Stimulants from Gardeniae Fructus for Cultured Endothelial Cell Proliferation

Toshimitsu HAYASHI,*,[a] Toshiyuki KAJI,[b] Masami TAKEBAYASHI,[a] Rie SOEJIMA,[a] Naokata MORITA,[a] Michiko SAKAMOTO,[b] and Nobuo SAKURAGAWA[c]

Faculty of Pharmaceutical Sciences[a] and Faculty of Medicine,[c] Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930–01, Japan and Faculty of Pharmaceutical Sciences, Hokuriku University,[b] Ho-3 Kanagawa-machi, Kanazawa 920–11, Japan. *Received August 29, 1991*

Bioassay-guided fractionation of Gardeniae Fructus extract (GFE), which stimulates the proliferation of cultured endothelial cells, led to the isolation of glycerol and D-mannitol. Both compounds significantly increased the incorporation of [^{3}H]thymidine and [^{14}C]leucine into the acid-insoluble fraction of bovine aortic endothelial cell layers in culture. This clearly indicated that glycerol and D-mannitol are active components of GFE on endothelial cell proliferation. On the other hand, they did not change the number of cultured vascular smooth muscle cells from bovine aorta. Glycerol and D-mannitol may be benefical drugs for vascular disorders.

Keywords Gardeniae Fructus; *Gardenia jasminoides*; endothelial cell; proliferation; glycerol; D-mannitol

Gardeniae Fructus (fruit of *Gardenia jasminoides* ELLIS, Rubiaceae) is an important crude drug which has been used in prescriptions of Chinese medicine such as Orengedoku-to (黄連解毒湯), Shishi-shi-to (梔子豉湯), Shishi-kankyo-to (梔子乾姜湯) and Inchin-ko-to (茵蔯蒿湯). The drug is expected to have antiinflammatory, choleretic, diuretic, antipyretic, sedative and hemostatic effects. Gardeniae Fructus was found to contain a number of iridoid glycosides as well as D-mannitol[1] and yellow pigments.[2] In recent years, several cinnamic acid derivatives were also isolated.[3]

Endothelial cells are located at the luminal surface of vessels and consist of a single layer of flattened, fairly uniform, polygonal and elongated cells. Recently, it has become evident that endothelial cells produce various biologically active substances influencing the blood coagulation-fibrinolytic system.[4] As endothelial cells compose a barrier between circulating blood and subendothelial tissues containing vascular smooth muscle cells and fibroblasts, an injury and a retarded growth of endothelial cells may cause pathologic changes such as thrombosis and smooth muscle cell hyperplasia with the eventual development of an atherosclerotic lesion.[4a,5] A search for stimulants of endothelial cell growth is therefore clinically important.

In our preliminary studies on the effects of oriental traditional medicines on blood coagulation and fibrinolysis, Gardeniae Fructus extract (GFE) was observed to accelerate fibrinolysis *in vitro*.[6] Furthermore, we found that GFE stimulates the proliferation of cultured endothelial cells.[7] Thus, it is suggested that a structural elucidation of the active compounds which stimulate the endothelial cell proliferation will contribute to a development of drugs for the prevention of vascular disorders, such as atherosclerosis and wound healing.

This paper deals with the isolation and identification of stimulants from GFE for cultured bovine aortic endothelial cell proliferation.

Materials and Methods

Materials Gardeniae Fructus was supplied by Tsumura Co., Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), ASF 301 medium and fetal bovine serum (FBS) were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), Ajinomoto Co., Ltd. (Tokyo, Japan) and Bioproducts, Inc. (Walkersville, MD, U.S.A.), respectively. Tissue culture plates and dishes were from Costar (Cambridge, MA, U.S.A.). [Methyl-^{3}H]thymidine (740 GBq/mmol) and L-[^{14}C(U)]leucine (11.8 GBq/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Endothelial cells and vascular smooth muscle cells from bovine aorta were gifts from Drs. Katsuo Sueishi and Yutaka Nakashima (First Department of Pathology, Faculty of Medicine, Kyushu University, Fukuoka, Japan).

Apparatus High performance liquid chromatography (HPLC) was performed using a Shimadzu LC-6A pump with a YMC-Pack Diol-120 column (300 × 8 mm i.d., YMC Co., Ltd., Kyoto, Japan) and a Shimadzu RID-6A refractive index detector.

Extraction and Fractionation Gardeniae Fructus (80 g) was cut into small pieces and extracted with boiling water (4.8 l) for 1 h. After filtration through paper, the water soluble extract was lyophilized to give a brownish powder (GFE, 21.2 g). GFE was then dialyzed against distilled water at room temperature for 1 d. The dialyzable portion was concentrated under reduced pressure and the residue was lyophilized to yield brownish powder (GFE-L, 15.4 g). GFE-L was applied on a column of charcoal activated (60—150 mesh, Nacalai Tesque Inc. Kyoto, Japan), followed by elution with H_2O, MeOH and $CHCl_3$, successively. The fractions eluted with H_2O were lyophilized to give a colorless powder (GFE-L-W, 4.6 g) which was then chromatographed over Toyopearl HW-40F (Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan) with H_2O to afford two fractions, GFE-L-W-I (3.8 g) and GFE-L-W-II (28 mg).

Isolation of Stimulants for Proliferation of Endothelial Cells GFE-L-W-I (1.2 g) was repeatedly chromatographed over DEAE Toyopearl 650 M (Toyo Soda), Bio Gel P-4 and Bio Gel P-2 (200—400 mesh, Bio Rad Laboratories, CA, U.S.A.) with H_2O to give compounds **1** (70 mg), **2** (94 mg) and **3** (37 mg). The compounds **1** and **2** were identified as glycerol and D-mannitol, respectively, by direct comparison of spectral data of **1** and **2** with those of authentic samples. Compound **3**: colorless powder; phenol–H_2SO_4 test: +; Fehling test: −.

Cell Culture and Cell Counting Endothelial cells or smooth muscle cells were each plated in 24-well culture plates and cultured for 24 h in DMEM supplemented with 10% FBS at 37 °C in a humid atmosphere of 5% CO_2 in air. The medium was then discarded and the cell layer was washed twice with a serum-free ASF 301 medium; the cells were cultured for 72 h in serum-free ASF 301 medium in the presence of the sample. After culture, the cell layer was dispersed with 0.25% trypsin–0.02% ethylenediaminetetraacetic acid in Ca, Mg-free phosphate-buffered saline (CMF-PBS). The cell suspension was well-pipetted and the cell number was counted with a hematocytometer.

Deoxyribonucleic Acid (DNA) and Protein Synthesis The endothelial cells or smooth muscle cells were each plated in 6-well culture plates and cultured for 24 h in DMEM supplemented with 10% FBS. The medium was then discarded and the cell layer was washed twice with a serum-free ASF 301 medium. The cell layer was incubated at 37°C for 72 h in the presence of the sample at 0.1 μg/ml and labeled with 10 kBq/ml [^{3}H]-thymidine combined with or without 25 kBq/ml [^{14}C]leucine during the last 3 h of the incubation. After incubation, the medium was discarded and the cell layer was washed twice with CMF-PBS; the cell layer was 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 content by the method of Kissane and Robins.[8] The incorporation of [^{3}H]thymidine and [^{14}C]leucine into the

5% trichloroacetic acid (TCA)-insoluble fraction of the cell homogenate was measured by a liquid scintillation counter using a portion of the homogenate.

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

Results and Discussion

In a previous paper, we reported that the low molecular mass fraction of GFE (GFE-L) stimulated the proliferation of cultured endothelial cells from the bovine aorta.[7] Thus, bioassay-guided fractionation of GFE-L was carried out, and three active compounds **1**—**3** were isolated (Chart 1). Compounds **1** and **2** were identified as glycerol and D-mannitol, respectively, by direct comparison of spectral data of **1** and **2** with those of authentic samples.

Compound **3** was obtained as a colorless powder which was positive on phenol–H_2SO_4 test and negative on Fehling test. The molecular weight of compound **3** was estimated to be about 8000 by HPLC using a series of polyethyleneglycols as marker substances. When compound **3** was treated with 2N trifluoroacetic acid (TFA) or 2N H_2SO_4, only a starting material was recovered, suggesting the absence of glycosidic linkage in compound **3**. A further detailed structure of compound **3** is currently under investigation.

Generally, glycerol exists as glycerides which are widely distributed in vegetoanimal oils and fats. It was originally found in olive oil from fruits of *Olea europaea* L. Glycerol has now been isolated from Gardeniae Fructus for the first time. It is one of the drugs clinically used as a laxative, enema, osmotic diuretic and as a glaucoma remedy. The drug was reported to show various biological effects such as stimulation of the assembly of microtubules,[9] induction of differentiation of erythroleukemia cells,[10] suppression of spermatogenesis[11] and heat shock protein induction during heat shock conditions.[12] On the other hand, D-mannitol, which had already been isolated as a major ingredient of Gardeniae Fructus by H. Inouye *et al.*,[1c] is one of the most frequently used medicaments for the prevention of renal failure and in neurosurgical practice. It was revealed that D-mannitol has a protective effect in myocardial ischemia and necrosis[13] and dilating effects on vascular smooth muscle in the cerebral micro circulation.[14] Lung injury following abdominal aortic aneurysmectomy was also prevented by D-mannitol.[15] Some of these biological effects of glycerol and D-mannitol were explained by their hypertonicity or functions as a hydroxyl radical scavenger.[16]

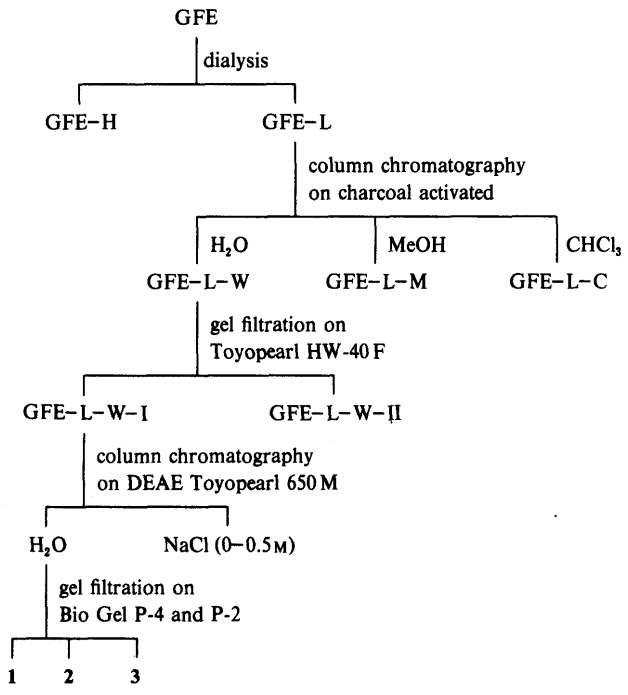

Chart 1. Fractionation of Gardeniae Fructus Extract (GFE)

To assess the proliferation of endothelial cells, we examined the effect of glycerol, D-mannitol and compound **3** on the number of endothelial cells. As shown in Table I, all of these isolates significantly increased the number of endothelial cells, suggesting that they stimulated the proliferation of endothelial cells. We then investigated the effect of these compounds on the incorporation of [^{3}H]-thymidine and [^{14}C]leucine into the acid-insoluble fraction of the endothelial cell layer to confirm this suggestion. As indicated in Table II, they significantly accelerated both incorporations, suggesting that they increased both DNA and protein synthesis by endothelial cells. On the other hand, the number of vascular smooth muscle cells was not increased by these compounds (data not shown); no increase of the incorporation of [^{3}H]thymidine into the TCA-insoluble fraction of the cultured vascular smooth muscle cell layer by these compounds was observed (Table III). These results suggest that the stimulatory effect of glycerol, D-mannitol and compound **3** is selective for endothelial cell proliferation. These experimental results are consistent with those of crude GFE,[17] suggesting that these compounds may stimulate the proliferation of endothelial cells through a stimulation of the synthesis of basic fibroblast growth factor (bFGF), an autocrine of

Table I. Effect of Glycerol, D-Mannitol and Compound **3** on the Number of Cultured Endothelial Cells

Sample	Cell number ($\times 10^{-4}$ cells/cm^2)
Control	4.31±0.15
Glycerol	4.93±0.04[a]
D-Mannitol	5.24±0.23[b]
Compound **3**	5.39±0.42[a]

Endothelial cells were plated at 3.0×10^4 cells/well and cultured for 24 h in 10%FBS–DMEM. After culture, the cell layer was washed twice with serum-free ASF301 medium and cultured for 72 h in serum-free ASF301 medium in the presence of 0.1 μg/ml glycerol, D-mannitol or compound **3**. Values are means±S.E. of 5 samples. Significantly different from control, *a*) $p<0.05$; *b*) $p<0.01$.

Table II. Effect of Glycerol, D-Mannitol and Compound **3** on the Incorporation of [^{3}H]Thymidine and [^{14}C]Leucine into 5% TCA-Insoluble Fraction of Cultured Endothelial Cell Layer

Sample	[^{3}H]Thymidine (dpm/μg DNA $\times 10^{-2}$)	[^{14}C]Leucine (dpm/μg DNA $\times 10^{-1}$)
Control	254±26	398±37
Glycerol	307±21[a]	450±38[a]
D-Mannitol	344±172[a]	492±13[a]
Compound **3**	334±14[a]	502±27[a]

Endothelial cells were plated at 5.0×10^4 cells/well and cultured for 24 h in 10%FBS–DMEM. After culture, the cell layer was washed twice with serum-free ASF301 medium and cultured for 72 h in serum-free ASF301 medium in the presence of 0.1 μg/ml of glycerol, D-mannitol or compound **3**. Values are means±S.E. of 4 samples. *a*) Significantly different from control, $p<0.05$.

TABLE III. Effect of Glycerol, D-Mannitol and Compound 3 on the Incorporation of [^{3}H]Thymidine into 5% TCA-Insoluble Fraction of Cultured Vascular Smooth Muscle Layer

Sample	[^{3}H]Thymidine incorporation (dpm/μg DNA)
Control	1477±94
Glycerol	1123±51[a]
D-Mannitol	1292±120[a]
Compound 3	1478±82

Vascular smooth muscle cells were plated at 2.0×10^4 cells/well and cultured for 24 h in 10%FBS–DMEM. After culture, the cell layer was washed twice with serum-free ASF301 medium and cultured for 72 h in the presence of 0.1 μg/ml glycerol, D-mannitol or compound 3. Values are means±S.E. of 5 samples. Significantly different from the control, *a*) $p<0.05$.

TABLE IV. Effect of Glycerol and D-Mannitol on the Number of Cultured Endothelial Cells

Sample	(*n*)	Cell number ($\times 10^{-4}$ cells/cm^2)		
			Glycerol	D-Mannitol
Control	(8)	3.59±0.15		
0.001 μg/ml	(4)		3.90±0.08	3.38±0.25
0.01 μg/ml	(4)		3.93±0.15	3.57±0.18
0.1 μg/ml	(4)		3.99±0.07[a]	3.97±0.07[a]
1.0 μg/ml	(4)		3.62±0.17	3.83±0.23

Endothelial cells were plated at 3.0×10^4 cells/well and cultured for 24 h in 10%FBS–DMEM. After culture, the cell layer was washed twice with serum-free ASF301 medium and cultured for 72 h in serum-free ASF301 medium in the presence of glycerol or D-mannitol. Values are means±S.E. *a*) Significantly different from control, $p<0.05$.

endothelial cells for their proliferation. This speculation is supported by the selective stimulation of endothelial cell proliferation by these compounds. Some other assumptions on the mechanism by which these compounds stimulated endothelial cell proliferation can be made. One possible mechanism is that they potentiated the activity of bFGF by their anionic structure, like polyanionic heparin.[18] Also, we speculate that these compounds may have a capacity to stimulate a receptor for bFGF production which has not been elucidated yet. Recently, J. P. Wiebe and C. J. Dinsdale showed that glycerol inhibited the proliferation of baby hamster kidney (BHK), Chinese hamster ovary (CHO), mammary cancer fibroblast (MCF-7), human breast line (HBL) and human glioma cells in culture at much higher concentrations (2—4% in the culture medium).[19] However, it is noteworthy that the stimulation with glycerol as well as D-mannitol for endothelial cell proliferation was selective for endothelial cells and the effective dose was as low as 0.1 μg/ml (Table IV). It is reported that β-glycerophosphate, a derivative of glycerol, may stimulate the proliferation of osteogenic cells.[20] In addition, R. I. Levin *et al.* noted that nitroglycerin, also a glycerol derivative, stimulated the synthesis of prostacyclin (PGI_2) by cultured human endothelial cells.[21] These findings indicate that polyalcoholic compounds and their analogues may exert influence on some key enzyme systems relating to the cell proliferation and physiologic functions of endothelial cells. Therefore, we examined the effect of other sugar alcohols and related compounds on the incorporation of [^{3}H]thymidine into a TCA-insoluble fraction of the endothelial cell layer. Among the compounds illustrated in Table V, only glycerol

TABLE V. Effect of D-Mannitol, Sorbitol, Xylitol, Ribitol, DIP-Mannitol, Glycerol and β-Glycerophosphate on the Incorporation of [^{3}H]Thymidine into a 5% TCA-Insoluble Fraction of Cultured Endothelial Cell Layer

Sample	[^{3}H]Thymidine incorporation (dpm/μg DNA $\times 10^{-2}$)
Control	649± 6
D-Mannitol	850±43[b]
Sorbitol	493±45[b]
Xylitol	593±31
Ribitol	646±70
DIP-mannitol[a]	236±25[c]
Glycerol	787±83[b]
β-Glycerophosphate	546±33

Endothelial cells were plated at 3.0×10^4 cells/well and cultured for 24 h in 10% FBS–DMEM. After culture, the cell layer was washed twice with serum-free ASF301 medium and cultured for 72 h in serum-free ASF301 medium in the presence of 0.1 μg/ml each sample. *a*) 1,2;5,6-Di-*O*-isopropylidene-D-mannitol. Values are means±S.E. of 4 samples. Significantly different from the control, *b*) $p<0.05$; *c*) $p<0.001$.

and D-mannitol accelerated the incorporation. This suggested that the effect of glycerol and D-mannitol may be specific for their structures.

The isolated compounds 1—3 from Gardeniae Fructus might be useful for the prevention of arteriosclerosis and thrombosis or wound healing. We are continuing further elucidation of stimulants for endothelial cell proliferation from Gardeniae Fructus, including compound 3.

References

1) *a*) T. Endo and H. Taguchi, *Chem. Pharm. Bull.*, **18**, 1066 (1970); *b*) *Idem, ibid.*, **21**, 2684 (1973); *c*) H. Inouye, Y. Takeda, S. Saito, H. Nishimura, and H. Sakurai, *Yakugaku Zasshi*, **94**, 577 (1974); *d*) H. Inouye, Y. Takeda, and H. Nishimura, *Phytochemistry*, **13**, 2219 (1974); *e*) Y. Takeda, H. Nishimura, O. Kadota, and H. Inouye, *Chem. Pharm. Bull.*, **24**, 2644 (1976).
2) T. Munesada, *Yakugaku Zasshi*, **42**, 666 (1922); R. Kuhn, A. Winterstein, and W. Wiegand, *Helv. Chim. Acta*, **11**, 716 (1928).
3) M. Nishizawa and Y. Fujimoto, *Chem. Pharm. Bull.*, **34**, 1419 (1986); M. Nishizawa, R. Izuhara, K. Kaneko, and Y. Fujimoto, *ibid.*, **35**, 2133 (1987).
4) *a*) G. Thorgeirsson and A. L. Robertson, *Am. J. Pathol.*, **93**, 803 (1978); *b*) I. Maruyama, *Acta Haematol. Jpn.*, **49**, 146 (1986); *c*) C. T. Esmon, *Science*, **235**, 1348 (1987); *d*) R. S. Cotran, *Am. J. Pathol.*, **129**, 407 (1987); *e*) M. S. Bajaj, M. N. Kuppuswamy, H. Saito, S. G. Spitzer, and S. P. Bajaj, *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 8869 (1990).
5) R. Ross, *N. Engl. J. Med.*, **314**, 488 (1986); A. Sumiyoshi and Y. Asada, *Ann. N. Y. Acad. Sci.*, **598**, 248 (1990).
6) N. Sakuragawa, K. Yuasa, M. Niwa, and S. Kondo, *Acta Med. Biol.*, **32**, 107 (1984).
7) T. Kaji, N. Miezi, K. Kaga, N. Ejiri, and N. Sakuragawa, *Planta Med.*, **56**, 353 (1990).
8) J. M. Kissane and E. Robins, *J. Biol. Chem.*, **233**, 184 (1958).
9) H. W. Detrich, S. A. Berkowitz, H. Kim, and R. C. Williams, *Biochem. Biophys. Res. Commun.*, **68**, 961 (1976).
10) H. D. Preisler and G. Lyman, *Cell Differentiation*, **4**, 179 (1975).
11) J. P. Wiebe and K. J. Barr, *Life Sci.*, **34**, 1747 (1984).
12) B. V. Edington, S. A. Whelan, and L. E. Hightower, *J. Cell Physiol.*, **139**, 219 (1989).
13) J. T. Willerson, W. J. Powell, T. E. Guiney, J. J. Stark, C. A. Sanders, and A. Leaf, *J. Clin. Invest.*, **51**, 2989 (1972); W. J. Powell, D. R. DiBona, J. Flores, and A. Leaf, *Circulation*, **54**, 603 (1976); G. J. Vlahakes, S. R. Giamber, K. O. Rothaus, and W. J. Powell, *J. Surg. Res.*, **47**, 438 (1989).
14) M. Takayasu and R. G. Dacey, *Neurosurg.*, **25**, 747 (1989).
15) I. S. Paterson, J. M. Klausner, G. Goldman, R. Pugatch, H. Feingold, P. Allen, J. A. Mannick, C. R. Valeri, D. Shepro, and H. B. Hechtman, *Ann. Surg.*, **210**, 796 (1989).
16) R. F. D. Maestro, H. W. Thaw, J. Bjork, M. Planker, and K. E.

Arfors, *Acta Physiol. Scand.*, Suppl., **492**, 43 (1980); R. P. Kaufmann, J. M. Klausner, H. Anner, H. Feingold, L. Kobzik, C. R. Valeri, D. Shepro, and H. B. Hechtman, *J. Trauma*, **28**, 458 (1988).
17) T. Kaji, T. Hayashi, N. Miezi, K. Koga, N. Ejiri, and N. Sakuragawa, *Chem. Pharm. Bull.*, **39**, 1312 (1991).
18) S. C. Thornton, S. N. Mueller, and E. M. Levine, *Science*, **222**, 623 (1983).
19) J. P. Wiebe and C. J. Dinsdale, *Life Sci.*, **48**, 1511 (1991).
20) T. Kaji, R. Kawatani, T. Hoshino, T. Miyahara, H. Kozuka, Y. Kurashige, and F. Koizumi, *Bone Mineral*, **9**, 89 (1990).
21) R. I. Levin, E. A. Jaffe, B. B. Weksler, and K. T. Goldman, *J. Clin. Invest.*, **67**, 762 (1981).