

## Differential Effects of Sodium Spirulan on the Secretion of Fibrinolytic Proteins from Vascular Endothelial Cells: Enhancement of Plasminogen Activator Activity

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(Received June 13, 2003; Accepted June 24, 2003)

Sodium spirulan (Na-SP) is a sulfated polysaccharide with  $M_r \sim 220000$  isolated from the blue-green alga *Spirulina platensis*. Na-SP influences the blood coagulation-fibrinolytic system by activation of heparin cofactor II *in vitro*, although it has been incompletely understood whether the polysaccharide can act on vascular endothelial cell functions. In the present study, we investigated the effects of Na-SP on the fibrinolytic activity of human coronary endothelial cells in a culture system. It was found that Na-SP enhances the activity of tissue-type and urokinase-type plasminogen activators in the conditioned medium of the cells through induction of urokinase-type plasminogen activator secretion and inhibition of plasminogen activator inhibitor type 1 (PAI-1) secretion. The inhibitory effect of Na-SP on PAI-1 secretion was maintained even when sodium ion was removed or replaced by calcium ion, while it disappeared with desulfation, indicating that metal ion is not required but the sulfate group is essential for the inhibition of endothelial PAI-1 secretion by Na-SP. The present study revealed that Na-SP not only shows a strong antithrombin effect through activation of heparin cofactor II but also exhibits a fibrinolytic property through differential effects on endothelial fibrinolytic protein secretion.

**Key words** — sodium spirulan, endothelial cell, fibrinolysis, plasminogen activator, plasminogen activator inhibitor

### INTRODUCTION

Sodium spirulan (Na-SP) is a sulfated polysaccharide isolated from a hot-water extract of the blue-green alga *Spirulina platensis*.<sup>1,2)</sup> Na-SP is composed of rhamnose, 3-*O*-methylrhamnose (acofriose), 2,3-di-*O*-methylrhamnose, 3-*O*-methylxylose, uronic acids, sulfate, and sodium ion, and the backbone consists of 1,3-linked rhamnose and 1,2-linked 3-*O*-methylrhamnose units with some sulfate substitution at the 4-position; the polymer is terminated at the nonreducing end by 2,3-di-*O*-methylrhamnose and 3-*O*-methylxylose residues.<sup>3)</sup> There are two types of disaccharide repeating units, *O*-hexuronosylrhamnose (aldobiuronic acid) and *O*-rhamnosylacofriose with sulfated groups.<sup>4)</sup>

Na-SP exhibits a strong antithrombin activity by activation of heparin cofactor II,<sup>5)</sup> which is a physiologic inhibitor of thrombin, in a different mechanism from that of heparin.<sup>6)</sup> In addition, Na-SP can stimulate the release of anticoagulant heparan<sup>7)</sup> and dermatan<sup>8)</sup> sulfate proteoglycans from vascular endothelial cell layers.<sup>9)</sup> These results indicate that Na-SP may have beneficial effects as an anticoagulant agent on the blood coagulation-fibrinolytic system through not only activation of heparin cofactor II but also influence upon vascular endothelial cell functions.

Vascular endothelial cells are importantly involved in the regulation of fibrinolysis. The cell type synthesizes and secretes fibrinolytic proteins such as tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activators,<sup>10)</sup> and their common inhibitor plasminogen activator inhibitor type 1 (PAI-1).<sup>11)</sup> Since both t-PA and u-PA convert plasminogen to plasmin which degrades fibrin, the fibrinolytic activity in circulating blood depends on the balance

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between t-PA, u-PA, and PAI-1 secreted from vascular endothelial cells.

Na-SP selectively stimulates the synthesis and secretion of t-PA in human fetal lung fibroblasts,<sup>12)</sup> suggesting that the polysaccharides may enhance the fibrinolytic activity in the blood. However, the response of vascular endothelial cells to exogenous agents on fibrinolytic protein synthesis is different from that of other cell types such as vascular smooth muscle cells and fibroblasts.<sup>13,14)</sup> The present study was undertaken to address the questions of whether Na-SP can influence the secretion of fibrinolytic proteins and their activities in human coronary endothelial cells in culture.

## MATERIALS AND METHODS

**Materials** — Human coronary endothelial cells and HuMedia-EG2, a growth medium for endothelial cells, were purchased from Kurabo (Osaka, Japan); tissue culture dishes and plates were from Iwaki (Chiba, Japan); a commercially available enzyme immunoassay kit for PAI-1 and the standards of PAI-1 were from Biopool (Umea, Sweden); plasminogen-rich fibrinogen was from Daiichi Pure Chemicals (Tokyo, Japan); 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrasolium bromide (MTT) was from Chemicon International (Temecula, CA, U.S.A.); human thrombin was from Sigma (St. Louis, MO, U.S.A.); and other reagents were from Wako Pure Chemical Industries (Osaka, Japan). The method for isolation and purification of Na-SP, replacement of sodium ion by calcium ion (*i.e.*, preparation of Ca-SP), removal of sodium ion from Na-SP (*i.e.*, preparation of H-SP), and desulfation of Na-SP (*i.e.*, preparation of desulfated SP) were described previously.<sup>1,15,16)</sup>

**Cell Culture and Determination of Fibrinolytic Proteins** — Vascular endothelial cells were cultured in Hu-Media-EG-2 in 24-well culture plates at 37°C in 5% CO<sub>2</sub> in air until confluent. The medium was discarded and the cell layer was washed twice with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cell layer was then incubated at 37°C for 24 hr in fresh DMEM supplemented with 10% FBS in the presence of Na-SP, Ca-SP, H-SP, or desulfated SP (5, 10, 25, 50, or 100 µg/ml). After incubation, the conditioned medium was harvested and used for either fibrin zymography or the deter-

mination of t-PA, u-PA, and PAI-1 by enzyme immunoassay. The cell layer was used for the MTT assay to determine the cell number. The secretion of the fibrinolytic proteins was expressed as ng/10<sup>6</sup> cells (t-PA and u-PA) or µg/10<sup>6</sup> cells (PAI-1).

**Fibrin Zymography** — To examine the fibrinolytic activity of the liquid phase of vascular endothelial cells after treatment with Na-SP, electrophoretic fibrin zymography was performed according to the method of Matsuo *et al.*<sup>17)</sup> Briefly, confluent cultures of endothelial cells were treated with Na-SP (5, 10, 25, 50, or 100 µg/ml) in DMEM supplemented with 10% FBS. The conditioned medium was harvested and incubated at 37°C for 1 hr with 0.125 M Tris-HCl buffer containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.002% bromophenol blue. SDS polyacrylamide gel electrophoresis of the medium was performed on a 7.5% polyacrylamide slab gel with fibrin matrix, plasminogen, and a 4.5% stacking gel. The slab gel was washed with 2.5% Triton X-100 for 1 hr and incubated at 37°C for 48 hr in 0.1 M glycine-NaOH buffer (pH 8.3). After incubation, the gel was stained with 0.1% Coomassie brilliant blue and destained with 7.5% acetic acid containing 5% methanol until the lytic zones became clear. The plasminogen-dependent protease activities were identified as t-PA and u-PA activities on the basis of the molecular weight of standard t-PA and u-PA.

**Statistical Analysis** — Data were analyzed for statistical significance using 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 AND DISCUSSION

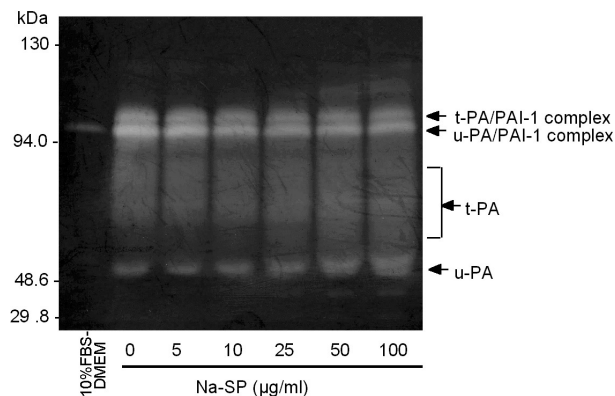
Vascular endothelial cells synthesize and secrete both t-PA and u-PA, although the activity of t-PA is markedly enhanced upon binding to fibrin<sup>18,19)</sup> whereas u-PA lacks fibrin-binding capacity, suggesting that t-PA rather than u-PA is implicated in intravascular fibrinolysis. On the other hand, PAI-1 associated with endothelium readily inhibits t-PA and u-PA, while that released into the liquid phase has little anti-activator activity.<sup>20)</sup> Since only the active form of PAI-1 can bind and inactivate t-PA and u-PA, plasminogen activator activity should be evaluated using qualitative methods such as fibrin zymography. Figure 1 shows the zymography of the

conditioned medium of vascular endothelial cells after treatment with Na-SP. It was found that Na-SP enhances the activity of both free t-PA and u-PA secreted from vascular endothelial cells. At the same time, complexes of PAI-1 with either t-PA or u-PA were decreased by Na-SP, suggesting that Na-SP decreases the endothelial secretion of PAI-1, resulting in higher activity of t-PA and u-PA.

Figure 2 shows the accumulation of t-PA, u-PA, and PAI-1 in the conditioned medium of vascular endothelial cells after treatment with Na-SP. As expected, Na-SP significantly decreased the accumulation of PAI-1. In addition, the accumulation of t-PA was unaffected but that of u-PA was significantly increased by Na-SP. Thus it is postulated that the Na-SP-enhanced activity of u-PA is due not only to

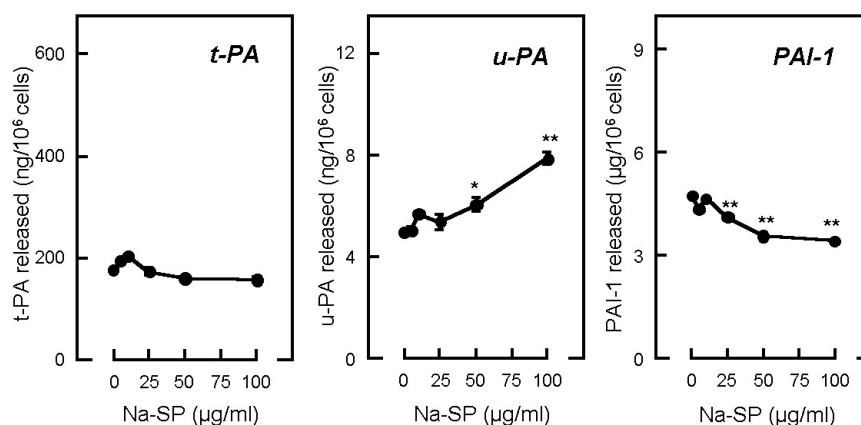
a decrease in PAI-1 secretion but also to an increase in u-PA secretion, whereas that of t-PA occurred only through a decrease in PAI-1 secretion in vascular endothelial cells. Although the regulation of t-PA, u-PA, and PAI-1 secretion from endothelial cells is not necessarily coupled,<sup>21)</sup> little is known about the differential regulation of fibrinolytic protein synthesis in vascular endothelial cells. The mechanism for the differential effects of Na-SP on endothelial fibrinolytic protein synthesis and secretion also remains to be elucidated.

There are two types of disaccharide repeating units, *O*-hexuronosyl-rhamnose and *O*-rhamnosyl-acofriose with sulfated groups in the Na-SP molecule,<sup>4)</sup> and the conformation is maintained by sodium ion. A specific primary structure or specific molecular conformation may be required for endothelial fibrinolytic protein secretion, because biological activities of Na-SP, including inhibition of vascular endothelial cell proliferation,<sup>1)</sup> induction of t-PA synthesis in human fetal lung fibroblasts,<sup>12)</sup> and antiviral effect,<sup>1)</sup> disappear by desulfation or removal of the metal ion. Figure 3 shows the accumulation of PAI-1 in the conditioned medium of vascular endothelial cells after treatment with Na-SP, Ca-SP, H-SP, and desulfated SP. It was revealed that the inhibitory effect of Na-SP on endothelial PAI-1 secretion was maintained even when sodium ion was removed or replaced by calcium ion; on the other hand, desulfated SP significantly increased PAI-1 secretion. It is suggested that sodium ion in the Na-SP molecule is not required but the sulfate group is essential for inhibition of endothelial PAI-1 secretion. However, it is unclear why desulfated SP has a stimulatory effect on PAI-1 secretion.



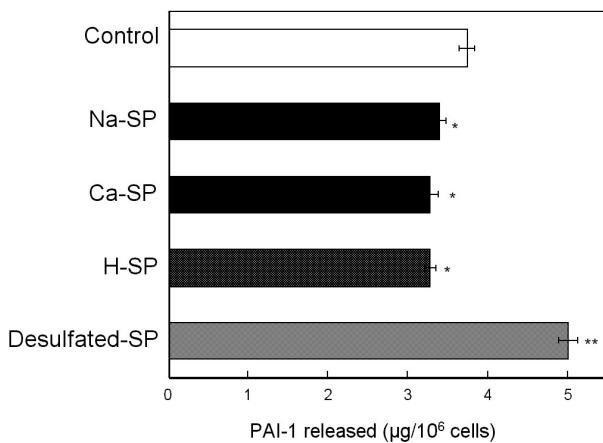
**Fig. 1.** Electrophoretic Enzymography (Fibrin Zymography) of the Conditioned Medium of Cultured Vascular Endothelial Cells after Treatment with Na-SP

Confluent cultures of human coronary endothelial cells were incubated at 37°C for 24 hr in the presence of Na-SP (5, 10, 25, 50, or 100 µg/ml).



**Fig. 2.** Secretion of t-PA, u-PA, and PAI-1 from Cultured Vascular Endothelial Cells after Treatment with Na-SP

Confluent cultures of human coronary endothelial cells were incubated at 37°C for 24 hr in the presence of Na-SP (5, 10, 25, 50, or 100 µg/ml). Values are mean  $\pm$  S.E. of six samples. Significantly different from the corresponding control, \* $p$  < 0.05; \*\* $p$  < 0.01.



**Fig. 3.** Secretion of PAI-1 from Cultured Vascular Endothelial Cells after Treatment with Na-SP, Ca-SP, H-SP, or Desulfated SP

Confluent cultures of human coronary endothelial cells were incubated at 37°C for 24 hr in the presence of Na-SP (100 µg/ml). Values are mean ± S.E. of six samples. Significantly different from the corresponding control, \* $p < 0.05$ ; \*\* $p < 0.01$ .

The present study demonstrates that Na-SP differentially acts on the secretion of fibrinolytic proteins and enhances the fibrinolytic activity of vascular endothelial cells. It is also shown that the response of endothelial cells to Na-SP is completely different from that of fibroblastic cells.<sup>12)</sup> In addition, it is indicated that the sulfate group in the Na-SP molecule plays an important role in the inhibition of endothelial PAI-1 secretion. Since sodium or calcium ion are in general essential for biological activities of Na-SP,<sup>1,12)</sup> the mechanism for Na-SP inhibition of endothelial PAI-1 secretion may be different from that for other biological activities. Further studies should be done to investigate the essential structure of Na-SP for differential action on endothelial fibrinolytic proteins and the receptor that mediates the Na-SP effects to clarify the specific and unique interactions of Na-SP with vascular endothelial cells.

**Acknowledgements** This work was supported in part by the Specific Research Fund of Hokuriku University (to T. K.) and a Grant-in-Aid for Young Scientists (B) from the Ministry of Education Culture, Sports, Science and Technology (to C. Y.).

## REFERENCES

- Hayashi, T., Hayashi, K., Maeda, M. and Kojima, I. (1996) Calcium spirulan, an inhibitor of enveloped virus replication, from a blue-green alga *Spirulina platensis*. *J. Nat. Prod.*, **59**, 83–87.
- Hayashi, K., Hayashi, T. and Kojima, I. (1996) A natural sulfated polysaccharide, calcium spirulan, isolated from *Spirulina platensis*: *in vitro* and *ex vivo* evaluation of anti-herpes simplex virus and anti-human immunodeficiency virus activities. *AIDS Res. Hum. Retroviruses*, **12**, 1463–1471.
- Lee, J. B., Hayashi, T., Hayashi, K., Sankawa, U., Maeda, M., Nemoto, T. and Nakanishi, H. (1998) Further purification and structural analysis of calcium spirulan from *Spirulina platensis*. *J. Nat. Prod.*, **61**, 1101–1104.
- Lee, J. B., Hayashi, T., Hayashi, K. and Sankawa, U. (2000) Structural analysis of calcium spirulan (Ca-SP)-derived oligosaccharides using electrospray ionization mass spectrometry. *J. Nat. Prod.*, **63**, 136–138.
- Hayakawa, Y., Hayashi, T., Hayashi, K., Hayashi, T., Ozawa, T., Niiya, K. and Sakuragawa, N. (1996) Heparin cofactor II-dependent antithrombin activity of calcium spirulan. *Blood Coagul. Fibrinolysis*, **7**, 554–560.
- Hayakawa, Y., Hayashi, T., Lee, J. B., Ozawa, T. and Sakuragawa, N. (2000) Activation of heparin cofactor II by calcium spirulan. *J. Biol. Chem.*, **275**, 11379–11382.
- Mertens, G., Cassiman, J. J., Van den Berghe, H., Vermeylen, J. and David, G. (1992) Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. Core protein characterization and antithrombin III binding properties. *J. Biol. Chem.*, **267**, 20435–20443.
- Whinna, H. C., Choi, H. U., Rosenberg, L. C. and Church, F. C. (1993) Interaction of heparin cofactor II with biglycan and decorin. *J. Biol. Chem.*, **268**, 3920–3924.
- Kaji, T., Shimada, S., Yamamoto, C., Fujiwara, Y., Lee, J. B. and Hayashi, T. (2002) Inhibition of the association of proteoglycans with cultured vascular endothelial cell layers by calcium and sodium spirulan. *J. Health Sci.*, **48**, 250–255.
- Levin, E. G. and Loskutoff, D. J. (1982) Cultured bovine aortic endothelial cells produce both urokinase and tissue-plasminogen activators. *J. Cell Biol.*, **94**, 631–636.
- Erickson, L. A., Schleef, R. R., Ny, T. and Loskutoff, D. J. (1985) The fibrinolytic system of the vascular wall. *Clin. Haematol.*, **12**, 513–530.
- Hayakawa, Y., Hayashi, T., Hayashi, K., Ozawa, T.,

- Niiya, K. and Sakuragawa, N. (1997) Calcium spirulan as an inducer of tissue-type plasminogen activator in human fetal lung fibroblasts. *Biochim. Biophys. Acta*, **1355**, 241–247.
- 13) Yamamoto, C. and Kaji, T. (1999) Effect of lead on the synthesis of tissue plasminogen activator by vascular endothelial cells in culture. *J. Health Sci.*, **45**, 119–125.
- 14) Yamamoto, C. and Kaji, T. (2002) Induction of plasminogen activator inhibitor type 1 synthesis by cadmium in human vascular endothelial cells in culture. *J. Health Sci.*, **48**, 55–61.
- 15) Hayashi, K., Hayashi, T., Morita, N. and Kojima, I. (1993) An extract from *Spirulina platensis* is a selective inhibitor of herpes simplex virus type 1 penetration into HeLa cells. *Phytother. Res.*, **7**, 76–80.
- 16) Kaji, T., Fujiwara, Y., Hamada, C., Yamamoto, C., Shimada, S., Lee, J. B. and Hayashi, T. (2002) Inhibition of cultured bovine aortic endothelial cell proliferation by sodium spirulan, a new sulfated polysaccharide isolated from *Spirulina platensis*. *Planta Med.*, **68**, 505–509.
- 17) Matsuo, C., Fukao, H. and Matsuo, O. (1988) Characterization of plasminogen activator produced by an established cell line from human ovary. *J. Cell Physiol.*, **134**, 253–260.
- 18) Hoylaerts, M., Rijken, D. C., Lijken, H. R. and Collen, D. (1982) Kinetics of the activation of plasminogen by human tissue plasminogen activator. *J. Biol. Chem.*, **257**, 2912–2919.
- 19) Ranby, M. (1982) Studies on the kinetics of plasminogen activation by tissue plasminogen activator. *Biochim. Biophys. Acta*, **704**, 461–469.
- 20) Levin, E. G. (1986) Quantitation and properties of the active and latent plasminogen activator inhibitors in cultures of human endothelial cells. *Blood*, **67**, 1309–1313.
- 21) Yamamoto, C., Kaji, T., Sakamoto, M. and Koizumi, F. (1992) Effect of endothelin on the release of tissue plasminogen activator and plasminogen activator inhibitor-1 from cultured human endothelial cells and interaction with thrombin. *Thromb. Res.*, **67**, 619–624.