A Stimulatory Effect of Artemisia Leaf Extract on the Proliferation of Cultured Endothelial Cells

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To investigate the effect of the hot water extract from Artemisia leaf (*Artemisia princeps* PANPANINI) (AFE) on the proliferation of endothelial cells, the cells from bovine aorta were cultured for up to 96 h in the presence of 1, 5, 10 or 50 μg/ml AFE in RPMI1640 medium supplemented with 10% fetal bovine serum. After a 72 h culture, the cell number was significantly increased by AFE at 1, 5 and 10 μg/ml. An increase in the cell number by 5 μg/ml AFE observed after a 72 or 96 h treatment. The incorporations of both [^{3}H]thymidine and [^{14}C]leucine by the growing cells were significantly increased by 5 μg/ml AFE after a 72 h treatment. In addition, the incorporation of [^{3}H]thymidine by either growing or confluent cells was significantly increased by 50 μg/ml AFE after a 72 h treatment. The stimulatory activity of AFE was recognized in the low-molecular-weight fraction (molecular weight ≦ 10000 dalton). These results clearly indicated that AFE contained some low-molecular-weight component(s) which stimulates the proliferation of vascular endothelial cells *in vitro*.

Keywords Artemisia leaf; endothelial cell; DNA synthesis; mitogen; plant extract; proliferation

Vascular endothelial cells are significantly involved in vascular homeostasis including hemostasis and prevention of thrombosis. The cells produce prostacyclin,[1] tissue plasminogen activator,[2] tissue plasminogen activator inhibitor[3] and von Willebrand factor.[4] A retardation of proliferation after injury to endothelial cells causes vascular pathology such as arteriosclerosis and thrombosis. Thus, it is important to study stimulators of endothelial cell proliferation.

Endothelial cell growth factors have been found in plasma,[5] bovine brain[6] and some cultured cell lines.[7,8] These factors may be physiologically involved in neovascularization resulting from the growth of fetus and neonate, recovery of injured vessels and inflammation. However, there is no drug so far available to induce the proliferation of endothelial cells.

We have studied the effect of traditional herbal drugs on blood coagulation and fibrolysis. We found that the water-soluble extract of Artemisia leaf inhibits both blood coagulation and fibrolysis,[9] and that of Gardenia fruit accelerates fibrolysis.[10] Moreover, we found that the extract of Gardenia fruit stimulates the proliferation of endothelial cells.[11] We considered that the Artemisia leaf extract may also contain components which have a stimulatory effect on endothelial cells.

In the present study, we found that the water soluble extract of Artemisia leaf (*Artemisia princeps* PANPANINI) (AFE) stimulates the proliferation of cultured endothelial cells.

Materials and Methods

Materials RPMI1640 medium and fetal bovine serum were purchased from Nissui Pharmaceutical Co., Ltd. (Japan) and Filtron (Australia), respectively. Tissue culture plates and dishes were from Costar (U.S.A.). [Methyl-^{3}H]thymidine (6.7 Ci/mmol and 20 Ci/ml) and L-[^{14}C(U)]leucine (317.8 mCi/mmol) were obtained from New England Nuclear Corp. (U.S.A.). A disposable ultrafiltration instrument, Centricut 10, was purchased from Kurabo (Japan).

Preparation of AFE A dried powder (150 g) extracted by hot water from 1 kg of Artemisia leaf (*Artemisia princeps* PANPANINI) was supplied by Tsumura Co., Ltd. (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.

Cell Culture and Cell Counting Endothelial cells were isolated from bovine aorta by scraping the surface of the intima. The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum in a 60 mm dish at 37 °C in a humid atmosphere of 5% CO_2 in air until confluent. Then, the cells were transferred into 24-well culture plates at about 10^5 cells/well and cultured in the presence of AFE (1, 5, 10 or 50 μg/ml) for 24, 48, 72 or 96 h. After culture, the medium was discarded and the cell layer was washed twice with Ca,Mg-free phosphate-buffered saline (CMF-PBS), and dispersed with 0.25% trypsin–0.02% ethylenediamine tetraacetic acid (EDTA) in CMF-PBS. The cell suspension was well pipeted and the cell number was counted with hematocytometer.

Examination of Deoxyribonucleic Acid (DNA) Synthesis Endothelial cells were cultured in 35 mm dishes for 72 h in the presence of 50 μg/ml AFE and labeled with 2 μCi/ml [^{3}H]thymidine during the last 3 h of the culture. In another experiment, cells were cultured until confluent, then treated with 50 μg/ml AFE for 72 h. The metabolic labeling with [^{3}H]thymidine was carried out in the same way. To clarify the effect of AFE on both DNA and protein syntheses, cells were cultured in 35 mm dishes for 72 h in the presence of 5 μg/ml AFE and labeled with both 0.13 μCi/ml [^{3}H]thymidine and 0.05 μCi/ml [^{14}C]leucine during the last 3 h of the culture. 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 DNA by the method of Kissane and Robins.[12] The incorporation of [^{3}H]thymidine or [^{14}C]leucine into the 5% trichloroacetic acid (TCA)-insoluble fraction of the cell homogenate was measured by liquid scintillation counting using a portion of the cell homogenate.

Separation of AFE into Low-Molecular-Weight (LMW) and High-Molecular-Weight (HMW) Fractions AFE (5.0 ml at 2.5 mg/ml) was ultrafiltered in a Centricut 10 at 1000 *g* for 3 h to separate the AFE into the LMW (molecular weight ≦ 10000 dalton (Da)) and the HMW (molecular weight ≧ 10000 Da) fractions. The LMW and HMW fractions from 1 g of AFE contained 678 and 230 mg, respectively, of the extracts. The effect of either 3.39 μg/ml LMW fraction or 1.15 μg/ml HWM fraction obtained from 5 μg/ml AFE on the cell number was examined after a 72 h treatment.

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

Results and Discussion

After a 72 h culture, AFE significantly increased the number of cultured endothelial cells at all tested concentrations (Fig. 1). A time course study showed that treatment of the cells with 5 μg/ml AFE tended to increase the cell number at 72 h and significantly increased it at 96 h (Fig. 2). These results clearly indicate that AFE stimulates endothelial cell proliferation. As Artemisia leaf is a com-

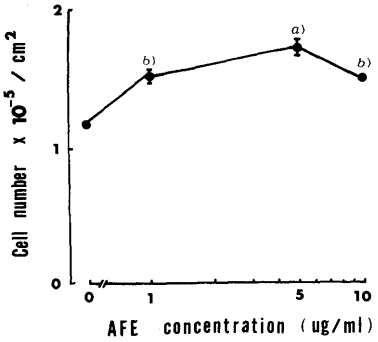

Fig. 1. Effect of AFE on the Number of Cultured Endothelial Cells

The cells were cultured in the presence of 1, 5 or 10 μg/ml AFE for 72 h and the number was counted. Values are means ± S.E. of 4 samples. *a*) Significantly different from the control, $p<0.01$; *b*) significantly different from the control, $p<0.001$.

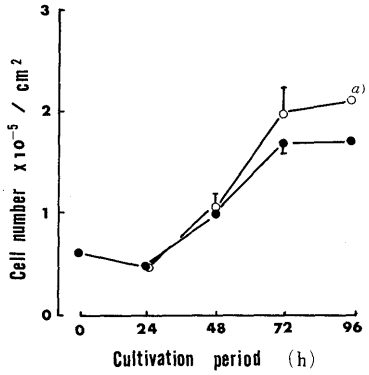

Fig. 2. Time Course Study of the Effect of AFE on the Number of Cultured Endothelial Cells

The cells were cultured in the presence of 5 μg/ml AFE for 24, 48, 72 or 96 h and the number was counted. Values are means ± S.E. of 4 samples. *a*) Significantly different from the control, $p<0.05$. ○, 5 μg/ml AFE; ●, control.

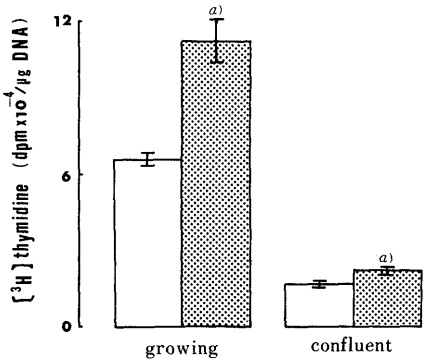

Fig. 3. Effect of AFE on the Incorporation of [^{3}H]Thymidine into the 5% TCA-Insoluble Fraction of Cultured Endothelial Cells in the Growing or Confluent State

The cells were cultured for 72 h in the presence of 50 μg/ml AFE (growing), or cultured until confluent then treated with 50 μg/ml AFE for 72 h (confluent). Metabolic labeling with [^{3}H]thymidine was carried out during the last 3 h of the culture. Values are means ± S.E. of 4 samples. *a*) Significantly different from the control, $p<0.01$. □, control; ▨, 50 μg/ml AFE.

ponent of Kyuki-kyogai-to (Xiong-gui-jiao-ai-tang), which is a formulation of herbal drugs for hemostasis, a significant role of Artemisia leaf in the formulation may be an acceleration of endothelium restoration by stimulation of endothelial cell proliferation after hemostasis.

It has been reported that endothelial cells at subconfluence are not the same as those at confluence with respect

TABLE I. Effect of 5 μg/ml AFE on the Incorporations of [^{3}H]Thymidine and [^{14}C]Leucine into the 5% TCA-Insoluble Fraction of Growing Endothelial Cell Layer

	[^{3}H]Thymidine (dpm × 10^{-2}/μg DNA)	[^{14}C]Leucine (dpm/μg DNA)
Control	94 ± 4	177 ± 4
5 μg/ml AFE	177 ± 6[a)]	225 ± 8[b)]

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

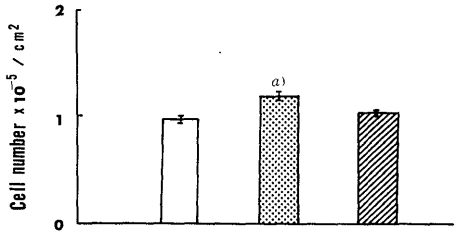

Fig. 4. Effect of the LMW or HMW Fraction of AFE on the Number of Cultured Endothelial Cells

The cells were cultured in the presence of the LMW or HMW fraction of AFE for 72 h and the number was counted. Values are means ± S.E. of 4 samples. *a*) Significantly different from the control, $p<0.05$. □, control; ▨, LMW; ▨, HMW.

to low density lipoprotein metabolism[13] and the production of growth factors for smooth muscle cells and fibroblasts was reported.[14] Accordingly, we examined whether AFE stimulation of DNA synthesis occurs not only in growing cells but also in confluent cells. As shown in Fig. 3. AFE at 50 μg/ml significantly increased the incorporation of [^{3}H]thymidine by both growing cells and confluent cells. No morphological abnormality was caused by the AFE treatment.

Greenburg *et al.*[15] reported that serum-free conditioned medium of cultured endothelial cells from bovine aorta was effective for the proliferation of the cells. This suggests that cultured endothelial cells produce a growth factor for themselves. It is possible that AFE stimulates the synthesis of such a growth factor by the cells. As shown in Table I, AFE at 5 μg/ml significantly increased both [^{3}H]thymidine and [^{14}C]leucine incorporations. Accordingly, the AFE-induced increase in the number of endothelial cells occurred with a stimulation of both DNA and protein syntheses. Thus, we postulate that AFE might enhance the growth factor production as well as directly stimulating DNA synthesis. However, the mechanism of the AFE stimulation of endothelial cell proliferation remains to be elucidated.

The HMW fraction of AFE did not increase the cell number, whereas the LMW fraction significantly increased it (Fig. 4). This suggests that the AFE stimulation of endothelial cell proliferation was due to LMW compound(s) but not to macromolecule(s) in AFE. It is likely that LMW compounds were more stable than macromolecules to hot water extraction.

In conclusion, it was demonstrated that AFE stimulated the proliferation of cultured endothelial cells. The stimulatory activity was present in the LMW fraction of AFE. AFE may be effective for promoting the restration of injured endothelium.

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