Homocysteine Inhibits Proteoglycan Synthesis in Cultured Bovine Aortic Smooth Muscle Cells

Yasuyuki Fujiwara, Chihiro Mikami, Michinori Nagai, Chika Yamamoto, Takashi Hirooka, Masahiko Satoh, and Toshiyuki Kaji

Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University, 1–100 Kusumoto-cho, Chikusa-ku, Nagoya, Aichi 464–8650, Japan, Department of Environmental Health, Faculty of Pharmaceutical Sciences, and Organization for Frontier Research in Preventive Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920–1181, Japan

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Homocysteine is a risk factor for vascular diseases such as atherosclerosis, and proteoglycans (PGs) derived from arterial smooth muscle cells are the key molecules in atherosclerosis progression. We investigated the effect of homocysteine on the synthesis of PGs in vascular smooth muscle cells in vitro. Homocysteine significantly decreased the accumulation of PGs, particularly in the conditioned medium, independent of cell density without non-specific cell damage. DEAE-Sephacel ion exchange chromatography of PGs showed that homocysteine selectively decreases chondroitin/dermatan sulfate PGs (CS/DSPGs) with low charge density, i.e., the small CS/DSPG biglycan and decorin, in the conditioned medium. Fluorophore-assisted carbohydrate electrophoresis analysis demonstrated that homocysteine selectively increases the disaccharide unit of iduronic acid-4-0-sulfated N-acetylgalactosamine in chondroitin/dermatan sulfate chains. However, no change was observed in heparan sulfate chains. Therefore, it is suggested that homocysteine inhibits the synthesis of small CS/DSPGs with low charge density in vascular smooth muscle cells. Furthermore, homocysteine affects the microstructure of the chondroitin/dermatan sulfate chains. These alterations may influence the progression of atherosclerosis.

Key words —— homocysteine, vascular smooth muscle cell, proteoglycan, glycosaminoglycan, atherosclerosis

INTRODUCTION

Proteoglycans (PGs) are macromolecules that consist of a core protein and one or more glycosaminoglycan (GAG) side chains such as chondroitin sulfate, dermatan sulfate, and heparan sulfate. These macromolecules are prominent constituents of both the extracellular matrix and the cell surface and are involved in the formation of the extracellular matrix, cell adhesion, and cytokine/growth factor interactions.1–5) Vascular PGs have been implicated in vascular properties such as viscoelasticity, permeability, lipid metabolism, hemostasis, and thrombosis as well as in the pathogenesis of vascular diseases, including atherosclerosis.4–6) Vascular smooth muscle cells are the major cell type in the arterial wall; they synthesize and secrete a large chondroitin sulfate PG (CSPG), versican,7) and small leucine-rich chondroitin/dermatan sulfate PGs (CS/DSPGs), decorin and biglycan,8,9) as well as a large heparan sulfate PG (HSPG), perlecan.10)

PGs excessively accumulate in the atherosclerotic vascular wall. The type of PGs and the microstructure of GAG chains vary; this alteration is postulated to be due to the regulation of PG synthesis by growth factors/cytokines7,9) and other factors such as thrombin.11)

Homocysteine, an intermediate compound formed during the metabolism of methionine, is normally present in the plasma of healthy people at up to 10 µM concentration; however, its plasma concentration can reach up to 500 µM and higher in patients suffering from hyperhomocysteinemia.12,13) Hyperhomocysteinemia is caused by genetic factors such as homozygous deficiency of cystathionine β-synthase12,14) and acquired conditions, including deficiency of folic acid, vitamin B6 and B12, impaired renal function, and several
medications. Elevated blood levels of homocysteine have been identified as an independent risk factor for atherosclerotic diseases in coronary, cerebral, and peripheral vessels. Moreover, there is evidence that hyperhomocysteinemia-induced atherosclerosis may be due to endothelial cell injury or dysfunction, activation of the coagulation system, and stimulation of proliferation, migration, and collagen production in vascular smooth muscle cells. In addition, it has been shown that homocysteine induces HSPG dysfunction in endothelial cells, resulting in decreased binding of antithrombin III and extracellular superoxide dismutase to the cell surface. Thus, it is possible that homocysteine influences PG synthesis and/or GAG chain formation in vascular smooth muscle cells.

The present study was undertaken to determine whether homocysteine alters PG synthesis and GAG chain formation in vascular smooth muscle cells. We found that homocysteine selectively inhibits the synthesis of small CS/DSPGs and alters the disaccharide composition of the GAG chains.

**MATERIALS AND METHODS**

**Materials** — Vascular smooth muscle cells derived from bovine aorta were gift from Dr. Yasuo Suda (Kagoshima University Graduate School of Sciences and Engineering, Kagoshima, Japan). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Tissue culture dishes and plates were purchased from Iwaki (Chiba, Japan). Fetal bovine serum (FBS), [35S]Na2SO4 (carrier free) and [methyl-3H]thymidine (2.7 TBq/mmol) were obtained from MP Biomedicals (Irvine, CA, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, and the cells were sub-cultured until confluence. They were transferred into 24-well culture plates at a density of 5 x 10^3 cells/cm^2 and further cultured for 24 hr (“sparse culture”) in DMEM supplemented with 10% FBS. After washing the cells with DMEM supplemented with 10% FBS, the medium was replaced with fresh DMEM supplemented with 10% FBS, and the cells were subsequently incubated at 37°C for 8, 24, or 48 hr with homocysteine (0.1, 0.2, 0.5, 1, or 2 mM) combined with or without copper sulfate (10 μM) in the presence of [35S]sulfate (1 MBq/ml). After incubation, the conditioned medium was harvested, and solid urea was added up to a concentration of 8 M. The cell layer was washed with ice-cold Ca2+- and Mg2+-free phosphate-buffered saline (CMF-PBS) and extracted with an 8 M urea solution containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 10 mM N-ethylmaleimide, 2 mM EDTA, 0.1 M phenylmethylsulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100 (pH 7.5) at 4°C for 15 min, and the cell extraction solution was harvested. The medium and cell extracts were used to determine the incorporation of [35S]sulfate into the GAGs by the CPC precipitation method as follows. Portions of the extracts were spotted on filter papers and washed 5 times for 1 hr in 1% CPC with 0.05 M NaCl. The radioactivity of the PGs pre-
cipitated on the dried filter paper was measured by liquid scintillation counting.

**DEAE-Sepharose Ion Exchange Chromatography** — Vascular smooth muscle cells cultured in 60-mm dishes were treated with homocysteine (1 mM) for 48 hr in the presence of [35S]sulfate (2 MBq/ml) and copper sulfate (10 µM). After treatment, the conditioned medium was harvested, and solid urea was added up to a concentration of 8 M. The medium was chromatographed on PD-10 columns equilibrated in 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.25 M NaCl, 0.5% Triton X-100, and 50 mM Tris base. Any unbound radioactivity was removed from the column by washing with 30 ml of the buffer. The bound radioactivity was eluted from the column with a linear gradient of 0.25–0.7 M NaCl in urea buffer (total volume of 50 ml).

**Analysis of Disaccharide Composition of GAGs** — Vascular smooth muscle cells cultured in 100-mm dishes were treated with homocysteine (1 mM) for 48 hr in the presence of copper sulfate (10 µM). After treatment, the conditioned medium was harvested, and solid urea was added up to a concentration of 8 M. Proteoglycans that accumulated in the conditioned medium were concentrated on 0.3-ml DEAE-Sepharose mini-columns and precipitated with 3.5 volumes of 0.01% bovine serum albumin and digested at 37°C for 18 hr with both heparinase II and III (30 mU/ml, each). The heparan sulfate hydrolase products were recovered using Microcon 3 ultrafiltration devices and subsequently dried. The dried heparan sulfate and chondroitin/dermatan sulfate samples were fluorotagged separately at 90°C for 30 min with 2-aminoacridone hydrochloride (0.1 M) as described previously.26,27 The fluorotagged disaccharide units of heparan sulfate were immediately separated on the separating gels (19.5% acrylamide, 0.52% N, N'-methylenbisacylamide, 2.5% glycerol, 0.1 M Tris base, 0.09 M boric acid, 0.05% ammonium persulfate, and 0.1% TEMED) with a stacking gel (7.5% acrylamide, 0.2% N, N'-methylenbisacylamide, 2.5% glycerol, 0.1 M Tris base, 0.09 M boric acid, 0.05% ammonium persulfate, 0.6% agarose, and 0.1% TEMED). Similarly, the fluorotagged disaccharide units of chondroitin/dermatan sulfate were separated on the separating gels (19.5% acrylamide, 0.52% N, N'-methylenbisacylamide, 2.5% glycerol, 0.05% ammonium persulfate, 0.1% TEMED, and 0.045 M Tris-acetate buffer of pH 7.0) with a stacking gel (7.5% acrylamide, 0.2% N, N'-methylenbisacylamide, 2.5% glycerol, 0.05% ammonium persulfate, 0.6% agarose, 0.1% TEMED, and 0.045 M Tris-acetate buffer of pH 7.0). Electrophoresis was carried out in a running buffer (0.1 M Tris-borate, pH 8.3) at 4°C. The fluorescent images were obtained using a gel documentation system (Atto, AE-6914). The bands of unsaturated heparan sulfate and chondroitin/dermatan sulfate disaccharides were quantitatively analyzed by National Institutes of Health Image analysis software that uses the bands of GlcA-GalNAc(6S) and gal6S, respectively, as internal standards. The disaccharides were identified by comigration by comparison with unsaturated disaccharide standards. The following disaccharide units generated by digestion of heparinase II and III were detected: glucuronic acid/iduronic acid- N-acetylgalcosamine (GlcA/IdoA-GlcNAc), glucuronic acid/iduronic acid- N-sulfated glucosamine (GlcA/IdoA-GlcNS), and 2-O-sulfated glucuronic acid/iduronic acid- N-sulfated glucosamine [GlcA/IdoA(2S)-GlcNS]. In chondroitin/dermatan sulfate chains, the following disaccharide units were generated by digestion with chondroitinase ABC and ACII: unsaturated glucuronic acid/iduronic acid- N-acetylgalactosamine (GlcA/IdoA-GalNAc), glucuronic acid/iduronic acid-4-O-sulfated-N-acetylgalactosamine [GlcA/IdoA-GalNAc(4S)],
glucuronic acid/iduronic acid-6-O-sulfated-N-acetylgalactosamine [GlcA/IdoA-GalNAc(6S)], and glucuronic acid/iduronic acid-4-O-,6-O-sulfated-N-acetylgalactosamine [GlcA/IdoA-GalNAc(4S,6S)]. The amounts of iduronic acid-containing disaccharide units, IdoA-GalNAc(4S), derived from dermatan sulfate were estimated by subtracting the amount of disaccharide units generated by digestion with chondroitinase ACII alone from that of the corresponding disaccharide units generated by digestion with both chondroitinase ABC and ACII.

**Determination of Cell Number and Leakage of LDH** —— Dense cultures of vascular smooth muscle cells were prepared in 24-well plates and incubated at 37°C for 48 hr with homocysteine (0.5, 1, or 2 mM) in the presence of copper sulfate (10 µM). After incubation, the medium was collected and used for the determination of LDH activity as a marker of nonspecific cell damage. The cell layer was washed twice with CMF-PBS and then dispersed using 0.25% trypsin-0.02% EDTA in CMF-PBS; the trypsinized cell suspension was then harvested. The cultured well was washed with CMF-PBS containing 10% FBS, and the washings were pooled with the corresponding cell suspension. The harvested cell suspension was used for the determination of cell number by a particle counter (Sysmex CDA-500).

**Incorporation of [3H]Thymidine** —— Dense cultures of vascular smooth muscle cells were treated with homocysteine (0.5, 1, or 2 mM) in the presence of copper sulfate (10 µM) in 6-well culture plates and labeled with [3H]thymidine (20 kBq/ml) during the last 6 hr of the treatment. After labeling, the medium was discarded, and the cell layer was washed twice with CMF-PBS. The cells were harvested with a rubber policeman in the presence of CMF-PBS. Cell homogenate was prepared by sonication, and incorporation of radioactivity into the 5% trichloroacetic acid-insoluble fraction of the cell homogenate was measured by liquid scintillation counting. A portion of the cell homogenate was used for the determination of DNA content by the fluorometric method28) to express the incorporated radioactivity as dpm/µg DNA.

**Statistical Analysis** —— Data were analyzed for statistical significance by analysis of variance and Bonferroni’s multiple t-test, where possible. P values of less than 0.05 were considered to indicate statistically significant differences.

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**RESULTS**

Figure 1 shows the incorporation of [35S]sulfate into PGs accumulated in the cell layer and conditioned medium of dense and sparse vascular smooth muscle cells after exposure to homocysteine in the presence or absence of copper sulfate. After a 48-hr incubation period, [35S]sulfate incorporation was significantly decreased by homocysteine (2 mM and below) in the cell layer and conditioned medium of both dense and sparse cells in a concentration-dependent manner. Since several studies have shown that copper ion augments the effect of homocysteine,18, 20, 24) we tested the effect of copper sulfate on the inhibition of PG synthesis by homocysteine and observed that copper sulfate enhances the inhibitory effect of homocysteine (Fig. 1). In a time course study, the decrease in

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![Fig. 1. The Incorporation of [35S]Sulfate into GAGs Accumulated in the Cell Layer and Conditioned Medium of Dense and Sparse Vascular Smooth Muscle Cells after Exposure to Homocysteine](image-url)
Fig. 2. Time Course of the Incorporation of $[^{35}\text{S}]$Sulfate into GAGs Accumulated in the Cell layer and Conditioned Medium of Dense and Sparse Vascular Smooth Muscle Cells after Exposure to Homocysteine

Dense (upper panels) and sparse (lower panels) cultures of bovine aortic smooth muscle cells were incubated at 37°C for 8, 24, or 48 hr with homocysteine (2 mM) in the presence of copper sulfate (10 µM) and $[^{35}\text{S}]$sulfate (1 MBq/ml). Values are means ± S.E. of 4 samples. **Significantly different from the corresponding control, $p < 0.01$.

$[^{35}\text{S}]$sulfate incorporation in both dense and sparse cells occurred after 8 hr and longer when the cells were treated with both homocysteine and copper sulfate (Fig. 2). These results suggest that homocysteine inhibits complete PG synthesis in vascular smooth muscle cells independent of the cell density and that the inhibitory effect is enhanced by copper ion.

The $[^{35}\text{S}]$sulfate-labeled PGs derived from the conditioned medium of dense vascular smooth muscle cells treated with homocysteine in the presence of copper sulfate were applied to a DEAE-Sephacel column to separate the PGs into HSPGs and CS/DSPGs based on charge density differences (Fig. 3). In cells with or without homocysteine treatment, the incorporated $[^{35}\text{S}]$sulfate radioactivity was eluted from the column by the NaCl gradient in 3 peaks, at approximately 0.40, 0.48, and 0.55 M NaCl (peaks I, II, and III, respectively). Peaks I, II, and III have been previously confirmed to contain a large HSPG, perlecan; small CS/DSPGs, decorin and biglycan; and a large CSPG, versican, respectively.11,29,30) It was revealed that homocysteine particularly decreased the radioactivity of peak II in the conditioned medium, suggesting the selective inhibition of decorin and/or biglycan synthesis.

It is possible that homocysteine not only inhibits the biglycan/decorin core protein synthesis but also influences the formation of GAG chains during the PG synthesis. The disaccharide composition of heparan sulfate and chondroitin/dermatan sulfate chains synthesized by vascular smooth muscle cells before and after treatment with homocysteine was measured by fluorophore-assisted carbohydrate electrophoresis (Fig. 4). In the conditioned medium, the disaccharide units of heparan sulfate chains were detected as unsaturated GlcA/IdoA-GlcNAc, GlcA/IdoA-GlcNS, and GlcA/IdoA(2S)-GlcNS (Fig. 4A). The percentages of heparan sulfate disaccharide units calculated based on the gel image were unaffected by homocysteine (Table 1). On the other hand, chondroitin/dermatan sulfate disaccharide units were detected as unsaturated GlcA/IdoA-GalNAc, GlcA-GalNAc(4S), IdoA-GalNAc(4S), GlcA/IdoA-GalNAc(6S), and GlcA/IdoA-GalNAc(4S,6S) (Fig. 4B). Homocysteine increased the percentage of 4-O-sulfated disaccharide units, in particular IdoA-GalNAc(4S), with a decrease in the percentage of GlcA/IdoA-GalNAc(6S); this resulted in a decrease in the ratio of [GlcA/IdoA-GalNAc(6S)] to [GlcA/IdoA-
Dense cultures of bovine aortic smooth muscle cells were incubated at 37°C for 48 hr with homocysteine (1 mM) in the presence of copper sulfate (10 µM). A representative gel image is shown.

**Table 1.** Disaccharide Composition of Heparan Sulfate Chains Accumulated in the Conditioned Medium of Dense Vascular Smooth Muscle Cells after Exposure to Homocysteine

<table>
<thead>
<tr>
<th>Unsaturated disaccharide unit</th>
<th>Control (%)</th>
<th>Homocysteine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcA/IdoA-GlcNAc</td>
<td>53.7</td>
<td>54.7</td>
</tr>
<tr>
<td>GlcA/IdoA-GlcNS</td>
<td>29.6</td>
<td>27.3</td>
</tr>
<tr>
<td>GlcA/IdoA(2S)-GlcNS</td>
<td>16.7</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Digestion products separated by fluorophore-assisted carbohydrate electrophoresis gels (see Fig. 4A) were quantitated using NIH image software.

**Table 2.** Disaccharide Composition of Chondroitin/Dermatan Sulfate Chains Accumulated in the Conditioned Medium of Dense Vascular Smooth Muscle Cells after Exposure to Homocysteine

<table>
<thead>
<tr>
<th>Unsaturated disaccharide unit</th>
<th>Control (%)</th>
<th>Homocysteine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcA/IdoA-GalNAc</td>
<td>3.7</td>
<td>3.5</td>
</tr>
<tr>
<td>GlcA-GalNAc(4S)</td>
<td>25.1</td>
<td>27.8</td>
</tr>
<tr>
<td>IdoA-GalNAc(4S)</td>
<td>29.8</td>
<td>34.3</td>
</tr>
<tr>
<td>GlcA/IdoA-GalNAc(6S)</td>
<td>37.6</td>
<td>30.2</td>
</tr>
<tr>
<td>GlcA/IdoA-GalNAc(4S,6S)</td>
<td>3.8</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Digestion products separated by fluorophore-assisted carbohydrate electrophoresis gels (see Fig. 4B) were quantitated using NIH image software. Serum contains a small amount of chondroitin/dermatan sulfate. Therefore, the amount of disaccharide units derived from chondroitin/dermatan sulfate produced by vascular smooth muscle cells was calculated by subtracting the amount of corresponding disaccharides in the fresh culture medium from that in the conditioned medium.

Figure 5 shows the cell number, incorporation of [3H]thymidine into acid-insoluble fraction, and leakage of LDH in dense vascular smooth muscle cells treated with homocysteine at 2 mM or less for 48 hr. Homocysteine did not influence the cell number and LDH leakage, indicating that the inhibition of PG synthesis in the cells was neither due to decrease in the cell number nor due to nonspecific cell damage. The dose-dependent increase in [3H]thymidine incorporation suggests a stimulation of the cell growth by homocysteine without causing any change in the cell number.

**DISCUSSION**

Vascular PGs, particularly CS/DSPGs, accumulate in the atherosclerotic vascular wall with alterations in their GAG composition. Vascular smooth muscle cells are the predominant cell type responsible for these alterations. However, regulation of PG synthesis in vascular smooth muscle cells has been incompletely understood. Platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), 2 growth factors implicated in the pathogenesis of atherosclerosis, induce the synthesis of versican and biglycan with modification of their chondroitin/dermatan sulfate chains. We demonstrated that lead and cadmium, possible risk factors of atherosclerosis, influence PG synthesis...
Lead selectively inhibits the synthesis of versican in vascular smooth muscle cells at a high cell density, whereas cadmium specifically induces inhibition of biglycan and decorin synthesis at a low cell density; however, both metals do not alter the microstructure of GAG chains. In the present study, it was suggested that homocysteine significantly and selectively inhibits PG synthesis in vascular smooth muscle cells independent of the cell density without decrease in the cell number and nonspecific cell damage. In addition, it was also suggested that homocysteine influences the sulfation during the formation of dermatan sulfate chains in biglycan/decorin—small CS/DSPGs expressed in vascular smooth muscle cells. Homocysteine may contribute to the abnormal metabolism of PGs during atherosclerosis together with PDGF and TGF-β.

Homocysteine damages endothelial cells through copper-catalyzed generation of hydrogen peroxide. It also inhibits the binding of antithrombin III to the endothelial cell surface HSPGs by producing hydrogen peroxide. Furthermore, homocysteine has been shown to decrease the binding of extracellular superoxide dismutase to the endothelial cell surface due to modification of heparan sulfate chain microstructure; this effect of homocysteine is augmented in the presence of copper ion. The inhibitory effect of homocysteine on PG synthesis in vascular smooth muscle cells was enhanced in the presence of copper ion, suggesting that the inhibition is mediated by hydrogen peroxide. Recently, homocysteine was shown to target intracellular metallothionein by forming a mixed-disulfide conjugate, resulting in the loss of activities of vascular endothelial cells. Since metallothionein detoxifies heavy metals, regulates zinc/copper homeostasis, and scavenges reactive oxygen species, it is possible that inhibition of PG synthesis in vascular smooth muscle cells by homocysteine may result from imbalance of zinc and redox homeostasis.

Arterial CS/DSPGs are involved in various events occurring in the vascular wall, including collagen fibrinogenesis, retention of cytokines and lipoproteins, and activation of heparin cofactor II. Chondroitin/dermatan sulfate chains have high affinity for low-density lipoprotein (LDL), and versican and biglycan are responsible for the accumulation of LDL in the atherosclerotic vascular wall. The PG-LDL complexes in the atherosclerotic plaques enhance the uptake of LDL by macrophages. In addition, both versican and biglycan promote the proliferation of vascular smooth muscle cells. These results suggest that versican and biglycan contribute to atherosclerosis progression through LDL accumulation and vascular smooth muscle cell hyperplasia in expanded intima. On the other hand, another small CS/DSPG, decorin, is postulated to suppress atherosclerosis progression by inhibiting TGF-β, suppressing cell migration, and activating heparin cofactor II. Furthermore, overexpression of decorin prevents the intimal thickening in the vascular wall of apolipoprotein E-deficient mice, an experimental animal model of atherosclerosis. We did not identify the type of CS/DSPGs whose synthesis was inhibited by homocysteine. However, it is postulated that homocysteine might inhibit decorin synthesis because biglycan was not accumulated in the cell.
layer in our system (homocysteine decreased cell layer-associated PGs). The mechanism by which homocysteine promotes atherosclerosis progression may include inhibition of decorin synthesis.

In summary, the present data suggest that homocysteine inhibits the synthesis of CS/DSPGs in arterial smooth muscle cells independent of the cell density. Specifically, homocysteine inhibits the synthesis of small CS/DSPGs such as decorin along with stimulation of 4-O-sulfation during the formation of chondroitin/dermatan sulfate chains. The mechanisms by which homocysteine induces vascular diseases such as atherosclerosis and thrombosis may include inhibition of decorin synthesis and alteration of the microstructure of dermatan sulfate chains in vascular smooth muscle cells.

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