Cell Density-dependent Expression of Chondroitin Sulfate Proteoglycan in Cultured Human Monocytes*

Lars Uhlin-Hansen
From the Institute of Medical Biology, University of Tromsø, Tromsø, Norway
Svein O. Kolset†
From the Department of Tumor Biology, Norwegian Cancer Society, Institute of Medical Biology, University of Tromsø, Tromsø, Norway

Monocytes were isolated and established in vitro at different cell densities. The incorporation of [35S]sulfate into macromolecules in monocytes (day 1 in culture) and monocyte-derived macrophages (day 5 in culture) was found to increase with decreasing cell density in approximately the same way in both day 1 and day 5 cell cultures. [35S]Sulfate was found to be incorporated almost exclusively into chondroitin sulfate proteoglycan (CSPG) in both high and low density monocyte and monocyte-derived macrophage cultures. The molecular size of the [35S]CSPGs produced by the high and low cell density cultures were not found to differ as judged by gel chromatography elution patterns. The molecular size and the structure of the glycosaminoglycan chains were found to be almost similar in high and low density day 1 and day 5 cultures. Only a small degree of proteoglycan degradation could be observed in both high and low density cultures. Furthermore, cell density-dependent differences in CSPG biosynthesis could be observed already 2 h after the establishment of the cultures, indicating that a process of "down-regulation" in high density cultures was already in operation. The glycosaminoglycan synthesis in high cell density day 1 cultures could be increased slightly following exposure to 0.5 mm benzyl-β-d-xyloside, but not to the same level as that observed in untreated low cell density cultures. By contrast, the expression of 35S-macromolecules by cells cultured at high cell density for 5 days could be increased by xyloside treatment almost to the same level as that observed in the low density cultures.

Monocytes and macrophages are cells with high secretory potentials. Products released from these cells include neutral proteases, oxygen species, complement factors, prostaglandins, interleukins, fibronectin, etc. (1). The wide range of proteases, oxygen species, complement factors, prostaglandins, monocyte and monocyte-derived macrophage cultures. The molecular size of the [35S]CSPGs produced by the high and low cell density cultures were not found to differ as judged by gel chromatography elution patterns. The molecular size and the structure of the glycosaminoglycan chains were found to be almost similar in high and low density day 1 and day 5 cultures. Only a small degree of proteoglycan degradation could be observed in both high and low density cultures. Furthermore, cell density-dependent differences in CSPG biosynthesis could be observed already 2 h after the establishment of the cultures, indicating that a process of "down-regulation" in high density cultures was already in operation. The glycosaminoglycan synthesis in high cell density day 1 cultures could be increased slightly following exposure to 0.5 mm benzyl-β-d-xyloside, but not to the same level as that observed in untreated low cell density cultures. By contrast, the expression of 35S-macromolecules by cells cultured at high cell density for 5 days could be increased by xyloside treatment almost to the same level as that observed in the low density cultures.

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† To whom correspondence should be addressed.

† The abbreviations used are: CSPG, chondroitin sulfate proteoglycan; SDS, sodium dodecyl sulfate; DME, Dulbecco's modified Eagle's medium; MDM, monocyte-derived macrophages; GAG, glycosaminoglycan; HPLC, high pressure liquid chromatography; ΔDi-4S, 2-acetamido-2-deoxy-3-O-((β-D-glucosyl-4-ene-pyranosyluronic acid)-4-O-sulfo-D-galactose; ΔDi-4S+, ΔDi-4, 6-dis, 2-acetamido-2-deoxy-3-O-((β-D-glucosyl-4-ene-pyranosyluronic acid)-4, 6-di-O-sulfo-D-galactose.
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fluorescence microscope. More than 95% of the cells were found to be fluorescence-positive in both high and low density monocyte cultures, whereas macrophages from salmon pro nephros were completely negative. Only a minor population of cells (3-5%) could, by microscopic inspection, be seen to be loose or nonadherent in the monocyte cultures. During the transition of monocytes into MDM, some cells were seen to detach and die, but these cells and debris were eliminated efficiently by the adherent cells. Day 5 cells accordingly were found to contain almost exclusively adherent cells with typical MDM morphology (Fig. 6b).

Monocytes were cultured in vitro at densities ranging from two times to one-eighth of stock solution for 1 or 5 days. [35S]Sulfate (50 μCi/ml) was added on day 0 or day 4, and the cells were exposed for 20 h before medium fractions were harvested and subjected to papain digestion. [35S]Glycosaminoglycans were isolated by gel chromatography (3). In addition, parallel fractions were solubilized in 1% SDS, boiled for 5 min, and dialyzed extensively against 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4, prior to and after alkali treatment. [35S]Sulfate contents were determined by scintillation counting. [35S]Glycosaminoglycans were isolated by Sephadex G-50 gel chromatography on small columns (24 x 0.7 cm) run in phosphate-buffered saline, pH 7.4, with 0.1% SDS. V5 fractions were collected and counted for content of radioactive glucosamine. When controls with 50 μCi of [35S]sulfate in 1 ml of DME were chromatographed, only 100-300 cpm could be recovered in the V5 fraction. It has been demonstrated previously that [35S] sulfate is incorporated almost exclusively into CSPG in monocytes and MDM (3, 4, see also “Results”). The amount of [35S]macromolecules detected after dialysis therefore is taken as a measure of proteoglycan and glycosaminoglycan (GAG) de novo synthesis. Control Sepharose CL-6B gel chromatographies were done routinely on chosen samples after completed dialysis or Sephadex G-50 gel chromatography. No free [35S]sulfate could be detected in any of the samples tested. Furthermore, [35S(CSPGs from monocyte and MDM culture media were subjected to Sepharose CL-6B gel chromatography both prior to and after alkali treatment (0.5 M NaOH for 20 h at room temperature), which result in the liberation of free [35S]-GAG chains. The column (1 x 90 cm) was run in 0.5 M Tris-Cl, pH 8.0, with 0.1% SDS and 0.15 M NaCl. Fractions of approximately 1 ml were collected and analyzed for content of radioactivity. Markers for void (V) and total (V) volumes were dextran blue and 2,4-dinitrophenylalanine, respectively. Galactosaminoglycans were degraded with chondroitinase ABC (3), and the disaccharides obtained by Sephadex G-50 gel chromatography were chromatographed on a Lichrocart-54 column (Merck, Darmstadt, West Germany) in 0.05 M sodium acetate buffer, pH 5.0, with a salt gradient ranging from 0 to 0.1 M NaCl. The disaccharide standards used were chondroitin 4-sulfate (8).

Cells from day 1 and day 5 cultures were fixed in 2.5% glutaraldehyde, followed by 1% osmium tetroxide, both in 0.1 M cacodylate buffer, pH 7.5. The bottom of the wells were cut out, and the cells were dehydrated in ethanol and critical point-dried (Balzers Union CPD-020). The cells were coated with 250 Å gold (Polaron SEM coating Unit E500), and scanning electron microscopy was performed with a JEOL-840 scanning microscope (JEOL Ltd., Tokyo, Japan).

The number of adherent cells in the different wells at the end of the labeling periods was determined by counting cells along the diameter in all cultures using a Leitz Wetzlar inverted phase microscope, equipped with a net of 10 x 10 square routes in one of the condensers. The total area counted was 1.6 mm² out of a total area of 201 mm². By microscopic inspection the cells were found to be evenly distributed, even at the lowest densities.

RESULTS

Previous studies have demonstrated that both monocytes and MDM synthesize and release chondroitin sulfate proteoglycans (CSPGs) when cultured in vitro (3, 4). After a 20-h label with [35S]Sulfate approximately 80% of the CSPG is recovered from the medium fractions in both monocyte and MDM cultures (4). The cultivation of both cell types at high and low density did not affect the release of [35S]CSPG (result not shown). The main emphasis in this study has therefore been put on material deriving from the medium fractions under the various experimental conditions chosen.

Effect of Cell Density on CSPG Biosynthesis—Monocytes were established in culture at varying densities. Due to the fact that adherent monocytes and MDM can not be released by trypsinization, the number of adherent cells was deter-
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mined after completion of the radiolabeling. The amount of \[^{35}S\]CSPG synthesized and released per cell into the medium in both monocyte and MDM cultures showed a clear decrease with increasing cell density. Thus, an increase in cell number from \(1.9 \times 10^4\) to \(3.2 \times 10^5\) in the monocyte cultures resulted in a 4.4 times decrease in \(^{35}S\) incorporation into macromolecules per cell (see Fig. 1). Also, in MDM cultures an increase in cell number from \(1.1 \times 10^4\) to \(3.2 \times 10^5\) resulted in a 10 times reduction in synthesis. Accordingly, the CSPG synthesis is down-regulated similarly by increasing cell density in both monocytes and their in vitro-differentiated counterparts, MDM.

The Effect of Cell Density on Proteoglycan Turnover—The lower expression of CSPG in the high density cell cultures possibly could be due to a higher turnover of proteoglycan than in the low density cultures. It is conceivable that the use of 20-h labeling periods would conceal a higher rate of CSPG degradation in the high cell density monocyte and MDM cultures. Two different strategies were chosen to address this problem in further detail.

First, \[^{35}S\]CSPG was purified from MDM cultures by DEAE-ion exchange chromatography as previously described (4). New monocyte cultures (from the same blood donor) were allowed to differentiate in vitro into MDM by 5 days cultivation. At this time point, purified \[^{35}S\]CSPG was added to the cultures without medium changes. Both high and low density MDM received approximately 11,000 cpm/well. Incubations were continued for 20 h, whereafter medium and cell fractions from both high and low density cultures were harvested, solubilized in SDS, and subjected to Sepharose CL-6B gel chromatography. \[^{35}S\]CSPG recovered from the medium of high (Fig. 2A) and low density cultures (Fig. 2B) were eluted with patterns almost identical to that of the purified \[^{35}S\] CSPG (Fig. 2C). From Fig. 2, A and B, it is further evident that approximately 20% of the radioactivity, in both cell and medium fractions, could be recovered in retarded elution positions, indicating that some intra- or extracellular degradation had taken place. The same amount of \[^{35}S\]CSPG was chromatographed directly after purification (Fig. 2C) as was added to the cell cultures. A summation of the fractions eluting with a \(K_{av} < 0.4\) would suggest that the recovery of \[^{35}S\]CSPG from both high and low density cultures is approximately 80%. Based on this observation high density MDM do not seem to degrade endogenous proteoglycan to a larger extent than the corresponding low density cultures.

Second, the amount of medium-associated CSPG was found to increase with time in both high and low density cultures,
as can be seen in Fig. 3. At the earliest time point of measurement (2 h), the low cell density cultures (24,000 cells/well) synthesized and released approximately two times more [35S]CSPG than the corresponding high density cultures (240,000 cells/well), and they persisted to do so during the entire 10-h incubation period. Furthermore, after a 20-h incubation period low density monocytes (38,000 cells) expressed two times more [35S]CSPG than the high density culture (315,000) as is demonstrated in Fig. 1. Almost identical differences in cell density-dependent CSPG expression can, accordingly, be measured both after 2- and 20-h pulse periods.

The Effect of Cell Density on Proteoglycan Structure—The possibility that the lower amounts of [35S]CSPG detected in high density monocyte and MDM cultures could be due to changes in proteoglycan structure was investigated by gel chromatography of these macromolecules both prior to and after alkali treatment. The elution profiles of [35S]CSPG from both low and high density monocyte cultures were found to be almost identical (Fig. 4, A and B). The alkali- liberated 35S-GAG chains were also found to be almost identical in molecular size as demonstrated by their elution profiles in Fig. 4, A and B. Accordingly, differences in proteoglycan or GAG molecular size does not form the basis for the cell density dependent CSPG synthesis. However, the higher level of [35S]CSPG in low density cultures could reside in an increased sulfation of the GAG chains. 35S-Labeled disaccharides obtained by chondroitinase ABC digestions were analyzed by HPLC. Both high and low density monocyte GAGs were found to contain >90% disaccharide comigrating with the ADi-4S standard. No difference in the content of disulfated disaccharide units (ADi-diS) in the respective 35S-GAG fractions could be detected (Fig. 5A). Moreover, no differences could be observed in the disaccharide composition of GAGs from low and high density MDM (Fig. 5B). Both cultures expressed a higher amount of disaccharides comigrating with the ADi-diS standard than the corresponding monocyte cultures, in accordance with previous results (3, 4).

The disaccharides analyzed were obtained by chondroitinase ABC digestions and gel chromatographies. The elution profiles for 35S-labeled material from both monocytes and MDM (at both cell densities) revealed that >95% was depolymerized to disaccharides (result not shown). The cell density-dependent incorporation of [35S]sulfate into macromolecules in both monocyte and MDM cultures, therefore, should be due to differences in proteoglycan synthesis per se and not an increased sulfation of other macromolecules. Further confirmative evidence for this conclusion was obtained by comparing the amount of [35S]sulfate incorporated into CSPG by SDS solubilization followed by Sephadex G-50 gel chromatography, and proteolytic digestion and corresponding gel chromatography. The amount of radioactivity recovered from the void volume fractions by both experimental procedures revealed the same cell density dependence as displayed in Fig. 1 for monocyte cultures.

Cell Density Examined by Scanning Electron Microscopy—When monocytes and MDM cultured at various cell densities were subjected to scanning electron microscopy, it was evident that a large degree of cell-cell contacts were established at the highest cell densities, whereas such contacts were almost nonexistent in the lowest density cultures, as is demonstrated in Fig. 6, a-d. The typical enlargement of the monocytes following transition into MDM is best seen at high cell densities (Fig. 6, a and b, see also below).

Effect of Benzyl-β-D-xyloside on Cell Density-dependent Proteoglycan Biosynthesis—The apparent effect of high cell density on the CSPG synthesis both in monocytes and MDM may be an irreversible process, possibly involving effects on gene expression and/or rate-limiting steps in the biosynthesis of these distinct molecules. By using xylosides it is possible to circumvent the possible regulatory steps on the core protein level and to determine the cells' capacity for GAG synthesis (see Ref. 9 and references therein). Exogenous xylosides will compete with endogenous xylosylated core protein for access to the GAG synthesis machinery, and by using such agents it may be possible to establish whether the synthesis of GAG chains conforms to the cell density dependence displayed for the CSPG synthesis. Both monocytes and MDM were exposed to 0.5 mM benzyl-β-D-xyloside, and the incorporation of 35S sulfate into macromolecules was determined and compared with the synthesis by control cells cultured at both high and low density. The effect of xyloside treatment was strikingly different in the two cell systems. Monocytes cultured at high

**Fig. 6.** Scanning electron micrographs of monocytes (a and c) and MDM (b and d) at cell densities of approximately 300,000 (a and b) and 20,000 (c and d) per well. The bars displayed represent 10 μm.
density increased their \(^{35}\text{S}\) incorporation only minimally following exposure to 0.5 mM xyloside; and, on a cellular basis, the synthesis was still 3.5 times lower than that of untreated low density cultures (Table I). High density MDM cultures, however, responded to xyloside treatment with a 10-fold increase in \(^{35}\text{S}\) incorporation, restoring the synthesis almost to the level of untreated low density cultures (Table I). The latter cell cultures were found to operate almost at the maximum of their capacity for CSPG synthesis; xyloside-treated cells incorporated only 1.7 times more than control cells.

Expression of Oversulfated CSPG in High and Low Cell Density MDM—From the scanning electron micrographs displayed it was evident that low density cell cultures (Fig 6, c and d) did not display the conspicuous morphological changes shown for the high cell density cultures (Fig 6, a and b) after 5 days incubation in vitro. Previous studies (10) have demonstrated that the expression of oversulfated CSPG is correlated to the development of MDM, as cells kept on fibronectin substrates did neither develop into typical MDM nor synthesize oversulfated CSPG. In contrast, the low cell density cells kept for 5 days in vitro were found to express disulfated disaccharide units in amounts equal to those found in the high cell density cell cultures (Fig 5B). Therefore it may be valid to propose that 5 days incubation of monocytes on plastic surfaces (possibly activating the cells), independent of the development of typical MDM (see Fig. 6d), is sufficient for the shift in GAG structure previously reported (3).

**DISCUSSION**

The implication of cell density, cell division, and confluency of culture systems for the biosynthesis of proteoglycans in various culture systems has been the subject of a number of recent studies. In human skin fibroblasts it has been demonstrated that postconfluent cells synthesize a cell surface heparan sulfate proteoglycan with affinity for transferrin, whereas proliferating cells synthesize a structurally different heparan sulfate proteoglycan with no transferrin binding activity (11). In human fibroblasts, also, the lateral diffusion of major histocompatibility antigens was found to decrease with increasing cell density. This change occurred both as a consequence of increased cell-cell contact and age of cultures but was not observed in growth arrested or in sparse cultures with conditioned media from dense (confluent) cells (12). Furthermore, exponentially growing smooth muscle cells have been demonstrated to synthesize 1.5-3 times more heparan sulfate than postconfluent cells. The latter cells, however, expressed heparan sulfate on the cell surface with eight times more antiproliferative activity per cell (against smooth muscle cells) than the corresponding fractions from exponentially growing cells (13).

Studies on the GAG biosynthesis in relation to cell division have revealed that Chinese hamster ovary cells depress this synthesis 4-fold during mitosis and increase it 2-3-fold during early G1 phase (14). In contrast, confluent hepatocytes (cell line) incorporated 2.5 times more sulfate than log-phase growing cells. Moreover, the heparan sulfate expressed by confluent cells was found to be more highly sulfated than that from dividing cells (15). Most studies demonstrating a decrease in glycosaminoglycan biosynthesis have been performed on proliferating cells (16, 17). Monocytes cultured in vitro do not, however, proliferate to any significant extent. Monocytes and macrophages are blocked in the G0 phase of the cell cycle, and only a small subpopulation will go through one cycle of replication in vitro, and only when exposed to exogenously added mitogens (18). To our knowledge the present paper is the first demonstration of cell density-dependent proteoglycan biosynthesis in nondividing cells.

The changes in proteoglycan and glycosaminoglycan biosynthesis or structure referred to may only be suggestive as to the role of these macromolecules in the control of cell division and other cell processes and functions. The present study demonstrates that both monocytes (day 1 cells) and MDM (day 5 cells) increase the expression of CSPG with decreasing cell density. Short term labeling experiments clearly showed a higher release of \(^{35}\text{S}\)CSPG from the low density cultures than from the high density system already 2 h after establishment of the cultures, and that the level of \(^{35}\text{S}\) proteoglycans recovered from the medium was found to differ by a factor of 2 throughout the 10-h incubation period (see Fig. 3). Moreover, the high density MDM did not degrade \(^{35}\text{S}\)CSPG added to the cultures to any significant extent. The data presented, therefore, do not seem to lend support to different rates of turnover as a possible explanation for the cell density-dependent CSPG biosynthesis reported. Furthermore, we were able to exclude differences in the macromolecular properties of the CSPGs structure as a possible basis for the observed cell density difference in \(^{35}\text{S}\) incorporation. The sulfate density and the molecular size of the GAG chains were not found to differ in high and low density cultures. In addition, \(^{35}\text{S}\) sulfate was found to be incorporated almost exclusively into GAG chains in high and low density monocyte cultures, excluding possible sulfation of other macromolecules as an explanation for the observed differences.

Particularly in the MDM cultures, the "down-regulation" induced by a high cell density could be circumvented by triggering the GAG biosynthesis with benzyl-\(\beta\)-D-xyloside (Table I), clearly demonstrating the reversibility of the cell density-dependent decrease in CSPG biosynthesis. The
ference in the effect of xyloside in high density monocyte and MDM may suggest that the regulation of the observed cell density-dependent CSPG biosynthesis is different in the two cell systems. The data presented can only be suggestive as to the possible importance of core protein in the regulation of this cell density-dependent phenomenon. From Table I one might speculate that the protein component of the CSPG would be more important as a regulator in MDM than in monocyte cultures. The difference in the effect of xyloside in monocyte and MDM cultures may also suggest that additional regulatory “factors” may be obligatory for the cell density effect on CSPG expression to be operable. The expression of CSPG in megakaryocytes has been shown recently to be regulated by a protein isolated from rabbit plasma (19, 20).

Scanning electron microscopy (see Fig. 6) clearly demonstrated the lack of cell-cell contact in the low density cultures. This was in sharp contrast to the extensive communication observed between cells in the high density cultures. This observation, however, can only be indicative as to the importance of extensive cell-cell contact for the regulation of this particular phenomenon. By microscopic inspection it was evident that at the two lowest densities employed in this study almost no cell-cell contacts could be observed (see also Table I). All the same, both monocytes and MDM were found to express the highest amount of CSPG at the lowest of these two cell densities (Table I), indicating that other factors than cell-cell contact may be operating in the regulation of the cell density-dependent CSPG biosynthesis.

The biological relevance of function of the higher demand for CSPG synthesis in low density monocyte and MDM cultures has not been established. The CSPGs produced are evidently not deposited into any matrix as they are recovered from conditioned media of the respective cultures. Moreover, they are not cell-associated as observed with mast cell (21) and natural killer cell storage granule proteoglycans. This may not exclude the possibility, however, that these proteoglycans may be related from a functional point of view; secretion of CSPG from natural killer cells has been correlated with tumor cell killing (22). CSPGs produced by lymphocytes and monocytes have been implied as mediators in the immune system (23). Furthermore, lymphoid cells have been demonstrated to express CSPG associated with the major histocompatibility complex class II or Ia antigens (24, 25). The fact that CSPG biosynthesis and release in both monocyte and MDM cultures is cell density-dependent may provide new perspectives and possible approaches to the elucidation of the function(s) of these molecules in the monocyte/macrophage system.

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