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Possible Mechanism of the Stimulatory Effect of Artemisia Leaf Extract on the Proliferation of Cultured Endothelial Cells: Involvement of Basic Fibroblast Growth Factor

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To investigate the possible mechanism of the stimulatory effect of a hot water extract from Artemisia leaf (*Artemisia princeps* PANPANINI) (AFE) on the proliferation of endothelial cells, cells from bovine aorta were cultured for 72 h in RPMI1640 medium supplemented with 10% fetal calf serum in the presence of 5 μg/ml AFE. The AFE treatment significantly increased the cell number after culture, while in the presence of 10 μg/ml unfractionated heparin, AFE conversely decreased it. This implied that AFE enhanced the cell growth promotion by basic fibroblast growth factor (bFGF). The accumulation of bFGF was significantly increased in the culture medium, in the low-affinity (glycosaminoglycans-binding) fraction, and in the cell extract fraction, but was unchanged in the high-affinity (receptor-binding) fraction. The contents of [^{35}S]sulfate-labeled glycosaminoglycans in both cell layer and the medium were not increased by AFE treatment. The proliferation of A10 cells, an established cell line of smooth muscle cells from murine aorta, was not stimulated by AFE. A10 cells did not produce a significant amount of bFGF in the presence or absence of AFE. Thus, the production of bFGF was considered to be involved in AFE stimulation of cell proliferation. In conclusion, it was suggested that AFE stimulated endothelial cell proliferation by increasing the production of bFGF rather than by an increase in the number of bFGF receptors and the content of glycosaminoglycans in the cell layer. The enhanced reserve of bFGF in the low-affinity fraction of cell layer and in the medium would cause the AFE-stimulated proliferation of endothelial cells.

Keywords Artemisia leaf; basic fibroblast growth factor; endothelial cell; plant extract; proliferation; vascular smooth muscle cell

The growth of endothelial cells is involved in the repair process of the injured surface of vascular, and in some physiologic and pathologic processes of angiogenesis during early development, the growth of solid tumors and wound healing. Studies on the proliferation of endothelial cells will contribute to prevention of arteriosclerosis, thrombosis and metastasis of tumor.

We have studied the effect of traditional herbal drugs on blood coagulation and fibrinolysis. We found that the water-soluble extract of Artemisia leaf (AFE) inhibits both blood coagulation and fibrinolysis,[2] and that of Gardenia fruit (GFE) accelerates fibrinolysis[3] *in vitro*. Recently, we have found that both AFE and GFE individually stimulate the proliferation of cultured bovine aortic endothelial cells.[4,5] However, the following questions have remained: 1) Does AFE and/or GFE stimulate the proliferation of other cell lines, especially vascular smooth muscle cells? 2) Why do AFE and GFE stimulate the proliferation of endothelial cells?

These questions are very important since a drug which stimulates the proliferation of endothelial cells but not that of vascular smooth muscle cells is desirable, for example, in the prevention of arteriosclerosis and thrombosis. Clarification of the mechanism by which AFE and GFE stimulate endothelial cell proliferation will contribute to their evaluation as useful drugs.

Sato and Rifkin[6] reported that basic fibroblast growth factor (bFGF) exhibits an autocrine activity for endothelial cells. More recently, bFGF receptors have been identified in human vascular endothelial cells.[7] The proliferation of endothelial cells is regulated by bFGF. In the present study, we investigated the relationship between AFE-stimulated endothelial cell proliferation and bFGF. Moreover, we tested the effect of AFE on the proliferation of A10 cells, an established cell line of smooth muscle cells from murine aorta.

Materials and Methods

Materials RPMI1640 medium and fetal calf serum (FCS) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and Filtron (Australia), respectively. Tissue culture plates dishes were from Costar (Cambridge, MA., U.S.A.). [Methyl-^{3}H]thymidine (740 GBq/mmol), L-[^{14}C]leucine (11.8 GBq/mmol) and $Na_2[^{35}S]O_4$ (19.3 GBq/mmol) were obtained from New England Nuclear Corp. (Boston, MA., U.S.A.). Unfractionated heparin (UFH) from bovine lung was purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.). An ultrafilter unit, CENTRISART I was purchased from Sartorius GmbH (W. Germany). The bFGF [^{125}I]radioimmunoassay kit was from Amersham Corp. (U.K.). An established cell line of smooth muscle cells from murine aorta, A10 cells were obtained from the American Type Culture Collection (Rockville, MD., U.S.A.).

Preparation of AFE The same AFE that was examined in the previous report[4] was used in this study. A dried powder (150 g) extracted by hot water from 1 kg of Artemisia leaf (*Artemisia princeps* PANPANINI) was supplied by Tsumura Co., Ltd. (Tokyo, Japan). The powder was suspended in distilled water at the concentration of 16.7 mg/ml, and boiled for 60 min. After cooling to room temperature, the suspension was filtered through paper and the water-soluble fraction was lyophilized. The powder obtained was called AFE. The yield was 2.5 g of AFE from 10 g of the supplied extract. An aqueous solution of AFE was sterilized through a filter and added to the culture medium.

Cell Culture and Cell Counting Endothelial cells were isolated from bovine aorta by scraping the surface of the intima. The cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37 °C in a humid atmosphere at 5% CO_2 in air in a 100 mm dish until confluent. They were then transferred into 24-well culture plates at about 10^5 cells/well and cultured in the presence of 5 μg/ml AFE combined with 10 μg/ml UFH for 72 h. After culture, the medium was discarded and the cell layer was washed twice with Ca, Mg-free phosphate-buffered saline (CMF-PBS). The cell layer was dispersed with 0.25% trypsin–0.05% ethylenediaminetetraacetic acid (EDTA) in CMF-PBS. The cell suspension was well pipetted and the cell number was counted with a hematocytometer.

Accumulation of bFGF Endothelial cells were cultured in the presence of 5 μg/ml AFE in 24-well culture plates for 72 h. After culture, the medium was removed; cultures were washed with CMF-PBS (the wash was combined with the medium); twice with 0.5 ml of 2 M NaCl in 20 mM *N*-hydroxyethylpiperazine-*N*'-2-ethanesulfonate (Hepes), pH 7.5; twice with 0.5 ml of 2 M NaCl in 20 mM sodium acetate, pH 4.0; and were extracted with 1 ml of 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1. The bFGF in the medium; bFGF eluted with 2 M NaCl at neutral pH (the

low-affinity fraction), which represents bFGF bound to glycosaminoglycans; bFGF eluted with a subsequent wash with 2 M NaCl at pH 4.0 (the high-affinity fraction), which represents bFGF bound to receptors; and bFGF released by a subsequent extraction with 0.5% Triton X-100, which represents internalized bFGF were measured by radioimmunoassay. The sensitivity, as determined by the response at 50% displacement of tracer, was approximately 12 fmol. The fractionation of bFGF was carried out by the method of Moscatelli.[7]

Accumulation of [^{35}S]Sulfate-Labeled Glycosaminoglycans (^{35}S-Glycosaminoglycans) Endothelial cells were cultured with 370 kBq/ml [^{35}S] sulfate for 72 h in the presence of 5 μg/ml AFE in 24-well culture plates. After culture, the medium was removed and the cell layer was washed twice with CMF-PBS. The wash was combined with the medium. The cell layer was incubated at 37 °C with 0.25 ml of CMF-PBS containing 0.25% trypsin and 0.05% EDTA for 5 min. The trypsinized cell suspension was harvested and the well was washed with 0.25 ml of CMF-PBS. The wash was combined with the suspension and centrifuged at 3000 rpm for 5 min to obtain the supernatant ("trypsinate" fraction). The trypsinate fraction includes ^{35}S-glycosaminoglycans derived from the cell surface and the solubilized matrix.[9] The ^{35}S-glycosaminoglycans were isolated by the method of Wasteson *et al.*[10] as follows: the trypsinate fraction was incubated with 3 mg/ml pronase at 50 °C for 3 h. After cooling to room temperature, 2 mg/ml carrier chondroitin sulfate and 0.5% cetylpyridinium chloride (CPC) were added and incubated for 30 min at 37 °C. Then, the mixture was centrifuged at 3000 rpm for 15 min to obtain the precipitated ^{35}S-glycosaminoglycans–CPC complex. The precipitate was dissolved in 0.1 ml of 4 M NaCl and reprecipitated by addition of 1.4 ml of 80% aqueous ethanol. Then, the suspension was centrifuged at 3000 rpm for 15 min. The obtained precipitate was dissolved in 0.4 ml of distilled water and the radioactivity was measured by liquid scintillation counter. The collected medium was ultrafiltered with CENTRISART I to remove free [^{35}S]sulfate and low-molecular-weight [^{35}S]sulfate-labeled materials (mol wt. ≦ 5 kilodaltons). The ^{35}S-glycosaminoglycans were purified from the high-molecular-weight fraction by CPC precipitation and the radioactivity was measured.

Culture of A10 Cells A10 cells were cultured in the same way as endothelial cells for 48 h in the presence of 2.5, 5.0, 10, or 20 μg/ml AFE. The cell number transferred into wells was 2×10^4 cells/well. After culture, the cell number was counted as described above. In another experiment, the cells were cultured in 6-well culture plates for 72 h in the presence of 5 μg/ml AFE and labeled with 4.81 kBq/ml [^{3}H]thymidine and 3.33 kBq/ml [^{14}C]leucine during the last 3 h of the culture. At this time, endothelial cells were cultured and labeled in the same way. After culture, the cell layer was washed twice with CMF-PBS and the cells were scraped off with a rubber policeman in the presence of CMF-PBS. The cell homogenate was prepared by sonication and an aliquot was used for the determination of deoxyribonucleic acid (DNA) by the method of Kissane and Robins.[11] The incorporations of [^{3}H]thymidine and [^{14}C]leucine into the 5% trichloroacetic acid (TCA)-insoluble fraction of the cell homogenate were measured by liquid scintillation counter using a portion of the cell homogenate.

Statistical Analysis Data were analyzed for statistical significance by Student's *t* test.

Results

As previously reported,[4] the highest effect of AFE on endothelial cell proliferation occurred at 5 μg/ml, so that AFE at 5 μg/ml was principally used in the following experiments.

Table I shows the effect of UFH on AFE-stimulated proliferation of endothelial cells. In the absence of UFH, AFE at 5 μg/ml significantly increased the cell number of endothelial cells after a 72 h treatment. In contrast, in the presence of 10 μg/ml UFH, AFE significantly decreased the cell number. The cells were not grown in the presence of 100 μg/ml UFH.

The accumulation of bFGF in endothelial cell cultures was investigated as shown in Table II. The total accumulation of bFGF was significantly increased by 5 μg/ml AFE. The fractionation of bFGF revealed that AFE significantly increased this factor in the medium, the low-affinity and the cell extract fractions, however, it was unchanged in the high-affinity fraction.

Since glycosaminoglycans of endothelial cells serve as a low-affinity binding site in the cell layer,[12] the content of ^{35}S-glycosaminoglycans in both cell layer and medium were measured. As shown in Table III, AFE at 5 μg/ml did not increase the content of ^{35}S-glycosaminoglycans in either the cell layer or the medium.

Figure 1 shows the effect of AFE on the number of A10 cells, an established cell line of vascular smooth muscle cells from murine aorta. AFE at 20 μg/ml and below did not

Table I. Effect of 10 μg/ml UFH on AFE-Enhanced Number of Endothelial Cells

	Number of endothelial cells	
	−UFH	+UFH
Control	0.77±0.02	0.83±0.03
5 μg/ml AFE	0.87±0.02[a]	0.66±0.03[a]

Endothelial cells were cultured for 72 h in the presence of 5 μg/ml AFE combined with 10 μg/ml unfractionated heparin. Values are means±S.E. of 4 samples expressed as cell number × 10^{-5}/cm^2. *a*) Significantly different from control, $p<0.01$.

Table II. Effect of 5 μg/ml AFE on the Content and Distribution of Basic Fibroblast Growth Factor in Cultured Endothelial Cells

	Medium (fmol)	Low affinity (fmol)	High affinity (fmol)	Cell extract (fmol)	Total (fmol)
Control	143±6	117±1	133±2	138±2	534±2
5 μg/ml AFE	162±5[a]	133±1[b]	139±3	155±4[c]	571±9[c]

Endothelial cells were cultured for 72 h in the presence of 5 μg/ml AFE. Values are means±S.E. of 4 samples. Significantly different from control, *a*) $p<0.05$; *b*) $p<0.001$; *c*) $p<0.01$.

Table III. Effect of 5 μg/ml AFE on the Accumulation of ^{35}S-Glycosaminoglycans in the Cell Layer and the Medium

	Cell layer (dpm × 10^{-2})	Medium (dpm × 10^{-2})	Total (dpm × 10^{-2})
Control	73±4	793±31	865±35
5 μg/ml AFE	71±1	886±29	898±63

Endothelial cells were cultured for 72 h with 370 kBq/ml [^{35}S]sulfate in the presence of 5 μg/ml AFE. Values are means±S.E. of 4 samples.

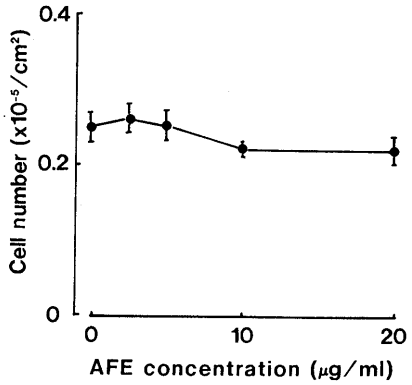

Fig. 1. Effect of AFE on the Number of Cultured A10 Cells

The cells were cultured in the presence of 2.5, 5.0, 10 or 20 μg/ml AFE for 48 h and the number counted. Values are means±S.E. of 4 samples.

TABLE IV. Effect of 5 μg/ml AFE on the Incorporation of [^{3}H]Thymidine and [^{14}C]Leucine into 5% TCA-Insoluble Fraction of the Layers of Endothelial Cells and A10 Cells

	Endothelial cells		A10 cells	
	[^{3}H]-Thymidine	[^{14}C]-Leucine	[^{3}H]-Thymidine	[^{14}C]-Leucine
Control	114±4	110±7	273±12	374±8
5 μg/ml AFE	174±4[a)]	209±5[a)]	267± 7	358±7

Endothelial cells or A10 cells were cultured for 72 h and labeled with 4.81 kBq/ml [^{3}H]thymidine and 3.33 kBq/ml [^{14}C]leucine during the last 3 h of the culture. Values are means±S.E. of 5 samples expressed as dpm × 10^{-1}/μg DNA in [^{3}H]thymidine and as dpm/μg DNA in [^{14}C]leucine. *a)* Significantly different from control, $p<0.001$.

increase cell number. As shown in Table IV, 5 μg/ml AFE significantly increased the incorporation of both [^{3}H]thymidine and [^{14}C]leucine by endothelial cells but did not change that by A10 cells. Although bFGF contents in the medium and the cell layer of A10 cells were measured by radioimmunoassay, there was no detectable amount of bFGF in either compartment.

Discussion

We previously reported that AFE has a stimulatory effect on the proliferation of cultured bovine aortic endothelial cells.[4)] Endothelial cells have bFGF receptors[7)] and produce bFGF as an autocrine which regulates DNA synthesis.[6)] Accordingly, AFE stimulation of endothelial cell proliferation was considered to be due to an enhanced production of bFGF by the cells or to a direct stimulation of DNA synthesis. In the present study, we showed that AFE treatment increased the number of endothelial cells but a combination of AFE with UFH conversely decreased it (Table I). Imamura and Mitsui[13)] reported that growth promotion of endothelial cells by bFGF was suppressed by heparin. Hence, it was suggested that AFE-stimulated proliferation of endothelial cells was dependent on the production of bFGF.

It was revealed that AFE increased total accumulation of bFGF in cultures of endothelial cells (Table II). The enhancement of accumulation occurred in the medium, the low-affinity and the cell extract fraction. Glycosaminoglycans derived from endothelial cells bind bFGF and protect it from thermal denaturation or proteolytic degradation.[14)] Furthermore, the bFGF bound to glycosaminoglycans is a source of long-term stimulation of DNA synthesis by endothelial cells.[15)] It was therefore postulated that bFGF increased by AFE contributed to AFE stimulation of endothelial cell proliferation.

However, the content of ^{35}S-glycosaminoglycans in the cell layer was not increased by AFE (Table III). This indicated that AFE-induced increase in bFGF in the low-affinity fraction may be due to the higher concentration of bFGF in the medium of AFE-treated cultures and/or to a facilitation of bFGF binding to glycosaminoglycans, but not due to an increase in glycosaminoglycan content. Since the accumulation of ^{35}S-glycosaminoglycans in the medium as well as in the cell layer was not changed by AFE. AFE might not affect the metabolism of glycosaminoglycans. Further, the increased accumulation of bFGF in the medium of AFE-treated cultures would be involved in the stimulation of endothelial cell proliferation as a source of supply of bFGF to the cell layer.

The accumulation of bFGF in the high-affinity fraction was not increased by AFE, suggesting that the primary effect of AFE on endothelial cells was the induction of bFGF production rather than bFGF receptor production or facilitation of bFGF binding to the receptors.

The bFGF bound to receptors is internalized and degraded in lysosome,[16)] however, the rate of the degradation of internalized bFGF may be slower than its rate of uptake, leading to an accumulation of bFGF within the cells. Thus, we speculate that AFE-induced increase in bFGF accumulation in the cell extract fraction may reflect an elevated rate of internalization of bFGF rather than an inhibition of its degradation. In other words, endothelial cells treated with AFE might use more bFGF than control cell did.

It was demonstrated that AFE did not increase the number of A10 cells (Fig. 1); it increase both DNA and protein systheses by endothelial cells but did not change those by A10 cells (Table IV). These results clearly indicate that AFE may not stimulate the proliferation of vascular smooth muscle cells and that protein synthesis might be involved in the AFE stimulation of endothelial cell proliferation. Vascular smooth muscle cells were also reported to have bFGF receptors and proliferation stimulated by bFGF.[17)] In addition, it was reported that vascular smooth muscle cells from bovine aorta express the bFGF gene.[18)] However, Clemmons and Wyk[19)] reported that DNA synthesis of vascular smooth muscle cells from porcine aorta is linked to the capacity of produce somatomedine-like peptide. A10 cells which were used in this study did not produce a detectable amount of bFGF. Thus, it is not conclusive whether or not the proliferation of vascular smooth muscle cells *in vivo* is regulated by bFGF. Nevertheless, an assumption can be made that the primary effect of AFE on cell proliferation is the stimulation of bFGF production, so that the proliferation of cells such as A10 cells which do not produce bFGF as an autocrine is not stimulated by AFE.

In conclusion, it was demonstrated that AFE stimulated the proliferation of endothelial cells through the stimulation of bFGF production. This effect on the cell function is considered beneficial in the evaluation of AFE as a drug for use in treating arteriosclerosis and thrombosis.

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