Arch. Pharm. Res. Vol.21, No.3, pp. 260-265, 1998

In vitro Angiogenic Activity of *Aloe vera* Gel on Calf Pulmonary Artery Endothelial (CPAE) Cells

Myoung-Jin Lee[1], Ok-Hee Lee[2], Soo-Hong Yoon[3], Seung-Ki Lee[4], Myung-Hee Chung[5], Young-In Park[6], Chung-Ki Sung[7], Jae-Sue Choi[8] and Kyu-Won Kim[2]

[1]*Institute of Biotechnology, Yeungnam University, Taegu,* [2]*Department of Molecular Biology, Pusan National University, Pusan,* [3]*College of Pharmacy, Catholic University of Taegu-Hyosung, Taegu,* [4]*College of Pharmacy,* [5]*College of Medicine, Seoul National University, Seoul,* [6]*College of Natural Resources, Korea University, Seoul,* [7]*College of Pharmacy, Chonman National University, Kwangju and* [8]*Department of Food and Nutrition, Pukyong National University, Pusan, Korea*

(Received December 26, 1997)

Angiogenic activity of *Aloe vera* gel was investigated by *in vitro* assay. We obtained the most active fraction from dichloromethane extract of *Aloe vera* gel by partitioning between hexane and 90% aqueous methanol. The most active fraction (F3) increased the proliferation of calf pulmonary artery endothelial (CPAE) cells. In addition, F3 fraction induced CPAE cells to invade type I collagen gel and form capillary-like tube through *in vitro* angiogenesis assay, and increased the invasion of CPAE cells into matrigel through *in vitro* invasion assay. Furthermore, the effect on the mRNA expression of proteolytic enzymes which are key participants in the regulation of extracellular matrix degradation was investigated by northern blot analysis. F3 fraction enhanced mRNA expression of urokinase-type plasminogen activator (u-PA), matrix metalloproteinase-2 (MMP-2), and membrane-type MMP (MT-MMP) in CPAE cells whereas the expression of plasminogen activator inhibitor-1 (PAI-1) mRNA was not changed.

Key words : Angiogenesis, CPAE cells, *In vitro* angiogenesis assay, Urokinase-type plasminogen activator (u-PA), Matrix metalloproteinase (MMP).

INTRODUCTION

Aloe plants, a member of the lily family, are useful as food, and occupy a prominent place in folk medicine for centuries (Bovik, 1966; Lushaugh and Hale 1953). Only a few of more than 300 known aloe species are currently used by the pharmaceutical and cosmetic industries. Among them, *Aloe barbadensis*, commonly called *Aloe vera*, has been one of the most used healing plants in the history of mankind. It was reported that *Aloe vera* gel improved wound healing in a dose dependent fashion (Davis *et al.*, 1987). We have tried to elucidate the pharmacological mechanism of wound healing effect of *Aloe vera* gel. Wound healing is presented as three overlapping series of events: inflammation, new tissue formation and matrix remodeling (Dunphy, 1974). To furnish the new tissue formed in wound healing with oxygen and metabolites, angiogenesis is an essential process (Thompson *et al.*, 1991). Angiogenesis is the growth of new capillaries from pre-existing capillaries and post-capillary venules (Bischoff, 1995). When angiogenesis is impaired, as in the aged or in the irradiated tissues, wound healing is retarded or unsuccessful (Phillips *et al.*, 1991). We have reported that *Aloe vera* gel stimulated angiogenesis in chick embryo chorioallantoic membrane (CAM) assay (Lee *et al.*, 1995). Angiogenesis is associated with dramatic changes in endothelial cell behavior. In response to angiogenic stimuli, the normally quiescent microvascular endothelial cells start to invade, and differentiate to tube-like structure (Ausprunk and Folkman, 1977). A crucial step in the sequence of events of leading to angiogenesis is the invasion of the perivascular matrix. To achieve extracellular matrix degradation, different enzymatic cascades must be cooperated. A number of participant proteins have been characterized. Plasminogen activators (PAs) and their inhibitors, plasminogen activator inhibitors (PAIs), are thought to be key participants in the balance of proteolytic and antiproteolytic activities that regulate the matrix turnover (Dano *et al.*, 1985). In addition, matrix metalloproteinases (MMPs) are a member of the unique family of proteolytic enzymes which contain a zinc ion at their active sites, and MMPs can degrade native collagens and other extracellular matrix com-

Correspondence to: Kyu-Won Kim, Department of Molecular Biology, Pusan National University, Pusan 609-735, Korea

ponents (Sato *et al.*, 1992).

In this study, we confirmed the angiogenic activity of *Aloe vera* gel by *in vitro* angiogenesis assay, and its angiogenic mechanism was investigated by a series of *in vitro* assays. The effects of *Aloe vera* gel on proliferation, differentiation, and invasion of CPAE cells were demonstrated. In addition, mRNA expression of proteolytic enzymes related to extracellular matrix degradation was observed by northern blot analysis.

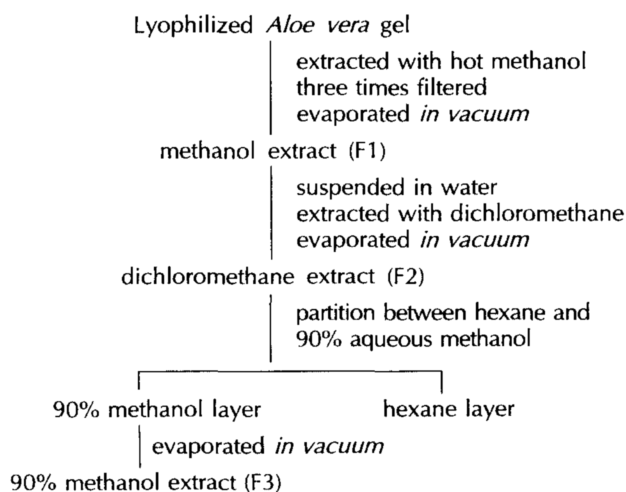
MATERIALS AND METHODS

Materials

Phorbol 12-myristate 13-acetate (PMA), (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide (MTT), and gelatin were purchased from Sigma Chemical Co.. Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO/BRL Laboratories. Transwell plates were purchased from Corning Costar Co.. Calf pulmonary artery endothelial (CPAE) cells and human u-PA cDNA were purchased from American Type Culture Collection. Human PAI-1 cDNA was obtained from Dr. J.J. Loskutoff, Research Institute of Scripps Clinic, USA. Human MMP-2 cDNA and MT-MMP cDNA were kindly provided from Dr. Motoharu Seiki, Department of Molecular Virology and Oncology, Cancer Research Inst., Kanazawa University, Japan.

Extraction and fractionation of *Aloe vera* gel

Lyophilized *Aloe vera* gel was refluxed with methanol three times for 3 h. The methanol layer was filtered and concentrated by rotary vacuum evaporator. Fractionation of *Aloe vera* gel was conducted as shown in Scheme 1. The methanol extract (F1) was fractionated with dichloromethane, filtered and concentrated. The dichloromethane extract (F2) was partitioned between hexane and 90% aqueous methanol, and F3 fraction was obtained by evaporation of 90% methanol layer.



Scheme 1. Fractionation procedure of *Aloe vera* gel

Cell culture

Calf pulmonary artery endothelial (CPAE) cells were maintained with DMEM supplemented with 15% (v/v) fetal calf serum, 100 U/ml penicillin, and 100 μg / ml streptomycin. CPAE cells were used between passage 19 and 26. The media were changed twice a week.

Cell proliferation assay

CPAE cells were seeded at 5×10^4 cells/well in 12-multiwell plates. After 1 day, media were changed, and the desired concentration of F3 fraction was added to each well. The cells were incubated in humidified incubator at 37°C for 3 days, then media were discarded, and 1 ml of new media and 100 °Cl of MTT solution were added to each well. After incubation at 37°C for 3 h, media and MTT solution were discarded, and 1 ml of acid-isopropanol solution (0.04 N HCl in isopropanol) was added to each well. Optical density was measured at 570 nm by spectrophotometer (Tada *et al.*, 1986).

In vitro angiogenesis assay

CPAE cells were seeded and grown on the surface of type I collagen gel in 24-multiwell plates. Media were changed every 2 days. After CPAE cells were grown to confluence, PMA (20 ng/ml) and F3 fraction (100 μg/ml) were treated for 5 days. For collagen gel formation, 8 volume of type I collagen stock solution (1 g of type I collagen in 300 ml of 0.1% acetic acid solution) was quickly mixed with 1 volume of 10x minimal essential medium and 1 volume of sodium bicarbonate (11.76 mg/ml) on ice, dispensed into 24-multiwell plates, and allowed to gel at 37°C for 20 min (Pepper *et al.*, 1993). The morphological changes of CPAE cells by treatment of PMA and F3 fraction were observed and photographed in phase contrast microscope.

In vitro invasion assay

In vitro invasion assay was carried out by the method of Saiki et al (Saiki *et al.*, 1993). Invasion was measured by the use of 24-transwell units with 8 μm porosity of polycarbonate filters (Corning Costar Co.). The lower side of filter was coated with 10 μl of 0.5 mg/ml type I collagen, and the upper side of filter was coated with 10 μl of 0.5 mg/ml matrigel (reconstituted basement membrane). CPAE cells pretreated with F3 fraction for 48h were resuspended in 100 μl of DMEM and were placed upper part of trans ell

unit, and the lower compartment contained 600 μl of DMEM containing 0.1 mg/ml bovine serum albumin (BSA). The same concentration of F3 fraction as being pretreated was added in the upper and lower part of transwell unit, and CPAE cells were incubated for 19 h in an humidified incubator at 37°C. Then, CPAE cells were fixed with methanol, and stained with hematoxyline and eosine (Sigma Chemical Co.). CPAE cells on the upper surface of the filter were removed by wiping with cotton swab, and invasion was determined by counting of the cells that had migrated to the lower side of the filter with optical microscopy at x400 magnification. Thirteen fields were counted and the mean value was obtained.

RNA preparation and northern blot analysis

Total RNA was prepared according to the procedure of the acid-guanidium thiocyanate-phenol-chloroform (AGPC) extraction method (Chomczynski and Sacchi, 1987). The total RNA was quantitated by absorbance at 260 nm. RNA samples were resolved on 1% agarose formaldehyde gel under denaturing condition, and transferred to nylon membrane (Zeta-Probe, Bio-Rad, CA). Then, the nylon membrane was UV cross-linked for 30 seconds for the fixation of RNA. The RNA transferred nylon membrane was hybridized with ^{32}P-labeled probes according to the modified method (Davis *et al.*, 1986; Sambrook *et al.*, 1989). Blot was then washed in 2x SSC and 0.1% SDS at room temperature and autoradiographed at -80°C. Then, the X-ray films were developed.

RESULTS

Stimulation of the proliferation of CPAE cells

The effect of F3 fraction on the blood endothelial cell proliferation was investigated by MTT assay. The proliferation of the endothelial cells is needed to perform angiogenesis. CPAE cells were treated with various concentrations (10, 100, 200 μg/ml) of F3 fraction for 3 days. The optical density values from F3 fraction-treated cells were compared to that from control cells. As shown in Fig. 1, the proliferation of CPAE cells was stimulated in a dose-dependent manner by treatment of F3 fraction.

Induction of vascular tube formation of CPAE cells grown on type I collagen

Confluent CPAE cells grown on the surface of three-dimensional type I collagen gel in 24-multiwell plates were treated with F3 fraction (100 μg/ml). PMA was used as a positive control. The morphology of control CPAE cells was cobblestone-like monolayer of polygonal cells (Fig. 2a). There was no change in morphology of control cells for 5 days of incubation. After the treatment of PMA for 24h, numerous CPAE cells started to infiltrate into collagen matrix. And CPAE cells differentiated to tube-like structure after 3 days of PMA treatment (Fig. 2b). When CPAE cells were treated with F3 fraction, the infiltration of CPAE cells

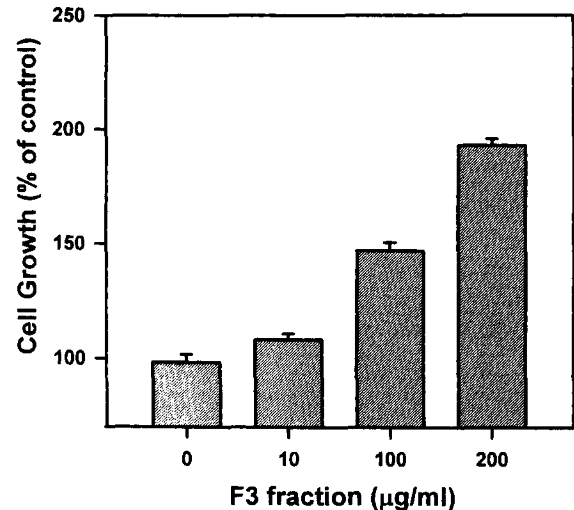


Fig. 1. Effect of F3 fraction on the proliferation of CPAE cells. CPAE cells were treated with various concentrations of F3 fraction for 3 days. Proliferation was determined by MTT assay. The OD values from F3 fraction-treated cells at 570 nm was compared to that from control cells.

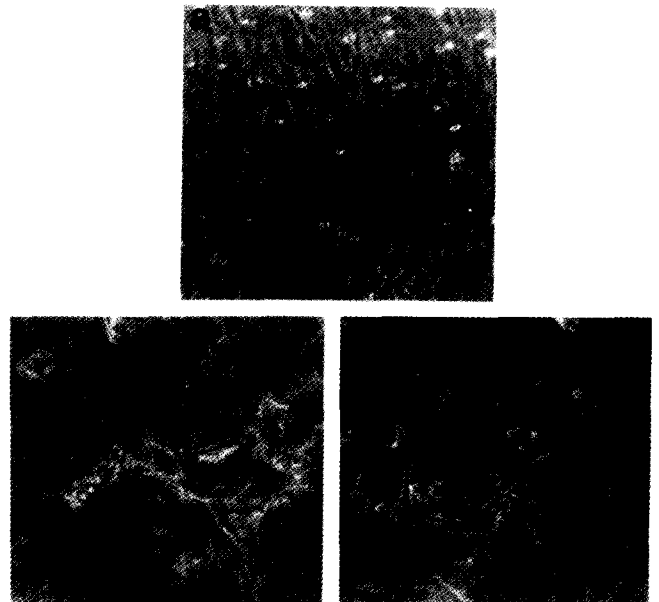


Fig. 2. Effect of F3 fraction on the morphology of CPAE cells grown on collagen gel. CPAE cells were grown to confluence on the surface of type I collagen gel in 24-multiwell plates and then treated with sample (a, control; b, 20 ng/ml of PMA for 3 days; c, 100 μg/ml of F3 fraction for 5 days). Control CPAE cells formed a cobblestone-like monolayer (a). The infiltration into collagen matrix and differentiation to tube-like structure were observed in (b) and (c).

into collagen gel was started after 2 days of treatment and the formation of tube-like structure was observed after 5 days of treatment (Fig. 2c).

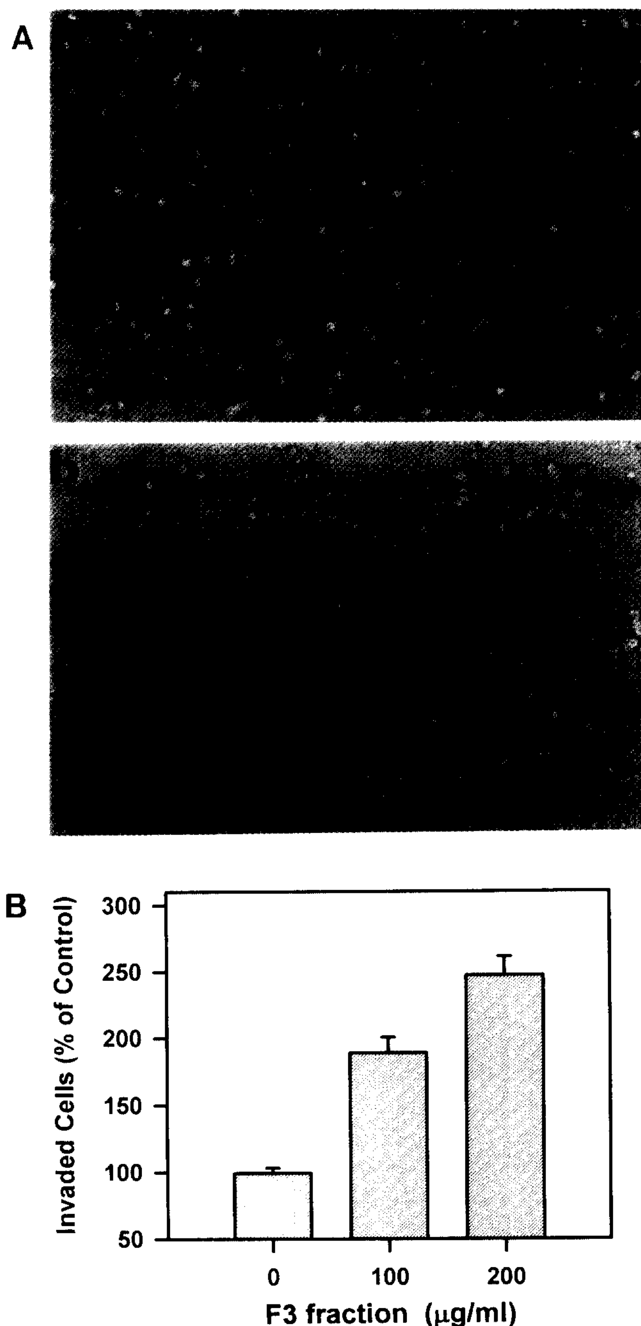


Fig. 3. Effect of F3 fraction on the invasiveness of CPAE cells into matrigel. (A) Confluent CPAE cells were treated with F3 fraction for 48 h, and 5×10^4 CPAE cells were inoculated in the upper part of transwell chamber (a, control; b, 100 μg/ml of F3 fraction). Magnification: x400. (B) Dose-dependent stimulation of F3 fraction. CPAE cells were treated with desired doses of F3 fraction for 48 h. Invasion was determined by counting of the cells that has migrated to the lower side of the filter with optical microscopy.

Stimulation of the invasion of CPAE cells into matrigel

The effect of F3 fraction on the invasiveness of CPAE cells was investigated. Matrigel was used as a barrier in transwell chamber. Confluent CPAE cells were treated with F3 fraction for 48 h prior to inoculation to transwell chamber. The invasive effect was determined by counting of the cells that had migrated to the lower side of the filter. As shown in Fig. 3A, the invasion into matrigel of CPAE cells treated with 100 μg/ml of F3 fraction was greatly stimulated when compared to control cells. In addition, F3 fraction stimulated the invasion of CPAE cells in a dose-dependent manner (Fig. 3B).

Effect on the mRNA expression of u-PA, PAI-1, MMP-2, and MT-MMP

Total RNA was separated from CPAE cells treated with F3 fraction, and the mRNA expression of proteolytic enzymes was examined by northern blot analy-

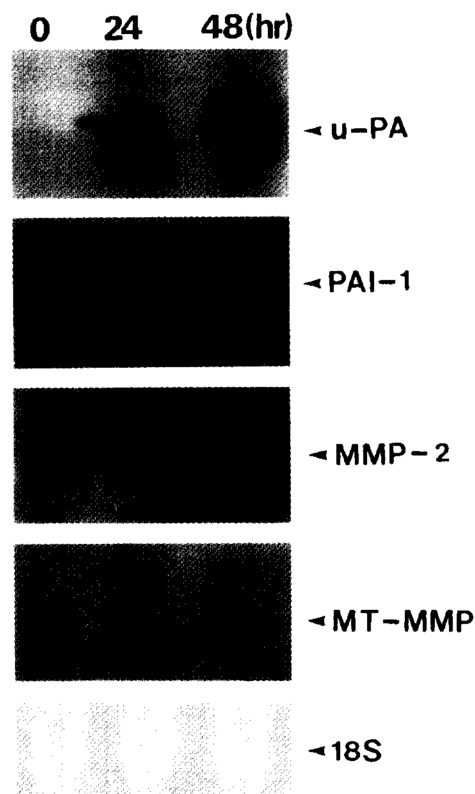


Fig. 4. Effect on the mRNA expression of u-PA, PAI-1, MMP-2, and MT-MMP in CPAE cells. Confluent CPAE cells were incubated with 100 μg/ml of F3 fraction for indicated time. Total RNA (20 μg) was fractionated on a 1% agarose gel and transferred to a nylon membrane. Northern blot hybridization was performed with ^{32}P- labeled u-PA, PAI-1, MMP-2, and MT-MMP cDNAs.

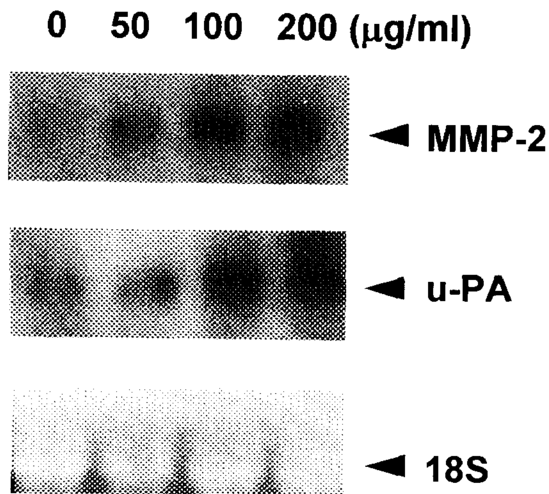


Fig. 5. Dose response of the mRNA expression of MMP-2 and u-PA in CPAE cells. Confluent CPAE cells were incubated with indicated doses of F3 fraction for 48 h. Total RNA (20 μg) was fractionated on a 1% agarose gel and transferred to a nylon membrane. Northern blot hybridization was performed with ^{32}P-labeled MMP-2 and u-PA cDNAs.

sis. u-PA and PAI-1 are thought to be key participants in the balance of proteolytic and antiproteolytic activities that regulate matrix turnover. As shown in Fig. 4, the expression of u-PA mRNA increased in a time-dependent manner for 48h whereas its inhibitor, PAI-1, showed no changes in mRNA level. Matrix metalloproteinases (MMPs) can degrade native collagens and other extracellular matrix components (Templeton and Stetler-Stevenson, 1991). The expression of MMP-2 mRNA in 100 μg/ml of F3 fraction-treated CPAE cells increased within 24 h, and that of MT-MMP mRNA increased slightly after 48 h (Fig. 4). In addition, the enhanced expression of MMP-2 and u-PA mRNA was dose dependent in CPAE cells treated with various concentrations of F3 fraction (50, 100, 200 μg/ml) as illustrated in Fig. 5.

DISCUSSION

Aloe vera has been one of the most used healing plants in the history of mankind. It was reported that *Aloe vera* gel promoted the healing of burns, other cutaneous injuries and ulcers (Klein and Penneys, 1988). We have tried to elucidate critical pharmacological mechanism for the healing effect of *Aloe vera* gel, and thus reported the angiogenic activity of *Aloe vera* gel by *in vivo* assay (Lee *et al.*,1995). Angiogenesis consists of at least three processes: 1) degradation of the extracellular matrix of the existing vessel, 2) migration of endothelial cells from the existing vessel toward the angiogenic stimulus, and 3) proliferation of endothelial cells to form a new vessel (Folkman and Shing, 1992). In this study, we confirmed the angiogenic activity of Aloe vera gel by *in vitro* angiogenesis assay. The most active fraction of Aloe vera gel (F3) induced CPAE cells to invade type I collagen and differentiate to tube-like structure (Fig. 2c). Type I collagen is a major constituent of the pericapillary connective tissue. Therefore, this phenomenon mimics the events occurring during angiogenesis *in vivo.* CPAE cells treated with F3 fraction did not form tube-like structure until 5 days of treatment whereas PMA-treated CPAE cells formed tube-like structure after 3 days of treatment. The result suggests that the effect of F3 fraction occurs slower than that of PMA on CPAE cells grown on collagen gel. Then, *in vitro* invasion assay was performed to assess the invasiveness of the endothelial cells. It is known that a crucial event during angiogenesis is the invasion of the perivascular extracellular matrix by sprouting endothelial cells, and the basement membrane creates a critical barrier to the passage of cells. As shown in Fig. 3, F3 fraction stimulated the invasion of CPAE cells into matrigel, reconstituted basement membrane. It has been proposed that cellular invasiveness in various biological processes including angiogenesis requires the elaboration of proteinases capable of degrading the extracellular matrix. A number of proteolytic enzymes have been characterized. Among them, plasminogen activators (PAs) and matrix metalloproteinases (MMPs) are major enzymes for the degradation of the extracellular matrix which is an essential first step in angiogenic process (Dano *et al.*, 1985). It has been reported that u-PA related to endothelial cell migration is synthesized by cultured endothelial cells derived from large vessels and capillaries (Pepper *et al.*, 1993), and PAI-1 rapidly binds with u-PA and thus can modulate u-PA activity (Mawatari *et al.*, 1991). Therefore, a tightly controlled balance between production of u-PA and its inhibitor, PAI-1, is required in angiogenesis (David *et al.*, 1987). We showed that F3 fraction increased the expression of u-PA mRNA in CPAE cells. However, PAI-1 mRNA expression was not changed. The enhanced expression of u-PA mRNA, but not PAI-1 mRNA, may result in net proteolytic activity for extracellular matrix and the stimulation of endothelial cell migration. In addition, F3 fraction enhanced mRNA expression of MMP-2 and MT-MMP in CPAE cells. It is known that the expression of MT-MMP on cell surface fits the requirements of an activator for pro-MMP-2 (Sato *et al.*, 1994). In the future, it will be necessary to investigate the effect of F3 fraction on the expression of other MMPs.

Taken together, it could be suggested that the angiogenic activity of the most active fraction of *Aloe vera* gel (F3) may be due to the stimulated proliferation and differentiation of endothelial cells, and the enhanced expression of proteolytic enzymes, especially u-PA and MMP-2, playing a major role in extracellular matrix degradation. In the future, further pu-

rification of F3 fraction for the development of a potent wound healing drug and the elucidation of the angiogenic mechanism of *Aloe vera* gel will be required.

ACKNOWLEDGEMENTS

This work was supported by the research grant from Namyang Aloe Co. Ltd., Korea.

REFERENCES CITED

Ausprunk, D. H. and Folkman, J., Migration and proleferation of endothelial cells in performed and newly-formed blood vessels during tumor angiogenesis. *Microvasc. Res.*, 14, 53-65 (1977).

Bischoff, J., Approaches to studying cell adhesion molecules in angiogenesis. *Trends on Cell Biol.*, 5, 69-73 (1995).

Bovik, E. G., Aloe vera panacer or old wives tales? *Texas Dent. J.*, 84, 13-16 (1966).

Chomczynski, P. and Sachi, N. M., Single-step method of RNA isolation by acid-guanidium thiocyanate-penol-chloroform extraction. *Anal. Biochem.*, 58, 541-548 (1987).

Dano, K., Andreasen, A., Grondahl-Hansen, J., Kristensen. P., Nielsen, S. and Skriver, L. , Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer. Res.*, 44, 139-146 (1985).

David, J., Linders, M., Keijer, J., Veerman, H., Hweikhuizen, H. and Annekoek, H., Structure of the human plasminogen activator inhibitor 1 gene: Nonrandom distribution of introns. *Biochemistry*, 26, 3763-3768 (1987).

Davis, J., Dibner, D., and Battey, J. F., *End labelling of synthetic probes*: *Basic method in molecular biology*. Elsevier press Inc., New York, pp. 72-74, 1986.

Davis, R. H., Kabbani, J. M. and Maro, N. P., Aloe vera and Wound healing. *J. Am. Pod. Med. Assoc.*, 77, 165-169 (1987).

Dunphy, J. E., *Modern biochemical concepts on the healing of wounds*: *Wound healing. Baltimore*, Williams and Wilkins, pp. 22-31, 1974.

Folkman, J. and Shing, Y., Angiogenesis. *J. Biol. Chem.*, 267, 10931-10934 (1992).

Klein, A. D. and Penneys, N. S., Aloe vera. *J. Ame. Aca. Dermatol.*, 18, 714-720 (1988).

Lee, M. J., Yoon, S. H., Lee, S. K., Chung, M. H., Park, Y. I., Jung, C. K., Choi, J. S. and Kim, K. W., *In vivo* angiogenic activity of dicholromethane extracts of Aloe vera gel. *Arch. Pharm. Res.*, 18, 332-335 (1995).

Lushbaugh, C. C. and Hale, D. B., Experimental acute radiodermatitis following beta irradiation. *Cancer* 6, 690-698 (1953).

Mawatari, M., Okamura, K., Matsuda, T., Hamanaka, R., Mizoguchi, H., Higashio, K., Kohno, K. and Kuwano, A., Tumor necrosis factor and epidermal growth factor migration of human microvascular endothelial cells and production of tissue-type plasminogen activator and its inhibitor. *Exp. Cell Res.*, 192, 574-580 (1991).

Pepper, M. S., Vassalli, J. D., Orci, L. and Montesano, R., Biphasic effect of transforming growth factor-β1 on *in vitro* angiogenesis. *Exp. Cell Res.*, 204, 356-363 (1993).

Phillips, G. D., Whitehead, R. A. and Kighton, D. R., Initiation and pattern of angiogenesis in wound healing in the rat. *Am. J. Anatomy.*, 192, 257-262 (1991).

Saiki, I., Fujii, H., Yoneda, J., Abe, F., Nakajima, M., Tsuruo, T. and Azuma, I. , Role of aminopeptidase N(CK13) in tumor-cell invasion and extracellular matrix degradation. *Int. J. Cancer,* 54, 137-143 (1993).

Sambrook, J., Fritch, E. F. and Maniatis, T., *Conditions for hybridization of oligonucleotide probes*: *Molecular cloning* (*2nd ed*). Cold Spring Harbor laboratory Press Inc., New York, pp. 1145-1157, 1989.

Sato, H., Kida, Y., Mai, M., Endo, Y., Sasaki, T., Tanaka, J. and Seiki, M., Expression of genes encoding type IV collagen-degrading metalloproteinase and tissue inhibitors of metalloprotinase in various human tumor cells. *Oncogene*, 7, 77-83 (1992).

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagwa, A., Yamamato, E. and Seiki, M., A matrix metalloproteinase expressed on the surface of invasive tumor cells. *Nature*, 370, 61-65 (1994).

Tada, H., Shiho, O., Kuroshima, K. I., Koyama, M. and Tsukamoto, K., An improved colorimetric assay for interleukin 2. *J. Immunol. Methods*, 93, 157-165 (1986).

Templeton, N. S. and Stetler-Stevenson, W.G., Identification of a basal promoter for the human Mr 72, 000 type IV collagenase gene and enhanced expression in a highly metastatic cell line. *Cancer Res.*, 51, 6190-6193 (1991).

Thompson, W. D., Harvey, J. A., Kazmi, M. A. and Stout, A. J., Fibrinolysis and angiogenesis in wound healing. *J. Phathol.*, 165, 311-318 (1991).