Blood Platelets Stimulate the Expression of Chondroitin Sulfate Proteoglycan in Human Monocytes

By Lars Uhlin-Hansen, Dagfinn Langvoll, Trude Wik, and Svein O. Kolset

Mononuclear phagocytes synthesize chondroitin sulfate proteoglycan (CSPG), which is constitutively secreted. Because mononuclear phagocytes are known to interact with blood platelets, the effect of platelets on the release of CSPG in cultured human monocytes was investigated. After 6 days in vitro, the monocytes were supplied with fresh medium with different additions and subsequently exposed to [35S]sulfate for 24 hours before the medium fractions were harvested and analyzed for content of [35S]CSPG. Indirect evidence for the release of stimulatory factors from blood platelets was found when the addition of medium containing 50% serum made from platelet-rich plasma increased the expression of [35S]CSPG almost sevenfold compared with serum-free medium, whereas medium containing 50% serum made from platelet-depleted plasma increased the expression of [35S]CSPG about fourfold. Further, direct evidence for the stimulatory effect of platelets was found as the addition of autologous platelets to serum-free medium increased the expression of [35S]CSPG about threefold, and addition of supernatant from a corresponding number of thrombin-stimulated platelets was almost as efficient. The effect of five different platelet-derived factors (which are all present in serum) was investigated. Both platelet-derived growth factor (PDGF), platelet factor 4 (PF 4), and prostaglandin E2 (PGE2) used in physiologic concentrations were found to stimulate the expression of [35S]CSPG twofold to threefold, whereas transforming growth factor-β had a slight inhibitory effect. 12-Hydroxyeicosatetraenoic acid had no significant effect on the expression of [35S]CSPG. Further evidence for the stimulatory effect of PDGF, PF 4, and PGE2 was found as serum depleted of these factors had significantly less stimulatory effect than control serum. The increased incorporation of [35S]sulfate into [35S]CSPG in cultures stimulated with serum or platelet-derived factors was not due to differences in molecular size or extent of sulfation of the proteoglycan molecules.

INTERACTIONS between blood platelets and mononuclear phagocytes are highly relevant to situations in vivo, such as the development of atherosclerotic plaques and thrombocytopenia. Histopathologic data have shown that in experimentally induced atherosclerosis platelets adhere to macrophages in the intimal lesions, and recently it has been shown that the adherence of platelets to mononuclear phagocytes is mediated by a specific membrane protein expressed on the surface of activated platelets. Platelets have been shown to induce cholesterol ester accumulation in cultured human monocytes and to stimulate the synthesis of thromboxane and tissue factor.

Although several studies have pointed to the stimulatory effect of blood platelets on mononuclear phagocytes, only few purified platelet-derived products have been shown to be effective. Blood platelets contain large amounts of different proteins, including platelet factor 4 (PF 4), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β), which are secreted from the platelets upon stimulation. Further, when platelets are activated, arachidonic acid is liberated from the membrane of the platelets and converted by lipoygenase and cyclooxygenase. The main lipoygenase products secreted from platelets is 12-hydroxyeicosatetraenoic acid (12-HETE), whereas prostaglandin E2 (PGE2) is a major cyclooxygenase pathway product. During preparation of serum from clotted blood these proteins and arachidonic acid metabolites are secreted from the platelets into serum. We have previously reported that mononuclear phagocytes cultured at low cell density express higher levels of chondroitin sulfate proteoglycan (CSPG) than high-density cultures. The "down-regulation" of CSPG expression in high-density cultures did not seem to be mediated through cell-cell contacts, but may partly depend on stimulatory serum factors either being consumed or degraded at higher rate. The present study was undertaken to elucidate which serum factors account for the stimulatory effect on the expression of CSPG in monocytes.

The results presented show that the major part of the stimulatory effect of serum is due to factors released from blood platelets, of which PF 4, PDGF, and PGE2 in physiologic concentrations were potent stimulators.

MATERIALS AND METHODS

Papain, dextran blue, 2,4-dinitrophenylalanine and chondroitin sulfate were purchased from Sigma Chemical Co (St Louis, MO). Diethyl aminoethyl (DEAE)-Sephacel, Sephadex G-50 superfine, and Sepharose CL-6B were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Lymphoprep was from Nycomed A/S (Oslo, Norway). Inorganic [35S]sulfate (carrier-free) was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Lymphoprep was from Nycomed A/S (Oslo, Norway). Inorganic [35S]sulfate (carrier-free) was purchased from Institut für Energiekonvertierung (Kjeller, Norway), whereas D-[(1-14C)glucosamine was from Du Pont de Nemours (Germany). Thrombin was obtained from F. Hoffmann-La Roche & Co Ltd., Diagnostica (Basel, Switzerland). Chondroitin ABC lyase (EC 4.2.2.4) was obtained from Seikagaku Kogyo Co (Tokyo, Japan). 12-Hydroxyeicosatetraenoic acid (12-HETE) and PGE2 were from Biomol, Biomol Research Laboratories Inc (Plymouth Meeting, PA). PF 4 (purified from human platelets) and antithrombin PF 4 rabbit serum (Assera PF4) were obtained from Diagnostica Stago (Asnières-sur-Seine, France). PDGF and TGF-β (both purified from human platelets), as well as rabbit polyclonal

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antibodies (IgG-fraction) against human PDGF, were kindly provided by Dr C.-H. Heldin, Ludwig Institute for Cancer Research, Biomedical Center (Uppsala, Sweden). Crude tissue thromboplastin from human brain was a gift from Dr E. Bjerklid, Institute of Medical Biology, University of Tromso (Tromso, Norway).

**Cell culture.** Human mononuclear leukocytes were isolated by Lymphoprep-centrifugation and seeded into 16-mm culture wells (Nuncleon Multidish from A/S Nunc, Intermed, Roskilde, Denmark) as previously reported. After incubation for 40 minutes, the cultures were washed to remove nonadherent cells, and 0.5 mL Dulbecco’s modified Eagle’s medium (DME) with penicillin 100,000 U/L, streptomycin 0.1 g/L, 20 mmol/L HEPES, and NaHCO₃ 2.2 g/L (GIBCO Laboratories, Grand Island, NY) with 20% AB serum was added to each well. This procedure showed about 2.5 x 10⁵ adherent cells, of which more than 95% were monocytes, as shown by histochemical staining, phagocytosis of IgG opsonized sheep red blood cells, and binding of a monocyte/macrophage-specific monoclonal antibody (1D5) as positive criteria.¹⁵,¹⁶

**Preparation of platelet-rich plasma (PRP).** Autologous blood was drawn into vacutainer tubes containing acid-citrate-dextrose (ACD; Becton Dickinson Vacutainer Systems, Rutherford, NJ) and centrifuged at 280g for 10 minutes in room temperature before plasma was collected. Both in the peripheral blood and in the PRP the concentration of platelets was usually about 2.5 x 10⁸/mL. No white blood cells were detected in the PRP by microscopic inspection or by using a Coulter Counter hemocytometer (Coulter Corp, Hialeah, FL).

**Preparation of platelet-depleted plasma (PDP).** Blood was drawn into vacutainer tubes containing either heparin or ACD and centrifuged at 2000g for 30 minutes before plasma was collected. No platelets were detected in the PDP.

**Preparation of blood platelets.** Platelets from PRP were washed twice by centrifugation in Tyrode’s buffer, pH 6.6, and resuspended in Tyrode’s buffer at a concentration of 2 x 10⁵/μL. Freshly isolated, autologous platelets were used in all experiments.

**Preparation of platelet releaser products.** Platelets suspended in Tyrode’s buffer were centrifuged and resuspended in DME. Release of platelet products was induced by adding 0.5 U/mL of thrombin to the platelets and incubating the suspension for 15 minutes at 37°C. Platelet-free supernatant was obtained after centrifugation at 2,000g for 10 minutes.

**Preparation of serum.** Autologous serum was prepared by three different procedures: (1) Serum made from clotted blood [S(CB)]. Blood was drawn into vacutainer tubes without additives (Becton Dickinson Vacutainer Systems) and incubated at 37°C. After 60 minutes, the tubes were centrifuged at 2,000g for 30 minutes before the serum was collected. (2) Serum made from PRP [S(PR)]. Serum was prepared from calcium-depleted (ACD containing) PDP by adding CaCl₂ to a final concentration of 12.5 mmol/L and crude tissue thromboplastin, incubating for 2 hours at 37°C, before centrifugation at 2,000g for 30 minutes. (3) Serum made from PDP (with PRP). The procedure was the same as for (2), but the serum was prepared from PRP instead of PDP.

**Preparation of PDGF-depleted serum.** Thirty milligrams of polystyrene beads (Dynabeads M-450; Dynal A/S, Oslo, Norway) in phosphate-buffered saline (PBS) were incubated with 150 μg of rabbit polyclonal antibodies (IgG-fraction) against human PDGF for 24 hours at 4°C under constant rolling. The beads were then incubated with 0.5% human serum albumin (HSA; Behring, Behringwerke AG, Marburg, Germany) for 24 hours at 4°C, before the beads were washed with PBS. Control beads were incubated only with HSA. One milliliter of autologous serum was incubated with 10 μg of Ab-coated beads or with control beads for 24 hours. The beads were then centrifuged and the PDGF-depleted serum collected.

**Preparation of PF 4-depleted serum.** Three milligrams of polystyrene beads precoated with sheep antirabbit IgG (Dynabeads M-280) was incubated with 0.5 mL rabbit serum containing about 60 μg antibodies against human PF 4 for 24 hours at 4°C under constant rolling. Thereafter, the beads were washed four times and then incubated with 1 mL autologous S(CB) for 24 hours at 4°C. The beads were then centrifuged and the PF 4-depleted serum collected.

**Stimulation/radiolabeling.** On day 6 of the cultivation period, the conditioned culture medium containing 20% AB-serum was removed and 300 μL fresh DME either without or with different additions (serum, plasma, platelets, or platelet-derived products) was added to each well. PGF₁ and 12-HETE were first dissolved in ethanol and then diluted with medium. Final concentration of ethanol in the cell cultures was less than 0.01%, and ethanol alone at this concentration did not influence the expression of [³⁵S]CSPG in control cultures. The cells were exposed to 50 μCi/mL of [³⁵S]sulfate for 24 hours. In one experiment, the cells were labeled with 50 μCi/mL of both [³⁵S]sulfate and [³⁴H]glucosamine. All experiments were performed with monocytes cultured for 6 to 7 days. Freshly isolated monocytes were variably contaminated with a small percentage of platelets. During the 6-day incubation period, these platelets were phagocytosed by the monocytes, and no contaminating platelets were present after this incubation period.

**Isolation of ³⁵S-macromolecules.** The medium fractions were harvested and sodium dodecyl sulfate (SDS) was added to a final concentration of 1% before they were boiled for 3 minutes. Free [³⁵S]sulfate was removed by applying 150 μL of the fractions to 2 mL syringes with Sephadex G-50 superfine gel. The syringes were then centrifuged at 1,000g for 5 minutes. When controls with 50 μCi/mL free [³⁵S]sulfate were applied to the syringes, less than 100 cpm could be recovered in the flow-through fraction. It has previously been shown that [³⁵S]sulfate is incorporated almost exclusively into CSPG in human monocytes, and the amount of ³⁵S-macromolecules detected after the syringe filtration is therefore taken as a measure of de novo synthesis of CSPG, which in the text is generally referred to as level of ³⁵S[CSPG expression. In some experiments, the cell fractions were also harvested by sonobilizing the cells in 1% SDS. Thereafter, the cell fractions were treated in the same way as described for the medium fractions.

**Gel chromatography.** ³⁵S-Labeled macromolecules from medium fractions (and in some experiments also the cell fractions) were subjected to Sepharose CL-6B gel chromatography both before and after alkaline treatment (0.5 mol/L NaOH for 8 hours at 20°C). The column (Econo-Column; Bio-Rad Laboratories, Richmond, CA) was run in 0.05 mol/L Tris/HCl with 0.1% SDS and 0.15 mol/L NaCl, pH 8.0, and fractions of 1 mL were collected and analyzed for content of radioactivity. Markers for void and total volume were dextran blue and 2,4-dinitrophenylalanine, respectively.

**High-voltage paper electrophoresis.** ³⁵S-Disaccharides were obtained after chondroitin ABC lyase treatment and gel chromatography of ³⁵S[CSPG from monocyte-conditioned media. The disaccharides were dissolved in water and applied to strips of Whatman 3MM paper (Whatman Inc, Clifton, NJ) and separated by high-voltage electrophoresis in 1.6 mol/L formic acid, pH 1.7, at 40 V/cm for 80 minutes. The paper was then dried and cut into 1-cm pieces and analyzed for radioactivity. Standard used were [³H]-labeled 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-0-sulfod-galactose (ΔDi-4S) and 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4,6-di-O-sulfod-galactose (ΔDi-4,6-diS) (kindly provided by Dr U. Lindahl, Upssala University, Uppsala, Sweden).

**High-performance liquid chromatography (HPLC).** Macromolecules labeled with both [³⁵S]sulfate and [³⁴H]glucosamine were...
isolated by ion-exchange chromatography and treated with chondroitin ABC lyase. The digest was then filtrated and reduced with sodium borohydride (200 mmol/L NaB\(_4\) for 60 minutes at room temperature) before the disaccharides were analyzed on a Dionex CarboPac PA1 (4 x 250 mm) column using a Dionex HPLC system (Dionex Corporation, Smyrna, GA). The disaccharides were eluted in a gradient of sodium tri-fluoro-acetate in 0.1 mol/L sodium hydroxide. Standards were used reduced ADi-OS, ADi-HA, and ADi-4S from Seikagaku Kogyo Co (Tokyo, Japan).

Statistics. Student's t-test was used, and P values below .05 were considered to show significant differences.

RESULTS

The effect of serum and plasma on the expression of \([^{35}S]CSPG\). Monocytes were cultured for 6 days, after which the culture medium was changed and medium containing various concentrations of autologous serum and plasma was added to the cultures. Thereafter, the cells were exposed to \([^{35}S]\)sulfate for 24 hours. As can be seen from Table 1, serum was found to be a very potent stimulator of the expression of \([^{35}S]CSPG\) in monocytes: incubating the cells in medium supplemented with 50% serum made from clotted blood [S(CB)] lead to an almost sevenfold increase in the expression of \([^{35}S]CSPG\), compared with cells incubated in serum-free medium. Heparinized platelet-depleted plasma [PDP(H)] was also found to stimulate the expression of \([^{35}S]CSPG\), but used in the doses of 20% and 50%, PDP(H) was only about half as efficient as S(CB) \((P < .005)\) (Table 1). However, at 5% level PDP(H) and S(CB) were equally efficient stimulators, indicating a low supply of stimulatory factors in such a serum concentration. Heparin alone, in concentrations up to 1 \(\mu\)g/mL, had no significant effect on the expression of \([^{35}S]CSPG\) (data not shown).

When serum prepared from recalcified platelet-rich plasma [S(PR)] was used, the same stimulatory effect on the \([^{35}S]CSPG\) expression as with S(CB) was observed, whereas serum made from recalcified platelet-depleted plasma [S(PDP)] had the same effect as PDP(H) (Table 1). This difference in stimulatory effect between S(PR) and S(PDP) is highly significant \((P < .005)\). When ACD and CaCl\(_2\) were added to the cultures in the same concentrations as used to prepare S(PR) and S(PDP) (see Materials and Methods), the secretion of CSPG was not significantly affected (result not shown). Altogether, these results clearly indicate that the difference between serum and plasma shown in Table 1 is due to factors released from blood platelets activated during the preparation of serum.

To investigate if the increased secretion of CSPG in cells incubated in medium supplemented with S(CB) was due to altered distribution of CSPG between extracellular and intracellular pools, the cell fractions were also harvested and analyzed in some experiments. These experiments showed that after a 24-hour labeling period the amount of cell-associated \([^{35}S]\)macromolecules (a combination of intact CSPG and glycosaminoglycan [GAG] chains) was about 20% of the total amount of \([^{35}S]\)macromolecules (cell fraction plus medium fraction). Further, there was no significant difference in the portion of cell-associated \([^{35}S]\)macromolecules in cells cultured in serum-free medium compared with cells cultured in medium supplemented with 20% S(CB). These results clearly indicate that the increased amount of CSPG secreted from cells cultured in S(CB) is not due to alteration between intracellular and extracellular pools of CSPG, but rather to an increased biosynthesis of CSPG. This is in accordance with what we previously have shown for monocytes stimulated with bacterial lipopolysaccharide and phorbol-12myristate-13acetate.\(^{16}\)

The effect of blood platelets and supernatant from thrombin-stimulated platelets on the expression of \([^{35}S]CSPG\). Subsequent to the medium change on day 6 of the cultivation period, various numbers of freshly isolated, autologous blood platelets were added to the serum-free culture medium, after which the cells were exposed to \([^{35}S]\)sulfate for 24 hours. The incorporation of \([^{35}S]\)sulfate into CSPG increased with increasing number of platelets added to the cultures \((P < .05)\) (Fig 1). The addition of \(3 \times 10^6\) platelets/well, which is about 12 platelets/monocyte, increased the expression of \([^{35}S]CSPG\) by about 2.5 times, whereas the addition of \(20 \times 10^6\) platelets led to a threefold increase. Higher numbers of platelets added to each well only variably increased the expression of \([^{35}S]CSPG\) more than threefold (result not shown). Medium fractions from cultures with only blood platelets did not contain \([^{35}S]\)macromolecules (data not shown), showing that the \([^{35}S]\)CSPG recovered from the cocultures is synthesized exclusively by the monocytes.

As described in Materials and Methods, platelets were stimulated with thrombin, and supernatants from 1, 3, 5, and \(20 \times 10^6\) platelets were added to the serum-free monocyte cultures. However, the supernatants did not stimulate the expression of \([^{35}S]CSPG\) as efficiently as intact platelets when used in corresponding concentrations \((P < .005\) for the concentrations \(3 \times 10^6/5 \times 10^6\), and \(.05 < P < .1\) for the concentrations \(1 \times 10^6/20 \times 10^6\)\) (Fig 1). Supernatant from \(20 \times 10^6\) platelets increased the

| Table 1. Expression of \([^{35}S]CSPG\) in Response to Serum and Plasma

<table>
<thead>
<tr>
<th>Additives</th>
<th>No. of Experiments</th>
<th>([^{35}S]CSPG) Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8</td>
<td>100 (25)</td>
</tr>
<tr>
<td>5% S(CB)</td>
<td>3</td>
<td>203 (27)</td>
</tr>
<tr>
<td>20% S(CB)</td>
<td>5</td>
<td>533 (64)</td>
</tr>
<tr>
<td>50% S(CB)</td>
<td>3</td>
<td>679 (116)</td>
</tr>
<tr>
<td>5% PDP(H)</td>
<td>3</td>
<td>212 (24)</td>
</tr>
<tr>
<td>20% PDP(H)</td>
<td>5</td>
<td>319 (48)</td>
</tr>
<tr>
<td>50% PDP(H)</td>
<td>3</td>
<td>417 (82)</td>
</tr>
<tr>
<td>20% S(PR)</td>
<td>5</td>
<td>502 (23)</td>
</tr>
<tr>
<td>20% S(PDP)</td>
<td>3</td>
<td>351 (31)</td>
</tr>
</tbody>
</table>

Culture medium containing various concentrations of autologous serum made from clotted blood [S(CB)] and PDP(H), as well as serum made from recalcified PRP [S(PR)] and serum made from recalcified platelet-depleted plasma [S(PDP)] were added to monocyte cultures simultaneously as the cells were exposed to \([^{35}S]\)sulfate for 24 hours. The incorporation of \([^{35}S]\)sulfate into CSPG was determined and expressed as percent of controls cultured in medium without serum ("none" under additives). The data given are the mean of three replicates from three or five different experiments, except for S(PR) and S(PDP), which were performed in duplicates. The data in the parentheses represent 1 SD.
expression 2.7 times, whereas $20 \times 10^6$ intact platelets increased the expression 3.1 times. Although the difference in stimulatory effect between supernatant from platelets and intact platelets is marginal, it may indicate that the stimulatory effect of blood platelets is not only due to factors released from the platelets, but that also signals mediated through cell/cell contact might be of importance. Indeed, we found that platelets fixed in 2.5% glutaraldehyde and subsequently washed, had a slight, but statistically significant, stimulatory effect on the expression of $[^{35}S]$CSPG (result not shown).

Thrombin alone, in the concentrations used to stimulate platelets, was not found to stimulate the expression of $[^{35}S]$CSPG (data not shown).

The effect of purified platelet-derived factors on the expression of $[^{35}S]$CSPG. Human serum has been reported to contain about 15 ng/mL of PDGF; by contrast, unclotted platelet-poor plasma contains only about 1 ng/mL of PDGF.\textsuperscript{19} As shown in Table 2, addition of 10 ng/mL of PDGF to the serum-free culture medium enhanced the expression of $[^{35}S]$CSPG twofold ($P < .005$), whereas 1 ng/mL had no significant stimulatory effect. Concentrations of PDGF up to 100 ng/mL did not give any higher level of stimulation than 10 ng/mL. PF 4 was also shown to stimulate the expression of $[^{35}S]$CSPG. At the concentration of $5 \mu$g/mL, which is about the concentration found in normal human serum,\textsuperscript{20} PF 4 enhanced the expression about threefold ($P < .005$). From Table 2 it is evident that PGE2 is also a potent stimulator of the expression of $[^{35}S]$CSPG in monocytes. At the concentration of 350 ng/mL, PGE2 increased the expression of $[^{35}S]$CSPG more than twofold ($P < .005$). In contrast to these three stimulatory factors, TGF-β, used at the physiologic concentration of 10 ng/mL,\textsuperscript{21} was shown to induce a slight reduction in the expression of $[^{35}S]$CSPG ($0.05 < P < .1$), whereas 12-HETE had no effect when used at concentrations up to 320 ng/mL (a concentration found in human serum).

To determine the contribution of PDGF and PF 4 to the stimulatory effect of serum on the expression of $[^{35}S]$CSPG, medium containing PDGF-depleted serum and medium containing PF 4-depleted serum (see Materials and Methods) were added to the monocyte cultures. As can be seen from Table 2, about 25% ($P = .05$) and 35% ($P < .01$) of the difference in effect between serum and plasma could be accounted for by PDGF and PF 4, respectively.

The effect of prostaglandins were also investigated by an indirect method in which blood for serum preparation was collected from the blood donors before and 24 hours after the intake of 600 mg acetylsalicylic acid (ASA). ASA is known to inactivate the cyclo-oxygenase by acetylation. In blood platelets, which have no de novo synthesis of proteins, the inhibition of the enzyme is irreversible. Accordingly, the intake of ASA will inhibit the production of prostaglandins in the platelets. From Table 2 it can be seen that the stimulatory effect of S(CB) was significantly reduced ($P < .005$) when the blood was collected after the intake of ASA, confirming the stimulatory effect of PGE2.

The t½ of ASA in plasma (in vivo) is about 15 minutes.

**Table 2. Expression of $[^{35}S]$CSPG in Response to Platelet-Derived Factors**

<table>
<thead>
<tr>
<th>Additives</th>
<th>No. of Experiments</th>
<th>$[^{35}S]$CSPG Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>23</td>
<td>100 (30)</td>
</tr>
<tr>
<td>PDGF 1 ng/mL</td>
<td>3</td>
<td>118 (31)</td>
</tr>
<tr>
<td>PDGF 10 ng/mL</td>
<td>3</td>
<td>206 (57)</td>
</tr>
<tr>
<td>PF 4 10 ng/mL</td>
<td>5</td>
<td>103 (34)</td>
</tr>
<tr>
<td>PF 4 100 ng/mL</td>
<td>5</td>
<td>171 (63)</td>
</tr>
<tr>
<td>PF 4 1,000 ng/mL</td>
<td>5</td>
<td>204 (64)</td>
</tr>
<tr>
<td>PF 4 5,000 ng/mL</td>
<td>5</td>
<td>209 (85)</td>
</tr>
<tr>
<td>PGE2 3.5 ng/mL</td>
<td>2</td>
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</tr>
<tr>
<td>PGE2 35 ng/mL</td>
<td>2</td>
<td>161 (34)</td>
</tr>
<tr>
<td>PGE2 350 ng/mL</td>
<td>2</td>
<td>219 (46)</td>
</tr>
<tr>
<td>PGE2 3,500 ng/mL</td>
<td>2</td>
<td>224 (33)</td>
</tr>
<tr>
<td>TGF-β 1 ng/mL</td>
<td>3</td>
<td>91 (24)</td>
</tr>
<tr>
<td>TGF-β 10 ng/mL</td>
<td>3</td>
<td>78 (16)</td>
</tr>
<tr>
<td>12-HETE 3.2 ng/mL</td>
<td>2</td>
<td>104 (23)</td>
</tr>
<tr>
<td>12-HETE 32 ng/mL</td>
<td>2</td>
<td>116 (21)</td>
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<td>12-HETE 320 ng/mL</td>
<td>2</td>
<td>101 (39)</td>
</tr>
<tr>
<td>20% S(CB) without PDGF</td>
<td>5</td>
<td>590 (75)</td>
</tr>
<tr>
<td>20% S(CB) without PF 4</td>
<td>2</td>
<td>514 (38)</td>
</tr>
<tr>
<td>20% S(CB) after ASA</td>
<td>5</td>
<td>478 (31)</td>
</tr>
<tr>
<td>20% PDP(h)</td>
<td>2</td>
<td>378 (77)</td>
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Different concentrations of PDGF, TGF-β, PF 4, PGE2, and 12-HETE, as well as S(CB), S(CB) without PDGF, and S(CB) without PF 4 were added to monocyte cultures. Further, S(CB) was prepared from blood collected before or 24 hours after the administration of ASA to the blood donor, and added to the monocyte cultures. The cells were then exposed to $[^{35}S]$sulfate for 24 hours. The incorporation of $[^{35}S]$sulfate into CSPG was determined and expressed as percent of controls cultured in serum-free medium alone ("none" under additives). The data are the mean of three replicates from two to five different experiments. The data in the parentheses represent 1 SD.
When the blood was collected 24 hours after the intake of ASA, there should therefore be virtually no ASA left in the plasma. The reduced stimulatory effect of S(CB) after intake of ASA can therefore hardly be due to any direct effect of ASA on the monocytes.

Structure of the \[^{35}S\]CSPG synthesized. The structure and the molecular size of the \[^{35}S\]labeled macromolecules was analyzed by Sepharose CL-6B gel chromatography. As shown in Fig 2, the proteoglycan nature of the \[^{35}S\]labeled macromolecules could be shown by a shift in elution pattern after alkali treatment (which results in the liberation of free \[^{35}S\]GAG chains). There was no difference in molecular size between molecules recovered from serum-free cultures and those recovered from cultures with 50% S(CB). Furthermore, more than 95% of the \[^{35}S\]labeled macromolecules from both types of cultures were degraded with chondroitin ABC lyase, demonstrating the chondroitin sulfate nature of the proteoglycan molecules (data not shown).

The possibility that the increased \[^{35}S\]sulfate incorporation observed in cultures grown in the presence of serum (or platelet-derived factors) was due to increased sulfation of the GAG-chains was investigated by subjecting the disaccharides obtained after chondroitin ABC lyase digestions to high-voltage paper electrophoresis. As can be seen from Fig 3, the disaccharides obtained from both serum-free cultures and cultures grown in 50% S(CB) were composed of a mixture of about 90% \(\Delta Di-4S\) and about 10% \(\Delta Di-diS\) (on a molar basis). Because nonsulfated disaccharides not were detected in this experiment, the data do not show whether or not S(CB) stimulate the formation of monosulfated disaccharides. Therefore, monocytes cultured in the absence or presence of S(CB), were exposed to both \[^{35}S\]sulfate and \[^{3H}\]glucosamine. The double-labeled disaccharides, obtained after chondroitin ABC lyase digestion and ultrafiltration, were then analyzed by HPLC. This showed that less than 0.5% of the disaccharides were nonsulfated. Further, the ratio between nonsulfated (\(\Delta Di-0S\)), monosulfated (\(\Delta Di-4S\)), and disulfated (\(\Delta Di-diS\)) disaccharides was the same in material derived from monocytes cultured in the absence or presence of S(CB). The increased expression of \[^{35}S\]CSPG observed in cultures grown in the presence of serum (or platelet-derived factors present in serum) is, accordingly, not due to increased sulfation or increased molecular size of the CSPG synthesized, but rather to the expression of an increased number of proteoglycan molecules.

**DISCUSSION**

Mononuclear phagocytes synthesize CSPG, which is constitutively secreted when the cells are cultured in vitro. We have previously reported that the expression of CSPG in mononuclear phagocytes is modulated by classical monocyte/macrophage-activating agents such as bacterial lipopolysaccharide, interferon-γ, and phorbol esters. In the present study, we show that serum is a potent stimulator of the expression of CSPG in monocytes, and that serum made...
from clotted blood has a much higher stimulatory effect than plasma. Further, serum made from PRP had almost the same stimulatory effect as serum made from clotted blood, whereas the stimulatory effect of serum made from PDP was at the level of plasma. These results clearly indicate that the difference in stimulatory effect between serum made from clotted blood and plasma is due to factors released from blood platelets during the preparation of serum. According to these initial results we studied the effect of isolated platelets, as well as several purified platelet-derived products, on the expression of CSPG in monocytes.

PF 4 has previously been shown to be chemotactic for monocytes. Although somewhat controversial, PDGF has also been shown to be chemotactic for monocytes, as well as to induce superoxide anion and lysozyme release. Our results show that both PF 4 and PDGF, when used in physiologic concentrations, stimulate the expression of proteoglycan twofold to threefold in cultured monocytes.

TGF-β has previously been shown to be a potent stimulator of proteoglycan synthesis in various mesenchymal and epithelial cells. However, in the present study we show that TGF-β induces a small decrease in the expression of proteoglycan in monocytes. The ability of TGF-β to stimulate proteoglycan expression is, accordingly, dependent on the type of target cell. It is noteworthy in this context that TGF-β recently has been shown to suppress the release of H₂O₂ and to downregulate the production of interleukin-6 (IL-6) in activated macrophages, indicating that TGF-β induces “deactivation” of mononuclear phagocytes.

Several studies have focused on the effect of PGE₂ on mononuclear phagocytes, and PGE₂ has been shown to have both stimulatory and inhibitory effects. It is now established that PGE₂ inhibits the expression of major histocompatibility complex (MHC) class II antigens and also inhibits the production of IL-1 and tumor necrosis factor (TNF). Other studies have shown that PGE₂ stimulates the production of collagenase and tissue thromboplastin in mononuclear phagocytes. In all these studies, the effect of PGE₂ was found to be dependent on additional signals, because PGE₂ alone had no effect. With respect to biosynthesis of proteoglycans, PGE₂ has been shown to have a minor stimulatory effect on aortic smooth muscle cells and granulosa cells. In the present study, we added PGE₂ to serum-free medium without any other known stimulating agents. Under these conditions, we found that physiologic concentrations of PGE₂ stimulated the expression of CSPG more than twofold. Additional evidence for the stimulatory effect of prostaglandins was the fact that administration of a cyclo-oxygenase inhibitor to the blood donors before the preparation of serum significantly reduced the stimulatory effect of serum.

In contrast to PGE₂, relatively few studies have dealt with the effect of 12-HETE on mononuclear phagocytes. However, 12-HETE has been reported to enhance procoagulant tissue factor activity in endotoxin-stimulated mononuclear leukocytes. With respect to the expression of proteoglycan, our results show that 12-HETE has no effect in cultured monocytes.

Although being particularly abundant in platelets, both PDGF, TGF-β, and PGE₂ are also released from mononuclear phagocytes. The modulatory effects of these factors might therefore imply that monocytes have the ability to modulate their own expression of CSPG by autocrine mechanisms. It is interesting to note that only TGF-β induced a decrease in proteoglycan expression in monocytes. Because this agent is known as a “deactivator” of mononuclear phagocytes, a deactivation might be correlated to a decrease in the level of CSPG released from these cells. In contrast, previous data and results presented here show that stimulation is correlated to increased release of CSPG. It therefore seems likely that release of proteoglycan from monocytes may be important for inflammatory responses or other processes involving stimulated mononuclear phagocytes, such as the development of atherosclerosis. A key event in this process is the accumulation of low-density lipoprotein (LDL) molecules may be modified in the arterial wall and that the modified LDL is internalized by the scavenger receptor. However, it has also been suggested that LDL is complexed to proteoglycans in the arterial wall, and that the LDL/proteoglycan complexes are internalized by the macrophages. Recently, it has been shown that the macrophage-like cell line P388D₁ synthesizes a CSPG that binds specifically to LDL. This may indicate that CSPG secreted from mononuclear phagocytes in atherosclerotic lesions is complexed to LDL and subsequently internalized by the mononuclear phagocytes.

The role of blood platelets as recruiter of mononuclear phagocytes to the atherosclerotic lesions has been known for several years. In the present study, we show that blood platelets also have the capability to stimulate the expression of proteoglycan in mononuclear phagocytes. Although the biologic functions of the CSPG secreted from mononuclear phagocytes remains to be established, the stimulatory effect of blood platelets may have implications for both physiologic and pathologic processes.

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Blood platelets stimulate the expression of chondroitin sulfate proteoglycan in human monocytes

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