Biotechnology and Bioprocess Engineering **2005**, 10: 372-377

Water Extracts of *Aralia elata* Root Bark Enhances Migration and Matrix Metalloproteinases Secretion in Porcine Coronary Artery Endothelial Cells

In Suk Oh[1], Ji Won Han[2], and Hwan Gyu Kim[2]*

[1] Institute of Basic Sciences, Chonbuk National University, Chonju, Chonbuk 561-756, Korea
[2] Division of Biological Sciences, Research Center of Bioactive Materials, Chonbuk National University, Chonju, Chonbuk 561-756, Korea

Abstract *Aralia elata* is an edible mountain vegetable. Angiogenesis, the formation of new blood vessels, is a process involving migration, proliferation and cell differentiation, as well as the formation of new capillary structures. Matrix metalloproteinases (MMPs) plays an important role in angiogenesis. The development of a functional vascular system requires a variety of growth factors, their receptors, and intracellular signals. This study examines the effects of water extracts from: (i) *A. elata* root bark (Aralia extracts); (ii) a combination of Aralia extracts and fibroblast growth factors (FGF-2) on cultured porcine coronary artery endothelial cells (PCAECs). Aralia extracts induced the migration of PCAECs, which was inhibited by MMPs inhibitors. Combining Aralia extracts and FGF-2 enhanced the migration and the secretion of MMP-2 and MMP-9 from PCAECs. We postulated that the Aralia extracts, which induced migrating activity in PCAECs, may be accomplished by increased secretion levels of MMP-2 and MMP-9.

Keywords: *Aralia elata*, migration, FGF-2, MMPs

INTRODUCTION

Since natural food provides nutritional sources of bioactive compounds, many screening attempts have been conducted to identify lead compounds that are clinically important. *A. elata* is an edible mountain vegetable in Korea, which contains saponin, alkaloid, palmitic acid, linoleic acid, methyl eicosanoate and hexacosol. It manifests an effect on cardiac infarction, gastric ulcer, colitis, and enervation [1-5].

FGF-2 is a member of a large family of proteins that binds heparin and heparan sulfate and modulates the function of a wide range of cell types [6]. FGF-2 modulates numerous cellular functions in various cell types, including cell proliferation, differentiation, survival, adhesion, migration, and in processes such as wound healing, angiogenesis and vasculogenesis [7]. FGF-2 also regulates the expression of several very large biomolecules that are believe to mediate critical steps during angiogenesis. These include interstitial collagenase, urokinase type plasminogen activators (uPA), plasminogen activator inhibitors, uPA receptors, and β1 integrins [8,9]. These very large biomolecules may contribute to the invasive phenotype displayed by endothelial cells during angiogenesis [10]. Angiogenesis induced by FGF-2 also involves αvβ3 integrin and extracellular matrix (ECM) [11].

Angiogenesis is a complex physiological process consisting of several distinct steps leading to the development of new blood cells. It begins when the surrounding basement membrane capillaries start to degrade [12,13]. Then, the endothelial cells migrate towards angiogenic stimuli and proliferate. New data suggests that MMPs play an important role in angiogenesis [14]. To encourage migrating and sprouting, endothelial cells must secrete protease to dissolve the adjacent ECM. One such family of enzymes is the MMPs.

There are currently 24 known variants of MMPs. They are classified into the following six groups based on their putative substrate specificities and sequence homologies [15]: interstitial collagenase, gelatinases (type IV collagenase), stromelysins, metalloelastases, secreted RXXR-containing MMPs (stromelysin-3-like), and the membrane-type MMPs (MT-MMPs). Since MMPs are inhibited by endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) form 1:1 complexes with MMPs [16,17]. It is believed that the balance between the levels of MMPs and TIMPs is critical in regulating the breakdown of connective tissues by migrating cells. During the angiogenesis process, a variety of growth factors and cytokines exert their functions through autocrine, paracrine or endocrine action [13].

Recently, several studies have examined therapeutic angiogenesis that uses growth factors as approaches to treat ischemic myocardium [18,19]. However, a single

***Corresponding author**
Tel: +82-63-270-3352 Fax: +82-63-270-3362
e-mail: hgkim@moak.chonbuk.ac.kr

growth factor during angiogenesis may be insufficient for therapeutic purposes, because the development of a functional vascular system requires a cascade of growth factors, their receptors, and intracellular signals [20]. This study examines the effects of water extracts from *A. elata* root bark (Aralia extracts) and a combination of Aralia extracts and FGF-2 on cell migration and secretion of MMPs from PCAECs. Aralia extracts, or a combined treatment of Aralia extracts and FGF-2, were shown to induce the migration of PCAECs, which was inhibited by MMPs inhibitors. Aralia extracts induced the secretion of MMP-2 and MMP-9. Also, a combined treatment of Aralia extracts and FGF-2 had an enhanced effect on the secretion of MMP-2 and MMP-9. It is suggested, therefore, that Aralia extract-induced migrating activity in PCAECs may be accomplished by increased secretion levels of MMP-2 and MMP-9.

MATERIALS AND METHODS

Materials

Recombinant human MMP-2, MMP-9, TIMP-1, and TIMP-2 enzyme immunoassay kits were purchased from Fuji Chemical Industries (Toyama, Japan). Media and sera were obtained from Life Technology, Inc. (Gaithersburg, MD, USA). An MMP standard was purchased from Calbiochem (La Jolla, CA, USA). Recombinant human FGF-2 was purchased from Sigma (St. Louis, MO, USA). Other biochemical reagents, including gelatin, fibrinogen, Giemsa staining solutions, antibiotics, antimycotics and trypsin-EDTA were purchased from Sigma, unless otherwise specified.

Cell Culture

PCAECs were prepared from porcine coronary arteries by collagenase digestion, as previously described [21-23]. The endothelial origin of the cultures was confirmed by immunofluorescent staining with an anti-von Willebrand Factor antibody (Acceptable cultures had >95% fluorescent cells). These endothelial cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with a 20% (v/v) heat inactivated fetal bovine serum. The primary cultured cells used were taken between passages 2 and 4.

Preparation of *A. elata* Root Bark Extract

Authentic samples of the *A. elata* root bark were obtained from Sun-chang Gun, JeonBuk, Korea. Water extracts of the *A. elata* root bark were obtained as described by Lee *et al.* [24,25]. The extracts were prepared from fresh root bark of *A. elata*. A sample (200 g) was ground into a fine powder, and stirred in distilled water (3 L) for 48 h. The resulting filtrate was saturated with vapor pressure (121°C, 15 pound/inch^2). The precipitates containing coagulated proteins were removed by centrifugation. The supernatant was mixed with chloroform (1 L) using a separatory funnel. The chloroform fraction that contained proteins, resin and fiber was removed and the water fraction further mixed with *n*-hexane (1 L). Then, the *n*-hexane-soluble fraction was removed. Talc (Sigma, St. Louis, MO, USA) was added to the water-soluble fraction and the mixture was subjected to filtration using a membrane filter apparatus. The resulting filtrate was freeze-dried (7 g) and resuspended with water.

Migration Assays

A migration assay was performed using microcarrier beads as previously described [26]. PCAECs grown in confluence to microcarrier beads (diameter 175 μm; Sigma) and placed in a gelatinized 24-well plate (30 to 40 beads/well) in DMEM containing 2% serum with various reagents and incubated for 20 h. The wells were washed with PBS, fixed with ethanol, dried and stained with a Giemsa staining solution for 5 min. The cells that had migrated from the beads and attached to the wells were counted at magnification of 100× using an inverted phase-contrast microscope.

Enzyme Immunoassay of MMPs and TIMPs

PCAECs were seeded to 24-well plates at a density of 5 × 10^4 cells/cm^2 and grown for 24 h in DMEM supplemented with 20% serum. Then, confluent PCAECs were incubated in serum- and phenol red-free DMEM for 12 h. After the cells were washed with a fresh medium, a control buffer or indicated reagents were applied for 12 h. The actual quantities of the MMPs were assayed by enzyme immunoassay, according to the manufacturer's protocol (Fuji Chemical Industries, Toyama, Japan).

Zymography

PCAECs were seeded in 24-well plates at a density of 5 × 10^4 cells/cm^2 and grown for 24 h in DMEM supplemented with 20% serum. Then, confluent PCAECs were incubated in serum- and phenol red-free DMEM for 12 h. After the cells were washed with a fresh medium, a control buffer or the indicated reagents were applied for 12 h. The hydrolytic activities of MMPs were evaluated by gelatin zymography [27]. Samples were mixed using a 5× sample buffer (4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol and 0.1% bromophenol blue) and were applied to a 10% SDS-PAGE containing 0.1% gelatin. Reference standards were MMP-2 and MMP-9 (Chemicon, Temecula, CA, USA). After running, the gels were incubated in 2.5% Triton X-100 for 1 h and incubated in enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM $CaCl_2$ and 0.02% Brij-35) for 24 h at 37°C. The gels were stained with a 0.5% Coomassie brilliant blue 250 solution and destained with several changes of 30% methanol and 10% acetic acid.

Data Analysis

Data is expressed as means ± standard deviation (SD).

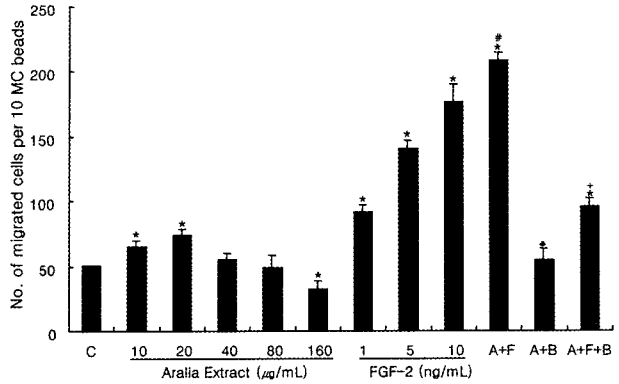

Fig. 1. Effects of single and combined treatment with Aralia extracts and FGF-2 on migratory in cultured PCAECs. To measure migratory activity, the cells grown to confluence on microcarrier beads, and the beads were placed into gelatinized 24-well plates in medium containing 2% serum and various amounts of Aralia extracts, FGF-2, Aralia extracts (20 μg/mL) plus FGF-2 (10 ng/mL) (A+F), BB94 (20 ng/mL) plus Aralia extracts (A+B), and BB94 (20 ng/mL) plus Aralia extracts plus FGF-2 (A+F+B) and incubated for 20 h. The number of endothelial cells that migrated from the beads and attached to the well per 10 beads were counted. Bars represent means ± SD from four independent experiments. Statistical significance was tested using one-way ANOVA followed by the Students *t* test. $^{*}P<0.05$ *vs* control buffer; $^{*}P<0.05$ *vs* Aralia extracts (20 μg/mL); $^{\#}P<0.05$ *vs* FGF-2 (10 ng/mL); and $^{+}P<0.05$ *vs* A+F.

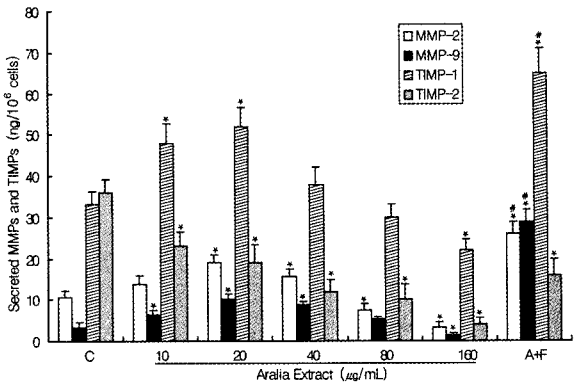

Fig. 2. Effects of single and combined treatment with Aralia extracts and FGF-2 on the secretion of MMPs and TIMPs in PCAECs. Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, the cells were incubated for 12 h after addition of a control buffer (C), various amounts of Aralia extracts, and Aralia extracts (20 μg/mL) plus FGF-2 (20 ng/mL) (A+F), and media were quantitatively assayed by enzyme immunoassay. Bars represent the means ± SD from three independent experiments. Statistical significance was tested using one-way ANOVA followed by the Students *t* test. $^{*}P<0.05$ *vs* control buffer; $^{\#}P<0.05$ *vs* Aralia extracts (20 μg/mL).

The statistical significance was tested using one-way ANOVA followed by the Student-Newman-Keuls Test. Statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Migration Assay of PCAECs

Placing microcarrier beads onto a confluent monolayer of PCAECs for 2 to 3 days produced beads covered by a confluent monolayer of cells with 25 to 30 cells per bead. When PCAECs bearing microcarrier beads were placed onto gelatinized plastic dishes with a control buffer for 20 h, they yielded a basal level of nondirectional migration (50 to 55 per 10 beads, Fig. 1). The number of migrating cells increased with the addition of Aralia extracts or FGF-2 stimulation in a dose-dependent manner. However, the addition of 160 μg/mL of Aralia extracts led to a significant decrease in cell migration. The migration potency of Aralia extracts was less than that of FGF-2. The doses of Aralia extracts (20 μg/mL) and FGF-2 (10 ng/mL) showing a submaximal effect on migratory activity were used in combined treatment FGF-2 is more potent than Aralia extracts. Our distinguished finding indicates that a combination of submaximal doses of Aralia extracts and FGF-2 is more potent than the maximal dose of Aralia extracts or FGF-2 alone. To enhance angiogenesis, endothelial cells have to increase their migration activity. Our results indicate that Aralia extracts are a suitable mitogen for PCAECs.

Aralia Extracts Induces MMP-2, MMP-9, and TIMP-1 Secretion but Suppresses TIMP-2 Secretion from PCAECs

A crucial step in angiogenesis is when proteases degrade the underlying basement membranes [24]. Endothelial cells release proteinases to degrade the ECM for their migration and proliferation *in vivo*. One family of such proteinases is the MMPs. The role of MMPs in angiogenesis has been demonstrated *in vitro* [15,17,29]. In culture, endothelial cells largely secrete MMP-2, which can disrupt the ECM and enable their migration and tube formation [30].

The step-wise activation processes of pro-MMPs suggest that MMP activities are controlled by endogenous inhibitors such as α_2-macroglobulin or tissue inhibitor of metalloproteinases (TIMPs) before MMPs are fully activated [31,32]. An enzyme immunoassay indicated that the culture media from PCAECs contained clearly detectable amounts of MMP-2, MMP-9, TIMP-1, and TIMP-2 (Fig. 2). The addition of Aralia extracts (20 μg/mL) on cultured PCAECs for 12 h produced increases of approximately 1.9-, 3.3-, and 1.6-fold in MMP-2, MMP-9 and TIMP-1 secretion, respectively, compared with the addition of a control buffer. On the other hand, the secretion of TIMP-2 was significantly suppressed compared with the addition of a control buffer (~52%). The interaction with TIMP-2 and pro-MMP-2 facilitated cell surface-mediated activation, whereas interactions with active MMP-2 resulted in inhibition. A critical factor is maintaining a balance between the levels of MMPs and TIMPs in regulating the break-

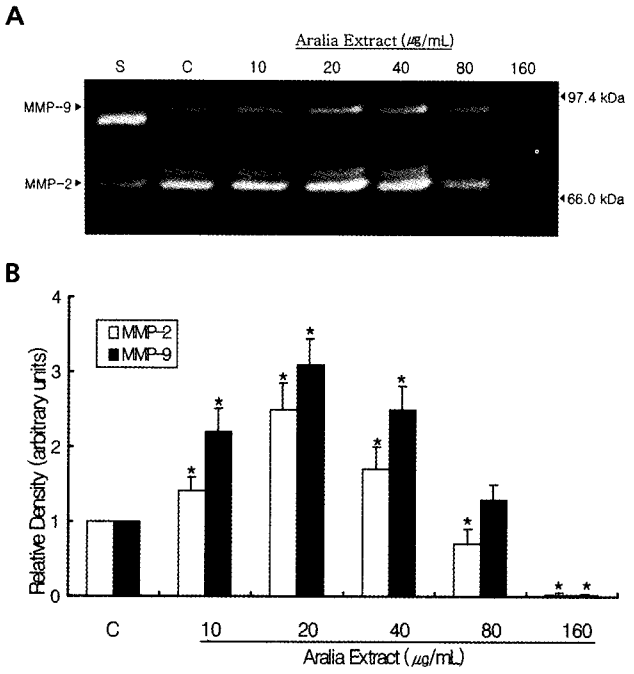

Fig. 3. Gelatin zymography of MMP-2 and MMP-9 in culture medium of PCAECs treated with Aralia extracts. (A) Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, the cells were incubated for 12 h after addition of control buffer (C) and various amounts of Aralia extracts. Equal amounts of proteins (10 μg/lane) from supernatants were loaded into each lane. Lane S contains standards of MMP-9 and MMP-2. (B) Densitometric analyses of the zymographs are presented as the relative ratio of induction of the MMP-2 or MMP-9 by addition of Aralia extracts. The MMP-2 or MMP-9 secretion by addition of control buffer for 12 h is arbitrarily presented as 1. Data are mean ± SD from three experiments. Statistical significance was tested using one-way ANOVA followed by the Student-Newman-Keuls test. $^*P<0.05$ *vs* control buffer.

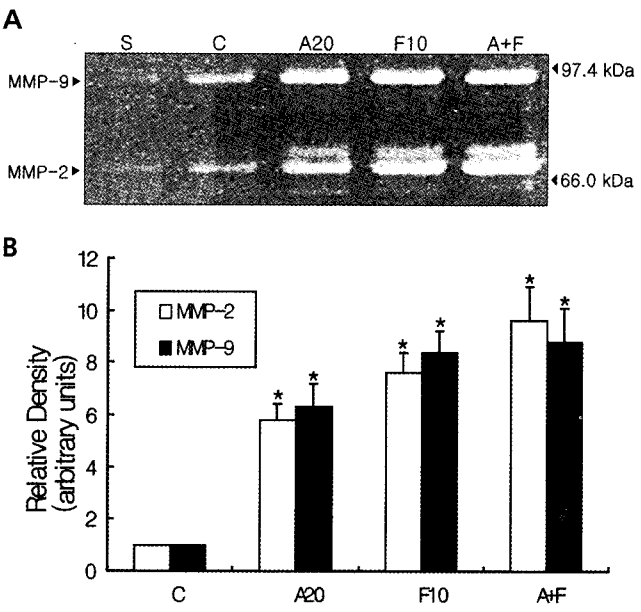

Fig. 4. Gelatin zymography of MMP-2 and MMP-9 in culture medium of PCAECs treated with Aralia extracts and FGF-2. (A) Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, the cells were incubated for 12 h after addition of control buffer (C), Aralia extracts (20 μg/mL) (A20), FGF-2 (10 ng/mL) (F10), and Aralia extracts (20 μg/mL) plus FGF-2 (10 ng/mL) (A+F). Equal amounts of proteins (10 μg/lane) from supernatants were loaded into each lane. Lane S contains standards of MMP-9 and MMP-2. (B) Densitometric analyses of the zymographs are presented as the relative ratio of induction of the MMP-2 or MMP-9 by addition of indicated reagents. The MMP-2 or MMP-9 secretion by addition of control buffer for 12 h is arbitrarily presented as 1. Data are mean ± SD from three experiments. Statistical significance was tested using one-way ANOVA followed by the Student-Newman-Keuls test. $^*P<0.05$ *vs* control buffer.

down of ECM by MMPs [33]. Therefore, an increased ratio of MMP-2 over TIMP-2 caused by Aralia extracts is positive for the degradation of ECM in PCAECs. However, higher concentrations (80 to 160 μg/mL) of Aralia extracts significantly suppress the secretion of MMP-2, MMP-9 and TIMP-1. The suitable effects of stimulation in endothelial cells with Aralia extracts were achieved with 20 μg/mL. However, 10 μg/mL also indicated an upregulation of MMPs secretion. A combination of the submaximal doses of Aralia extracts and FGF-2 produced an enhanced effect on the induction of MMP-2, MMP-9, and TIMP-1. These results suggest that Aralia extracts in synergy with FGF-2 may induce angiogenesis.

Aralia Extracts can Act as a Angiogenic Factor

The profiles of MMPs in the media were semi-quantitatively assayed by gelatin zymography. Consistent with the enzyme-immunoassay data (Fig. 2), Aralia extracts (20 μg/mL) produced increases of approximately 2.5- and 3.1-fold, respectively, in MMP-2 and MMP-9 secretion for 12 h, compared to the addition of a control buffer (Fig. 3). This suggests that Aralia extracts (10 to 20 μg/mL) may enhance angiogenesis moderately, in part, by stimulating MMP-2 and MMP-9 production. However, a higher concentration (160 μg/mL) of Aralia extracts nearly completely suppressed the secretion of MMP-2 and MMP-9. A combined treatment of Aralia extracts (20 μg/mL) and FGF-2 (10 ng/mL) on cultured PCAECs for 12 h produced increases of approximately 10.3- and 6.5-fold in MMP-2 and MMP-9 secretion, respectively, compared with addition of a control buffer (Fig. 4).

Aralia Extracts-Induced MMP-2 and MMP-9 Secretion is Suppressed by PI 3′-Kinase Inhibition

We examined the effect of PI 3′-kinase inhibitors when secreted of MMP-2 and MMP-9. The preincubation of LY294002 (100 nM) suppressed the Aralia extract-

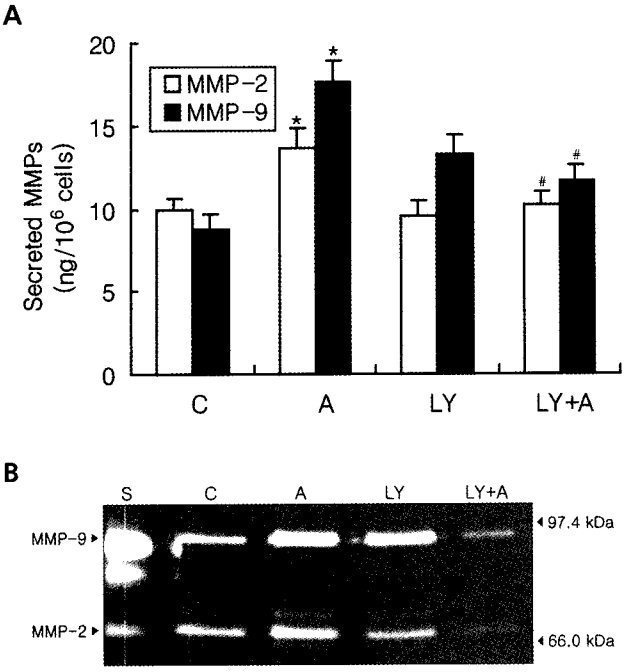

Fig. 5. Effect of Aralia extracts on secretion of MMP-2 and MMP-9 in the absence and presence of PI 3′-kinase inhibitors in PCAECs. Cells were incubated in serum- and phenol red-free DMEM for 12 h. Control buffer (C) and Aralia extracts (20 μg/mL) (A) was added to 0.5 mL of the same culture medium, cells were incubated for 12 h, and media were quantitatively assayed by enzyme immunoassay (A) and gelatin zymography (B). LY294002 (100 nM, LY) was pretreated 30 minutes before Aralia extracts addition. Data are mean ± SD from three experiments. Statistical significance was tested using one-way ANOVA followed by the Student-Newman-Keuls test. $^{*}P<0.05$ *vs* control buffer; $^{\#}P<0.05$ *vs* Aralia extracts (20 μg/mL).

induced MMP-2 and MMP-9 secretion. The addition of LY294002 (100 nM) on cultured PCAECs resulted in a 26- and 35% suppression of MMP-2 and MMP-9 secretion, respectively, for 12 h when compared with the addition of a control buffer (Fig. 5A). A gelatin zymography confirmed these results. Consistent with the enzyme-immunoassay data, Aralia extract-induced MMP-2 and MMP-9 secretion (20 μg/mL) was nearly suppressed (Fig. 5B). These results suggest that activation of PI 3′-kinase may be involved in Aralia extract-induced MMP-2 and MMP-9 secretion in PCAECs. The mechanisms by which PI 3′-kinase is involved in Aralia extract-induced MMP-2 and MMP-9 secretion will be examined in further studies.

Aralia Extracts Enhances Migration of PCAECs through Increased MMPs Secretion

Endothelial cell migration is an initial step in angiogenesis and neovascularization [12]. This process requires cell migration and invasion into the ECM beneath the basement membrane. Since migration activities were measured in gelatinized plates, Aralia extract-induced MMPs secretion could be a determinant for migration (Figs. 2~4). To test whether the increased MMPs were responsible for migration, the effect of the MMPs inhibitor was examined. Consistent with this idea, the addition of BB94 (20 ng/mL), a broad spectrum MMP inhibitor, on cultured PCAECs blocked the majority of the migratory effects of Aralia extracts (Fig. 1). Therefore, it is likely that, Aralia extracts induce the migration of cultured endothelial cells through the increase of MMP secretion.

The noteworthy finding from this research demonstrates that Aralia extracts induce the migration of PCAECs, which was inhibited by MMPs inhibitors. Aralia extracts also induced the secretion of MMP-2 and MMP-9. It is therefore suggested that Aralia extracts which induce migrating activity in PCAECs may be accomplished by increasing secretion levels of MMP-2 and MMP-9. The combination of Aralia extracts and FGF-2 enhance migration. Our results indicate that a combination of Aralia extracts and FGF-2 could be better than either a single growth factor for enhancing angiogenesis *in vitro*. Also, activation of PI 3′-kinase may be involved in Aralia extract-induced MMP-2 and MMP-9 secretion in PCAECs.

CONCLUSION

Angiogenesis is a process involving the formation of new capillary structures. In this study, we examined the effect of Aralia extracts and a combination of Aralia extracts with FGF-2 on cell migration and secretion of MMPs from PCAECs. The number of migrating cells increased with Aralia extracts or FGF-2 stimulation in a dose-dependent manner. Also, Aralia extracts (20 μg/mL) produced an increase of approximately 2.5- or 3.1-fold, respectively, in MMP-2 and MMP-9 secretion for 12 h, compared to the addition of a control buffer. Our notable finding is that a combination of submaximal doses of Aralia extracts and FGF-2 is more potent than the maximal dose of Aralia extracts or FGF-2 alone. It is likely that Aralia extracts induce the migration of cultured vascular endothelial cells through increased MMP-2 and MMP-9 secretion.

Acknowledgements This work was supported by the Korea Research Foundation Grant funded by the Korea Government (MOEHRD, Basic Research Promotion Fund) (Oh, I.S, R03-2004-000-10014-0). We thank the Research Center of Bioactive Materials at Chonbuk National University for kindly providing the facilities and materials for this research.

REFERENCES

[1] Chung, C. K. and M. E. Jung (2003) Ethanol fraction of *Aralia elata* Seemann enhances antioxidant activity and lowers serum lipids in rats when administered with benzo(a)pyrene. *Biol. Pharm. Bull.* 26: 1502-1504.

[2] Lee, E. B., O. J. Kim, S. S. Kang, and C. Jeong (2005) Araloside A, an antiulcer constituent from the root bark of

Aralia elata. *Biol. Pharm. Bull.* 28: 523-526.
[3] Song, S. J., N. Nakamura, C. M. Ma, M. Hatttori, and S. X. Xu (2001) Five saponins from the root bark of *Aralia elata*. *Phytochemistry* 56: 491-497.
[4] Yoshikawa, M., T. Murakami, E. Harada, N. Murakami, J. Yamahara, and H. Matsuda (1996) Bioactive saponins and glycosides. VII. On the hypoglycemic principles from the root cortex of *Aralia elata* seem: Structure related hypoglycemic activity of oleanolic acid oligoglycoside. *Chem. Pharm. Bull.* 44: 1923-1927.
[5] Hernandez, D. E., J. L. Hancke, and G. Wikman (1988) Evaluation of the anti-ulcer and antisecretory activity of extracts of *Aralia elata* root and *Schizandra chinensis* fruit in the rat. *J. Ethnopharmacol.* 23: 109-114.
[6] Basilico, C. and D. Moscatelli (1992) The FGF family of growth factors and angiogenesis. *Adv. Cancer Res.* 59: 115-165.
[7] Szebenyi, G. and J. F. Fallon (1999) Fibroblast growth factors as multifunctional signaling factors. *Int. Rev. Cytol.* 185: 45-106.
[8] Klein, S., F. G. Giancotti, M. Presta, S. M. Albelda, C. A. Buck, and D. B. Rifkin (1993) Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol. Biol. Cell* 4: 973-982.
[9] Mignatti, P. and D. B. Rifkin (1993) Biology and biochemistry of proteinases in tumor invasion. *Physiol. Rev.* 73: 161-195.
[10] Bikfalvi, A., S. Klein, G. Pintucci, and D. B. Rifkin (1997) Biological roles of fibroblast growth factor-2. *Endocr. Rev.* 18: 26-45.
[11] Brooks, P. C., R. A. Clark, and D. A. Cheresh (1994) Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 264: 569-571.
[12] Risau, W. (1997) Mechanisms of angiogenesis. *Nature* 386: 671-674.
[13] Folkman, J. and Y. Shing (1992) Angiogenesis. *J. Biol. Chem.* 267: 10931-10934.
[14] Brooks, P. C., S. Silletti, T. L. von Schalscha, M. Friedlander, and D. A. Cheresh (1998) Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. *Cell* 92: 391-400.
[15] Frederick, J. and J. F. Woessner Jr. (1988) The matrix metalloproteinase family. pp. 1-14. In: W. C. Parks and R. P. Mecham (eds.). *Matrix Metalloproteinases*. Academic Press, London, UK.
[16] Stetler-Stevenson, W. G. (1999) Matrix metalloproteinases in angiogenesis: A moving target for therapeutic intervention. *J. Clin. Invest.* 103: 1237-1241.
[17] Nagase, H. (1997) Activation mechanisms of matrix metalloproteinases. *Biol. Chem.* 378: 151-160.
[18] Ferrara, N. and K. Alitalo (1999) Clinical applications of angiogenic growth factors and their inhibitors. *Nat. Med.* 5: 1359-1364.
[19] Lee, J. B., J. S. Bae, J. H. Choi, J. H. Ham, Y. K. Min, H. M. Yang, T. Othman, and K. Shimizu (2004) Anticancer efficacies of doxorubicin, verapamil and quercetin on FM3A cells under hyperthermic temperature. *Biotechnol. Bioprocess Eng.* 9: 261-266.
[20] Carmeliet, P. (2000) Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* 6: 389-395.
[21] Kim, I., S. O. Moon, K. N. Koh, H. Kim, C. S. Uhm, H. J. Kwak, N. G. Kim, and G. Y. Koh (1999) Molecular cloning, expression, and characterization of angiopoietin-related protein induces endothelial cell sprouting. *J. Biol. Chem.* 274: 26523-26528.
[22] Haris, S. G. and M. L. Shuler (2003) Growth of endothelial cells on microfabricated silicon nitride membranes for an *in vivo* model of the blood-brain barrier. *Biotechnol. Bioprocess Eng.* 8: 246-251.
[23] Oh, I. S. and H. G. Kim (2004) Vascular endothelial growth factor upregulates follistatin in human umbilical vein endothelial cells. *Biotechnol. Bioprocess Eng.* 9: 201-206.
[24] Lee, Y., H. Kim, H. S. Choi, B. H. Kang, Y. B. Han, and S. J. Kim (2000) Water extract of 1:1 mixture of *Phellodendron* cortex and *Aralia* cortex has inhibitory effects on oxidative stress in kidney of diabetic rats. *J. Ethnopharmacol.* 73: 429-436.
[25] Choi, D. B., W. S. Cha, S. H. Kang, and B. R. Lee (2004) Effect of *Pleurotus ferulae* extracts on viability of human lung cancer and cervical cancer cell lines. *Biotechnol. Bioprocess Eng.* 9: 356-361.
[26] Rosen, E. M., L. Meromsky, E. Setter, D. W. Vinter, and I. D. Goldberg (1990) Quantitation of cytokine-stimulated migration of endothelium and epithelium by a new assay using microcarrier beads. *Exp. Cell Res.* 186: 22-31.
[27] Kleiner, D. E., I. M. K. Margulis, and W. G. Stetler-Stevenson (1998) Proteinase assay/zymography. pp. 5A7.1-5A7.8. In: A. Doyle, J. B. Griffiths, and D. G. Newell (eds.). *Cell and Tissue Culture: Laboratory Procedures*. John Wiley and Sons, Sussex, UK.
[28] Pepper, J. S., R. Montesano, S. J. Mandriota, L. Orci, and J. D. Vassalli (1996) Angiogenesis: A paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. *Enzyme Protein* 49: 138-162.
[29] Hiraoka, N., E. Allen, I. J. Apel, M. R. Gyetko, and S. J. Weiss (1998) Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell* 95: 365-377.
[30] Brown, M. D. and O. Hudlicka (2003) Modulation of physiological angiogenesis in skeletal muscle by mechanical forces: involvement of VEGF and metalloproteinases. *Angiogenesis* 6: 1-14.
[31] DeClerck, Y. A., Y. D. Yean, H. S. Lu, J. Ting, and K. E. Langley (1991) Inhibition of autoproteolytic activation of interstitial procollagenase by recombinant metalloproteinase inhibitor MI/TIMP-2. *J. Biol. Chem.* 266: 3893-3899.
[32] Ward, R. V., R. M. Hembry, J. J. Reynolds, and G. Murphy (1991) The purification of tissue inhibitor of metalloproteinases-2 from its 72 kDa progelatinase complex. Demonstration of the biochemical similarities of tissue inhibitor of metalloproteinases-2 and tissue inhibitor of metalloproteinases-1. *Biochem. J.* 278: 179-187.
[33] Nagase, H. and J. F. Woessner Jr. (1999) Matrix metalloproteinases. *J. Biol. Chem.* 274: 21491-21494.

[Received May 16, 2005; accepted July 26, 2005]