 which did not significantly increase in size after *in vitro* differentiation, and contained polysaccharide chains of similar M_r, whether isolated from monocytes or macrophage-like cells, support this conclusion. These data may also suggest that the increase in proteoglycan molecular weight is a capacity restricted to cultured monocytes from some blood donors, and not necessarily a phenomenon associated with monocyte differentiation *per se*.

The proteoglycans of monocytes and macrophage-like cells *in vitro* are largely secretory products [17], pointing to a function for these molecules in the extracellular space. The ability of the proteoglycans produced by macrophage-like cells to interact with fibronectin may have functional importance. Fibronectin has been implicated to function as a link between deposited fibrin and the macrophage cell surface, immobilizing the macrophage at its site of action [29], fibronectin contains binding sites for fibrin to which it becomes cross-linked *via* a transglutaminase reaction [30]. An interaction with highly sulfated chondroitin sulfate could, as has been shown for the fibronectin-heparin interaction [31, 32], possibly alter the conformational state of fibronectin, thereby enhancing its association with the macrophage cell surface [33].

Acknowledgements

This work was supported by grants from the Norwegian Council for Science and the Humanities, the Norwegian Cancer Society, the Swedish Medical Research Council (No. 2309, 6525), the Swedish Council for Forestry and Agricultural Research (No. A5861) and the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences.

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