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Stimulation of Proteoglycan Release from Cultured Vascular Endothelial Cell Layers by Sodium Spirulan

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Our previous study suggested that sodium spirulan (Na-SP) stimulated the release of proteoglycans from cultured vascular endothelial cell layers. In the present study, we characterized the stimulatory effect of Na-SP on endothelial proteoglycan release from the viewpoint of the Na-SP structure. The following results were obtained: Na-SP is a potent stimulator of endothelial proteoglycan release. The stimulatory effect of Na-SP disappears with desulfation or removal of sodium ion. Depolymerized Na-SPs (M_r ~14700 or greater) also exhibits stimulatory effects as does Na-SP (M_r ~220000). The stimulation occurs more potently in endothelial cells than in vascular smooth muscle cells. Therefore it is suggested that the stimulatory effect of Na-SP on proteoglycan release requires both the sulfate group and sodium ion, and the effect is achieved by an active structure in the molecule which interacts particularly with the endothelial cell extracellular matrix.

Key words —— sodium spirulan, endothelial cell, proteoglycan, extracellular matrix, vascular smooth muscle

INTRODUCTION

Sodium spirulan (Na-SP) is a sulfated polysaccharide with M_r ~220000 isolated from a hot-water extract of the blue-green alga *Spirulina platensis*.[1,2] The molecule of Na-SP contains two types of disaccharide repeating units, *O*-hexauronosyl-rhamnose (aldobiuronic acid) as the backbone structure and *O*-rhamnosyl-3-*O*-methylrhamnose (acofriose) with sulfate groups as the side chains.[3] The Na-SP molecule also contains sodium ions bound to sulfate groups, which may contribute to the maintenance of the molecular conformation.

Na-SP interacts with blood components and arterial cells. The polysaccharide exhibits antithrombin activity by activation of heparin cofactor II in a manner different from that of heparin.[4] Na-SP enhances the fibrinolytic activity in the liquid phase of cultured human coronary endothelial cells through inhibition of plasminogen activator inhibitor type 1 secretion.[5] Recent study has shown that Na-SP is a potent inhibitor of cultured vascular smooth muscle cell proliferation.[6] Those results indicate that Na-SP has anticoagulant, fibrinolytic, and antiatherogenic properties *in vitro*.

Vascular endothelial cells cover the inner surface of the blood vessel and exhibit anticoagulant activities by synthesizing and secreting anticoagulant and fibrinolytic substances. For example, the cells synthesize and secrete anticoagulant proteoglycans (PGs) such as the large heparan sulfate PG perlecan and the small chondroitin/dermatan sulfate PG biglycan, which exhibit antithrombin activity through activation of antithrombin III and heparin cofactor II by perlecan and biglycan, respectively.[7,8]

The release of PGs from vascular endothelial cells can contribute to the local anticoagulant activity in the liquid phase on the vascular endothelium. Endothelial PG release is promoted by thrombin,[9] plasmin,[10] and activation of the protein kinase C pathway.[11] In the previous study, we suggested that Na-SP promotes the release of PGs from cultured vascular endothelial cell layers.[12] The present study was undertaken to investigate the effects of Na-SP

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on the stimulation of PG release from cultured bovine aortic endothelial cells.

MATERIALS AND METHODS

Materials —— Vascular endothelial cells from bovine aorta were purchased from Dainippon Pharmaceutical (Osaka, Japan). Vascular smooth muscle cells from bovine aorta were a gift from Professor Yasuo Suda (Kagoshima University Graduate School of Science and Engineering, Kagoshima, Japan). The method for isolation and purification of Na-SP, removal of sodium ion from Na-SP (*i.e.,* preparation of H-SP), replacement of sodium ion by calcium ion (*i.e.*, preparation of Ca-SP), desulfation of Na-SP (*i.e.*, preparation of desulfated-SP) and depolymerization of Na-SP (*i.e.*, preparation of depolymerized Na-SP) were described previously.[1,2,12–15] Dulbecco's modified Eagle's medium and fetal bovine serum were from Nissui Pharmaceutical (Tokyo, Japan) and Equitech-Bio (Kerrville, TX, U.S.A.), respectively. ASF 301 medium was from Ajinomoto (Tokyo, Japan). Tissue culture plates and dishes were from Iwaki (Chiba, Japan). [^{35}S]$NaSO_4$ (carrier free) was from MP Biomedicals (Irvine, CA, U.S.A.). Heparin derived from porcine intestine (M_r ~10000) was from Sigma (St. Louis, MO, U.S.A.); chemically modified heparin derived from porcine intestine (desulfated heparin, *O*-desulfated heparin, and *N*-desulfated heparin, M_r ~10000 each), hyaluronan derived from porcine skin (M_r 100000–150000), pullulan (M_r ~73000), heparan sulfate derived from bovine kidney (M_r 20000–30000), chondroitin sulfate A derived from whale cartilage (M_r 40000–80000), and dermatan sulfate derived from porcine skin (M_r 11000–25000) were from Seikagaku (Tokyo, Japan); dextran sulfate (M_r ~10000) and dextran (M_r ~10500) were from Wako Pure Chemical (Osaka, Japan); and cetylpyridinium chloride (CPC) and other reagents were from Nacalai Tesque (Kyoto, Japan).

Release of [^{35}S]Sulfate-Labeled PGs from Vascular Endothelial and Smooth Muscle Cell Layers —— Vascular endothelial or smooth muscle cells were cultured in 24-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humid atmosphere of 5% CO_2 in air until confluence and then metabolically labeled with [^{35}S]sulfate (1 MBq/ml) for 24 hr in fresh medium. After labeling, the medium was discarded and the cell layer was washed twice with serum-free ASF 301 medium. The cell layer was incubated at 37°C for 1 hr with Na-SP, Ca-SP, H-SP, desulfated-SP, depolymerized Na-SP, heparin, desulfated heparin, *O*-desulfated heparin, *N*-desulfated heparin, dextran sulfate, dextran, pullulan, hyaluronan, heparan sulfate, chondroitin sulfate A, chondroitin sulfate C, or dermatan sulfate (10 μg/ml each) in fresh ASF 301 medium without [^{35}S]sulfate. After incubation, the conditioned medium was harvested and solid urea was added to a concentration of 8 M. The cell layer was washed twice with ice-cold Ca- and Mg-free phosphate-buffered saline and extracted with urea solution 8 M containing 6-aminohexanoic acid 0.1 M, EDTA 5 mM, phenylmethansulfonyl fluoride 0.1 M, NaCl 0.1 M, Tris base 50 mM, and 2% Triton X-100 (pH 7.5) at 4°C for 15 min and harvested by scraping with a rubber policeman. The [^{35}S]sulfate-labeled PGs were determined by the CPC precipitation method.[16] Briefly, portions of the medium and cell layer extracts were spotted on filter papers and washed five times for 1 hr in 1% CPC with NaCl 0.05 M. The radioactivity of precipitated PGs on the dried filter paper was measured by liquid scintillation counting. The percentage of the release of [^{35}S]sulfate-labeled PG was calculated by dividing the radioactivity in the conditioned medium by that found in both the conditioned medium and the cell layer.

Statistical Analysis —— Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) and Bonferroni's multiple *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

First, we evaluated the effect of Na-SP on the release of PGs from vascular endothelial cells as compared with the effects of other polysaccharides. As shown in Fig. 1, Na-SP strongly stimulated the PG release. Although heparin, dextran sulfate, and hyaluronan also had a stimulatory effect, Na-SP was the most potent stimulator of the tested polysaccharides. Desulfated heparin, *O*-desulfated heparin, *N*-desulfated heparin, dextran, pullulan, heparan sulfate, chondroitin sulfate A, chondroitin sulfate C, and dermatan sulfate failed to stimulate PG release, suggesting that the potent stimulatory effect of Na-SP on endothelial PG release is not general in polysaccharides but specific to Na-SP.

Since desulfation of heparin and dextran sulfate

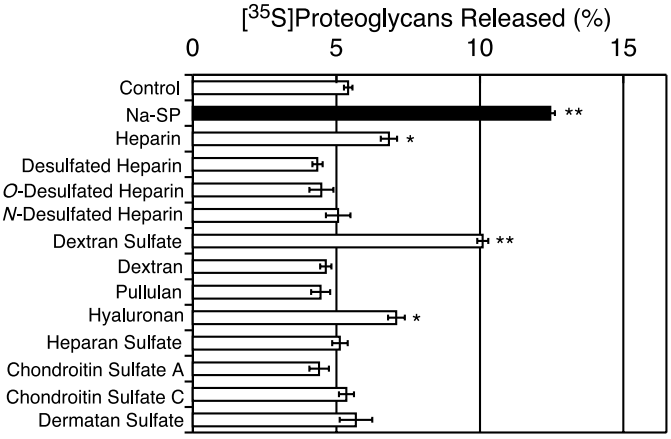

Fig. 1. Release of [^{35}S]Sulfate-Labeled PGs from Vascular Endothelial Cell Layers after Treatment with Na-SP or other Polysaccharides

Confluent cultures of bovine aortic endothelial cells were labeled with [^{35}S]sulfate and then incubated at 37°C for 1 hr with Na-SP, heparin, desulfated heparin, *O*-desulfated heparin, *N*-desulfated heparin, dextran sulfate, dextran, pullulan, hyaluronan, heparan sulfate, chondroitin sulfate A, chondroitin sulfate C, or dermatan sulfate (10 μg/ml each) in the absence of [^{35}S]sulfate. Values are means ± S.E. of four samples. Significantly different from the control, $*p < 0.05$; $**p < 0.01$.

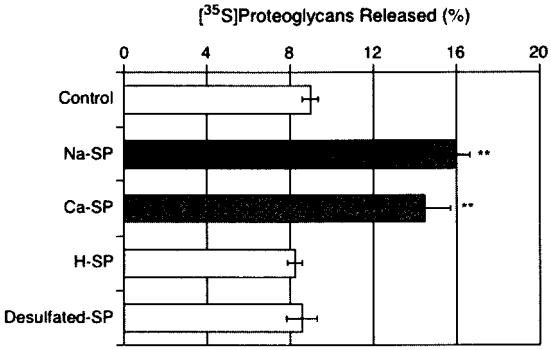

Fig. 2. Release of [^{35}S]Sulfate-Labeled PGs from Vascular Endothelial Cell Layers after Treatment with Na-SP, Ca-SP, H-SP, or Desulfated-SP

Confluent cultures of bovine aortic endothelial cells were labeled with [^{35}S]sulfate and then incubated at 37°C for 1 hr with Na-SP, Ca-SP, H-SP or desulfated-SP (10 μg/ml each) in the absence of [^{35}S]sulfate. Values are means ± S.E. of four samples. **Significantly different from the control, $p < 0.01$.

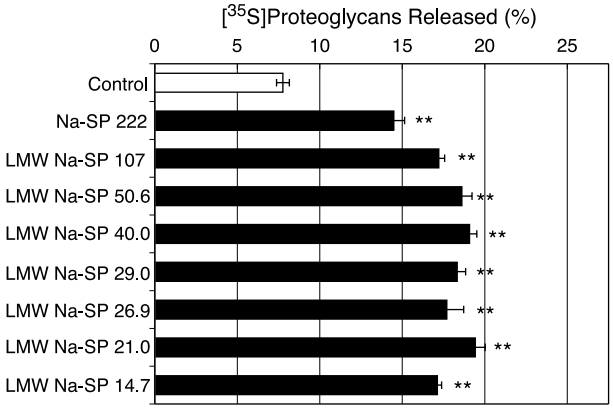

Fig. 3. Release of [^{35}S]Sulfate-Labeled PGs from Vascular Endothelial Cell Layers after Treatment with Na-SP or Depolymerized Na-SP

Confluent cultures of bovine aortic endothelial cells were labeled with [^{35}S]sulfate and then incubated at 37°C for 1 hr with Na-SP (M_r ~222000) or depolymerized Na-SP (M_r ~107000, M_r ~50600, M_r ~40000, M_r ~29000, M_r ~26900, M_r ~21000, M_r ~14700, 10 μg/ml each) in the absence of [^{35}S]sulfate. Values are means ± S.E. of four samples. **Significantly different from the control, $p < 0.01$.

diminished their stimulatory effects on the release of endothelial PGs, the effect of desulfated-SP on endothelial PG release was examined (Fig. 2). At that time, the effects of Ca-SP and H-SP were also tested to investigate the importance of sodium ion in Na-SP for endothelial PG release. Ca-SP as well as Na-SP stimulated the release of PGs from vascular endothelial cell layers. However, neither H-SP nor desulfated-SP had such a stimulatory effect, suggesting that Na-SP requires sodium ion bound to the sulfate group in the molecule for stimulation of endothelial PG release.

Figure 3 shows the effect of Na-SP and its depolymerized products on endothelial PG release. Na-SP maintained the stimulatory effect even when it was gradually depolymerized to M_r ~14700, suggesting that the effect on endothelial PG release is achieved by an active structure but not the whole structure of the Na-SP molecule.

To examine the possibility that vascular endothelial cells are the cell type that is sensitive to Na-SP with respect to PG release, the effect of Na-SP on vascular smooth muscle cell PG release was in-

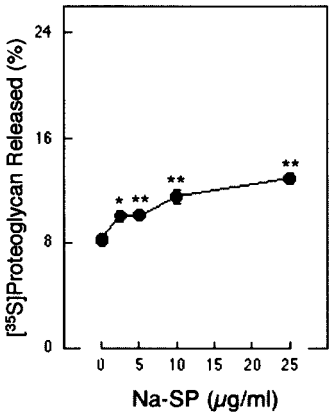

Fig. 4. Release of [^{35}S]Sulfate-Labeled PGs from Vascular Smooth Muscle Cell Layers after Treatment with Na-SP

Confluent cultures of bovine aortic smooth muscle cells were labeled with [^{35}S]sulfate and then incubated at 37°C for 1 hr with Na-SP (2.5, 5, 10, or 25 μg/ml) in the absence of [^{35}S]sulfate. Values are means ± S.E. of four samples. Significantly different from the control, $*p < 0.05$; $**p < 0.01$.

vestigated (Fig. 4). It was shown that Na-SP significantly stimulated PG release from the cells. However, the stimulation in vascular smooth muscle cells was weaker than that in vascular endothelial cells. Specifically, Na-SP at 10 μg/ml increased PG release by 2-fold or more in endothelial cells but only by 1.4-fold in vascular smooth muscle cells after 1-hr incubation.

DISCUSSION

In addition to the anticoagulant and fibrinolytic properties of Na-SP, stimulation of the release of PGs from vascular endothelial cell layers can contribute to local anticoagulant activity on the endothelium. In the present study, the effect of Na-SP on PG release was characterized. The results indicate that: Na-SP is a potent stimulator of endothelial PG release. The stimulatory effect of Na-SP disappears with desulfation or removal of sodium ion. Depolymerized Na-SPs (M_r ~14700 or greater) also exhibit stimulatory effects as does Na-SP (M_r ~220000). The stimulation occurs more potently in endothelial cells than in vascular smooth muscle cells. Taking these results together, it is suggested that the stimulatory effect of Na-SP on PG release requires both sodium ion and the sulfate group; in addition, the stimulation is achieved by an active structure in the molecule which interacts specifically with endothelial cell extracellular matrix.

The anticoagulant activity of heparin,[17,18] heparan sulfate,[19] and dermatan sulfate[19] requires the sulfate group in the molecule. The present data also show that the stimulation of endothelial PG release by heparin and dextran sulfate disappears with desulfation. The biological activities of Na-SP, including heparin cofactor II activation,[20] antiviral activity,[1] changes in fibrinolytic protein secretion in coronary endothelial cells[5] and fibroblastic cells,[21] and inhibition of endothelial[14] and smooth muscle cell proliferation,[6] require the sulfate group or sodium ion. These results suggest that whole or a part of the side chains in the Na-SP molecule, which contains sulfate groups, may be in general an active structure for interaction with heparin cofactor II and vascular cells. In the present study, it was shown that the stimulatory effect of Na-SP is diminished by either removal of sodium ion or desulfation, suggesting that the effect of Na-SP requires a polysaccharide structure with sulfate groups of which the molecular conformation is maintained by sodium or calcium ions bound to the sulfate groups.

Although the stimulatory effect of heparin on endothelial PG release disappears when depolymerized,[22] that of Na-SP was maintained even after depolymerization. Our previous study showed that depolymerization diminished the inhibitory effect of Na-SP on the proliferation of endothelial cells.[14] On the other hand, the inhibition of vascular smooth muscle cell proliferation by Na-SP is maintained even after depolymerization.[6] Although the mechanism for these differences is unclear, it can be assumed that the active structure of Na-SP on vascular cells depends on its occurrence in every cell type. In other words, Na-SP may interact with plural sites in vascular cells *via* different active structures with a particular molecular conformation. However, this remains to be elucidated.

It was observed that endothelial cells are more sensitive to Na-SP than vascular smooth muscle cells with respect to PG release. The predominant PGs in vascular endothelial cells are perlecan and biglycan,[23] whereas vascular smooth muscle cells synthesize and secrete the large chondroitin sulfate PG versican, biglycan, and another small chondroitin/dermatan sulfate PG decorin with a small amount of perlecan.[24] In these PGs, only versican forms aggregates with hyaluronan in the extracellular matrix, which is stabilized by link proteins.[25] Thus it is suggested that the release of versican from

the extracellular matrix of vascular smooth muscle cells by Na-SP is more difficult than that of perlecan, biglycan, and decorin. Identification of Na-SP-releasable PGs is under investigation in our laboratory.

The present data confirm the potent stimulatory effect of Na-SP on the release of PGs from vascular endothelial cell layers. In addition, it was found that the stimulation requires an active structure containing the sulfate group that binds sodium or calcium ions in the Na-SP molecule. Further investigations are necessary for clarification of both the Na-SP-releasable type of PGs and the active structure of Na-SP for stimulation of endothelial PG release.

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