Inhibition of Cultured Bovine Aortic Smooth Muscle Cell Proliferation by Colominic Acid

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Colominic acid (CA) is an α2,8-linked polymer of sialic acid, originally isolated from capsular Escherichia coli K1. Since inhibition of arterial smooth muscle cell hyperplasia is one of the effective strategies to prevent atherosclerosis, we investigated the effect of CA, purified as an α2,8-linked homopolymer of N-acetylneuraminic acid, on the proliferation of bovine aortic smooth muscle cells in culture. The results demonstrate that CA inhibits the proliferation of the cells without nonspecific cell damage. Sulfation did not modify the inhibitory effect of CA. Specifically, the inhibitory effect of sulfated CA was almost equal to that of CA in vascular smooth muscle cell proliferation. On the other hand, it was suggested that the inhibition of the proliferation by CA is in a degree similar to that by heparin but weaker than that by sodium/calcium-spirulans, known sulfated polysaccharides as the potent inhibitor of vascular smooth muscle cells. The present data suggest that CA with or without sulfate groups can be an origin of beneficial agents that prevents atherosclerosis through a moderate inhibition of arterial smooth muscle cell proliferation.

Key words colominic acid; smooth muscle cell; proliferation; sialic acid; sulfation; vascular

MATERIALS AND METHODS

Materials Vascular smooth muscle cells obtained from bovine aorta were a gift from Professor Yasuo Suda (Graduate School of Science and Engineering, Kagoshima University, Kagoshima, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were from Nissui Pharmaceutical (Tokyo, Japan) and MP Biomedicals (Irvine, CA, U.S.A.), respectively. Tissue culture plates and dishes were obtained from Nalge Nunc International (Aylesbury, U.K.). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were from Nissui Pharmaceutical (Tokyo, Japan) and MP Biomedicals (Irvine, CA, U.S.A.). C[14C]thymidine (3.3 TBq/mmol) was from Parkin Elmer Life Sciences (Boston, MA, U.S.A.). Lactate dehydrogenase kit was from Wako Pure Chemical (Osaka, Japan). CA (M, ca. 17000) and SCA 2) (M, ca. 22000) containing sulfur of 13.4% were isolated from capsular Escherichia coli K1 as sodium salt of an α2,8-linked homopolymer of N-acetylneuraminic acid with or without sulfation. Na-SP and Ca-SP were isolated and purified from the blue-green alga Spirulina platensis as described previously. ca) Heparin derived from porcine intestine (M, ca. 10000) was purchased from Sigma (St Louis, MA, U.S.A.). Other reagents were from Nacalai Tesque (Kyoto, Japan).

Morphological Procedure Vascular smooth muscle cells were cultured in DMEM supplemented with 10% fetal bovine serum in 100-mm dishes at 37 °C in a humid atmosphere of 5% CO2 until confluent. They were transferred into 24-well culture plates at 5×103 cells/cm2 and cultured for 24 h in DMEM supplemented with 10% fetal bovine serum. The cells were then incubated at 37 °C for 24, 48 or 72 h in the presence of CA, SCA, Na-SP, Ca-SP or heparin (50 µg/ml each) in fresh DMEM supplemented with 10% fetal bovine serum. After treatment, the medium was discarded and the cells were stained with Giemsa.

Determination of Cell Number Vascular smooth muscle cells were plated into 24-well culture plates at 5×103 cells/cm2 and then cultured for 24 h in DMEM supplemented with 10% fetal bovine serum. After culture, the cells were...
treated with CA or SCA (25, 50 or 100 μg/ml) for 24, 48 or 72 h in DMEM supplemented with 10% fetal bovine serum and then harvested with 0.2% trypsin containing 0.02% EDTA in Ca- and Mg-free phosphate-buffered saline (CMF-PBS). The harvested cell suspension was used for the determination of cell number by a particle counter (Sysmex CDA-500).

**Incorporation of [3H]Thymidine and Leakage of Lactate Dehydrogenase** Vascular smooth muscle cells were plated into 24-well culture plates at 5×10^3 cells/cm² and then cultured for 24 h in DMEM supplemented with 10% fetal bovine serum in 6-well culture plates. The cells were treated with CA, SCA, Na-SP, Ca-SP or heparin (50 μg/ml each) in fresh DMEM supplemented with 10% fetal bovine serum, and labeled with [3H]thymidine (20 kBq/ml) during the last 6 h of the treatment. After labeling, the medium was collected and used for the determination of lactate dehydrogenase activity as a marker of nonspecific cell damage. The cells were gently washed with CMF-PBS and harvested with a rubber policeman in the presence of CMF-PBS. Cell homogenate was prepared by sonication and incorporation of the radioactivity into 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 method to express the incorporated radioactivity as dpm/μg DNA.

**Statistical Analysis** Data were analyzed for statistical significance by ANOVA and Bonferroni’s multiple t-test when possible. p-Values of less than 0.05 were considered to indicate statistically significant differences.

**RESULTS**

Figure 1 shows the morphological appearance of vascular smooth muscle cells after treatment with CA or SCA. Both CA and SCA (50 μg/ml each) inhibited the growth of the cells after 24 h and longer without any degenerative changes. In addition, the inhibitory effect of CA and SCA on the cell growth was not different in degree.

Figure 2 shows the number of vascular smooth muscle cells after treatment with CA or SCA. Both CA and SCA at 25 μg/ml and more decreased the cell number in a dose-dependent manner. Time course study showed that the inhibition of cell growth occurred after 24 h and longer. These results agreed with the morphological observation showing a similar inhibitory effect of CA and SCA on vascular smooth muscle cells after 24 h and longer.

Table 1 shows the incorporation of [3H]thymidine into the acid-insoluble fraction and the leakage of lactate dehydrogenase into the medium of vascular smooth muscle cells after treatment with CA or SCA.

**Table 1. Incorporation of [3H]Thymidine into the Acid-Insoluble Fraction and the Leakage of Lactate Dehydrogenase into the Medium of Vascular Smooth Muscle Cells after Treatment with CA or SCA**

<table>
<thead>
<tr>
<th></th>
<th>[3H]Thymidine (dpm×10^-4/μg DNA)</th>
<th>Lactate dehydrogenase (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.24±0.23</td>
<td>42.2±0.5</td>
</tr>
<tr>
<td>CA</td>
<td>5.33±0.45**</td>
<td>39.0±0.3*</td>
</tr>
<tr>
<td>SCA</td>
<td>5.55±0.39**</td>
<td>38.9±0.1**</td>
</tr>
</tbody>
</table>

Sparse cultures of bovine aortic smooth muscle cells were incubated at 37°C with CA or SCA (25, 50 or 100 μg/ml each) for 48 h and labeled with [3H]thymidine during the last 6 h of the incubation. Values are means±S.E. of four samples. Significantly different from the corresponding control, * p<0.05; ** p<0.01.

(50 μg/ml each) inhibited the growth of vascular smooth muscle cells without any degenerative changes after a 48-h incubation (Fig. 3); a marked inhibition was observed in the cells treated with Na-SP and Ca-SP. When proliferative activity of the cells was evaluated by the [3H]thymidine incorpo-
The inhibitory effect of Na-SP (Ca-SP) on vascular smooth muscle cell proliferation requires sulfate groups in their molecules. In addition, the effects of Na-SP on vascular endothelial cell functions, including inhibition of the proliferation, inhibition of the secretion of plasminogen activator inhibitor type 1 and stimulation of the release of proteoglycans, disappear after desulfation. It has been shown that several biological activities of SCA also require the sulfate groups. Thus, the sulfation appears to be in general essential for exhibition or intensification of the biological activities of polysaccharides. In contrast, the present data showed that the inhibitory effect of CA on vascular smooth muscle cell proliferation is unaffected by the sulfation, suggesting that the polysaccharide chain structure of CA interacts for itself with the cells and the sulfate group does not influence the interaction. However, the minimum chain length of CA required for inhibition of vascular smooth muscle cell proliferation remains to be elucidated.

Proliferation of vascular smooth muscle cells is largely influenced by growth factors that regulate the formation of extracellular matrix. For example, platelet-derived growth factor (PDGF) stimulates the proliferation of vascular smooth muscle cells in concert with increased synthesis of versican, a large chondroitin sulfate proteoglycan. The proteoglycan interacts with hyaluronan and accumulates in the pericellular matrix when vascular smooth muscle cells proliferate and migrate. The newly formed versican-hyaluronan-rich extracellular matrix allows the cells to prepare them for proliferation and migration. In fact, inhibition of the versican-hyaluronan pericellular coat by short oligosaccharides of hyaluronan results in blocking PDGF-induced proliferation of vascular smooth muscle cells. It is possible that CA and SCA may disturb the formation of the versican-hyaluronan-rich extracellular matrix as does the short oligosaccharides of hyaluronan. In addition, the possibility that CA and SCA serve as a scavenger of autocrine growth factors, including PDGF, fibroblast growth factors, transforming growth factor-β and tumor necrosis factor-α, cannot be excluded.

In summary, the present data demonstrate that CA inhibits the proliferation of cultured vascular smooth muscle cells without inducing nonspecific cell damage. The inhibitory activity was not influenced by the sulfation. Recently, we found that CA strongly inhibits the proliferation of vascular endothelial cells and destroys the monolayer of the cells whereas SCA does not have such injurious activities (in preparation). SCA differs from CA, Na-SP and Ca-SP in that it inhibits the proliferation of vascular smooth muscle cells but not that of endothelial cells. Based on these results, further studies should be performed to clarify whether or not: (1) CA and SCA change the formation of extracellular matrix of vascular smooth muscle cells. (2) CA and SCA interact with growth factors/cytokines that regulates vascular smooth muscle cell behavior. (3) CA and SCA influence the regulation of vascular endothelial cell functions such as fibrinolytic activity. (4) There is a minimum chain length of CA and SCA that shows beneficial activities to prevent atherosclerosis by modulation of vascular cell functions. (5) There are chemical modifications, other than sulfation, or counter ions of CA that selectively intensify the beneficial effects of the polysaccharide on vascular cells. These studies will be
helpful for the possible clinical application of CA and SCA to prevention of atherosclerosis.

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