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Identification of Anti-Angiogenic and Anti-Cell Adhesion Materials from Halophilic Enterobacteria of the *Trachurus japonicus*

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Abstract The halophilic enterobacteria, *Enterobacteria cancerogenus*, was isolated from the intestines of the fusiform fish (*Trachurus japonicus*) to yield a protein-like material termed PLM-f74. PLM-f74 was characterized by strong inhibition ratios to angiogenesis (82.8% at the concentration of 18.5 µg/ml) and elevated antioxidative capacities with low toxicity. The PLM-f74 is a glycoprotein comprised of saccharides and amino acids. PLM-f74 inhibited cell adhesion that non-activated U937 monocytic cell adhesion to HUVECs activated with IL-1β by 78.0%, and the adherence of U937 cells treated with the PLM-f74 and stimulated with IL-1β to unstimulated HUVECs decreased by 102%. When both cell types were pretreated with PLM-f74, the adhesion of U937 cells to IL-1β-stimulated HUVECs was completely suppressed by 121% at a concentration of 18.5 µg/ml. PLM-f74 blocked signal pathways from VEGFR2, PI3K, β-catenin, and VE-cadherin to NF-kB, based on western bolt analysis. It also inhibited IL-1-stimulated HUVEC expression of the adhesion molecules, ICAM-1 by 40%, VCAM-1 by 60%, and E-selectin by 70% at the same concentration noted above. New anti-angiogenic and anti-cell adhesion materials showing elevated antioxidative capacities, and non-toxicity may be expected from these results.

Key words: Anti-angiogenesis, antioxidative capacity, cytotoxicity, fusiform fish, cell adhesion, protein-like material (PLM)

Angiogenesis is the process of forming new vasculogenesis from blood vessels [38, 29] and occurs in the healthy body to heal wounds and restore blood flow to tissues after injury [2, 28]. In females, angiogenesis also occurs during monthly reproductive cycles to rebuild the uterine lining [26, 33, 35], to mature the ovum during folliculogenesis and ovulation [1, 3] and during pregnancy to develop the placenta and aid circulation between the mother and developing fetus [16–17, 39]. When angiogenic growth factors are produced in excess of angiogenesis inhibitory factors, the balance is tipped in favor of vasculature growth, and when inhibitors are present in excess of stimulators, angiogenesis is arrested. Normally, a healthy body maintains a perfect balance of the angiogenesis modulators. In general, angiogenesis is turned off by the production of more inhibitors than stimulators [37]. The body loses control over angiogenesis in many serious states of disease. Angiogenesis-dependent diseases result when new blood vessels either grow excessively or insufficiently. Excessive angiogenesis is associated with diseases such as cancer [24], diabetic blindness [18], age-related macular degeneration [12], rheumatoid arthritis [20], and psoriasis [31], because the new blood vessels feed the diseased tissues at the expense of the normal tissues, which are destroyed in the process [34]. In the case of cancer, the new vessels allow tumor cells to escape into the circulation and seed other organs, *i.e.*, metastasize [13]. Alternatively, as a result of insufficient angiogenesis, coronary artery disease [11], stroke [21], and delayed wound healing [4] will occur because of the inadequate growth of blood vessels and a failure to restore proper circulation, which leads to the increased risk of tissue death [30].

In cases of excessive angiogenesis, there is a dependence upon "on" switches, known as angiogenesis-stimulating growth factors [9], *e.g.*, angiogenin, angiopoietin-1, interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-alpha), and vascular endothelial growth factor (VEGF), and for the insufficient case, it depends on "off" switches, known as angiogenesis inhibitors [6], *e.g.*, angiostatin, the interferons (alpha, beta, and gamma), interleukin-12, retinoid, and transforming growth factor-beta (TGF-b).

Many additional switches likely will be found in nature, including among animals, plants [25], and prokaryotes.

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Currently, novel and inexpensive anti-angiogenic switches have been under investigation for curing cancer, and ideally without side effects [19]. A major problem with such anti-angiogenic switches at present is that it is very difficult to obtain a sufficient amount of material from animal and plant resources.

Although marine organisms, including marine bacteria, have been very useful in providing materials for human health, they vary greatly from their terrestrial counterparts. These differences are likely because marine organisms have adapted in a more extreme environment than that of terrestrial bacteria in terms of nutrients, salt levels, a higher osmotic pressure, etc.

For these reasons, new components bearing anti-angiogenic effects will be expected from these marine organisms. Eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) from fusiform fish like Trachurus japonicus are known as inhibitors of cancer cell growth and of atherosclerosis [5, 27]. These unsaturated fatty acids are not oxidized in the body of fusiform fishes because of unknown factors that prohibit fatty acid oxidation. We suggest that such fishes may perhaps provide and be the source of new switches with novel anti-angiogenic effects and with reduced toxicity. We also propose that a large amount of such substances can be produced through fermentation using the marine bacteria that bear the higher antioxidative capacities.

MATERIALS AND METHODS

Selection of λ-Bacteria from Fusiform Fish of *Trachurus japonicus*

The intestines of freshly caught *Trachurus japonicus* were dissected, homogenized with PBS (phosphate buffered saline), and then cultured in a 250-ml Erlenmeyer flask containing 100 ml PYG medium (trypticase peptone, 5.0 g; peptone, 5.0 g; yeast extract, 10.0 g; beef extract, 5.0 g, KH_2PO_4, 2.0 g; tween 80, 1.0 ml; resazurin, 1.0 mg; salt solution, 40.0 ml; distilled water, 960 ml) in an anaerobic environment. Then, 200 μl of the cultured broth was transferred onto a solid PYG medium by the spread method in a Gaspack for 48 h at 25°C. Selected single colonies were cultured with PYG media in 250-ml flasks treated with nitrogen gas, and agitated at an rpm of 120 strokes for 50 h and at 25°C. Their antioxidative capacity was measured with an oxidative-reduction potential (ORP) system [20]; the higher antioxidative species were selected, and named λ-bacteria.

Cultivation of λ-Bacteria

Λ-28 strain reisolated from λ-bacteria was cultured under aerobic conditions with marine LB (Luria-Bertani, tryptone, 10.0 g; yeast extract, 5.0 g; NaCl, 10.0 g; distilled water,

1,000 ml) medium in a 5-l jar fermentor (Kobiotech., Incheon, Korea) with a working volume of 3 l at 25°C, 250 rpm, 0.5 vvm for 45 h. The inoculum size was 10% (v/v) and pH was controlled at 7.0 with 2 N HCl and 2 N NaOH. These conditions were chosen because in a prior experiment, the anaerobic cultured solution was toxic (data not shown) to human umbilical vein endothelial cells (HUVECs).

Extraction of Protein-Like Materials (PLM)

Supernatants from the jar fermentor with λ-28 strains was extracted by centrifugation (5,000 rpm, 15 min) and salted out with 80% ammonium sulfate in a 5-l beaker for 2 days at 4°C, and the pellets collected by centrifugation at 12,000 rpm for 20 min. The pellets were dialyzed in a cellulose membrane (MWCO 3,500; Spectrum Lab.) with 0.02 M MOPS (pH 7.0) and buffered with 0.02 M phosphate buffer (pH 7.0), and lyophilized [36].

Separation of Protein-Like Materials (PLM-74)

To control for anti-angiogenesis, 5.0 g of lyophilized PLM was dissolved in 10.0 ml of 0.02 M phosphate buffer (pH 7.0), filtered with a 0.20-μm syringe filter (MFS-25, Advantec MFS Inc.), and then subjected to size exclusion chromatography (SEC) using a 2-cm diameter, 1.5-m height glass column packed with 45.0 g of Sepadex G-100 (Pharmacia Biotech), equilibrated with 0.02 M phosphate buffer (pH 7.0) and eluted with the same buffer at a flow rate of 1 ml/min. The 74th fraction (PLM-f74) carrying the higher anti-angiogenic effect was used in this experiment.

Cell Culture

The HUVECs used in this study were obtained from Young Science Inc. (Seoul, Korea) with cells from passages 3 to 5. HUVECs were cultured in EBM-2 growth medium (Cambrex, Hopkinton, MA, U.S.A.) and 2% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. The cells were seeded in culture flasks coated with 2% gelatin (Sigma, St. Louis, MO, U.S.A.) and allowed to grow to confluence before experimental treatment.

Antioxidative Capacity of PLM

The antioxidant capacity of PLM was measured at 593 nm with a spectrophotometer and by the ferric reducing ability assay of plasma (FRAP method) [31]. To determine comparative antioxidant capacity, PLM was used at a non-toxic concentration. One unit of antioxidant capacity was designated as the equivalent antioxidant capacity of 1 mM Fe (II).

In Vitro Anti-angiogenesis

The formation of tubular structures by HUVEC in a matrigel was used to assess the effect of PLM on anti-angiogenesis. Culture plates (24-well) were coated with 150 μl of matrigel,

which was allowed to solidify at 37°C for 1 h. HUVECs were seeded on matrigel-coated wells (25,000 cells/well) and the cells treated with PLM at different concentrations for 24 h at 37°C and in a 5% CO_2 humidified atmosphere. Digital images were used to capture randomly selected areas of the wells (5 each). The network of tube formation was quantified by summing up the arbitrary length of tube from each image using the NIH Image program.

Identification of λ-28 Strains
Genomic DNA was isolated from the screened bacteria by the modified Rochelle method [34]. After chromosomal DNA was isolated with a Wizard genomic DNA purification kit (Promega, Madison, WI, U.S.A.), the DNA was amplified by a polymerase chain reaction (GenAMP PCR System 97000, Perkin-Elmer, Boston, MA, U.S.A.) using two oligonucleotide primers, 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-GGATACCTTGTTACGACTT-3'. The PCR product was purified using a Wizard PCR Prep DNA Purification System (Promega Co., Madison, WI, U.S.A.), and direct sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Boston, MA, U.S.A.) and BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Boston, MA, U.S.A.). The result was compared with a ribosomal DNA sequence of GenBank using the BLASTN program [23].

Cell Adhesion
Cell culture.   Cultured U937 cells were concentrated by centrifugation at 1,000 rpm for 5 min and suspended at a concentration of 10^6 cells/mL. The U937 cells were then cultured in the presence or absence of PLM-f74 for the necessary experiments.
U937 cell adhesion assay.   HUVECs were cultured to confluence in 24-well plates and then treated with different concentrations of PLM-f74 at 37°C for 20 h. After washing with PBS containing 1% FBS, cells were stimulated with 10 ng/ml of recombinant human IL-1β for 6 h. Then, U937 cells (2.5×10^5 cells/well) were seeded over the HUVEC monolayer and incubated for 30 min at 37°C. After incubation, nonadherent U937 cells were removed by washing each well three times with PBS+1% FBS. Cell adhesion was verified by obtaining five random photomicrographs from each well using an inverted phase contrast microscope and by counting the adherent cells using the NIH image analyzer program.
   To test the effect of PLM-f74 on monocyte adherence to HUVEC, U937 cells were incubated with PLM-f74 for 20 h, activated with IL-1β for 2 h, and then added to the HUVEC monolayer and incubated for 30 min. To test the effect of PLM-f74 on the adhesion of monocytes to HUVECs when both cells were treated with PLM-f74, we treated both cell types with the same concentration of PLM-f74 at different doses as described above, and then

stimulated HUVEC with IL-1β. After incubation of the monocytes with HUVEC for 30 min, the total number of adherent monocytes was determined.

Immunoprecipitation and Western Blot Analysis
For immunoprecipitation of signal molecules [8], HUVECs were seeded on 100-mm Petri dishes, and incubated in EBM2 medium with 2% FBS and grown to a confluent stage. PLM-f74 was treated with the different doses for 24 h and cells were washed with serum-free EBM2 medium. Control and PLM-f74-supplemented cells were starved overnight, and the cells then stimulated with recombinant human VEGF (BD Sciences, Bedford, MA, U.S.A.) at a concentration of 50 ng/ml for 30 min, 37°C. For protecting phosphorylation to protein [19], cells were pretreated for the last 7 min before lysis with a dilution of 1:1,000 from an equal volume mixture of vanadate (100 μmol/l) and hydrogen peroxide (200 μmol/l).
   Cells were quickly rinsed with ice-cold PBS+0.1 mM Na_3VO_4 and then solubilized on ice for 20 min with ice-cold lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1 mM vanadate, 1 mM EDTA, 1 mM FGTA, 0.2 mM PMSF, 0.5% NP-40) with a gentle side-to-side rocking motion. Cells were scraped and the lysates centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were collected and immunoprecipitated with protein G-Sepharose coupled to the polyclonal antibody VE-cadherin (1 μg/ml) for 12 h at 4°C. Immunoprecipitates were washed four times with lysis buffer containing 1 mM Na_3VO_4 and boiled in Laemmli sample buffer. Samples were separated by 7.5% SDS-PAGE gel and transferred to 0.2 μm nitrocellulose membranes (Bio-Rad Lab. Hercules, CA, U.S.A.). The membranes were blocked with 5% NFDM (non-fat dry milk) in PBS containing 0.1% Tween-20 and probed for 1 h at room temperature with either of the following antibodies: VEGFR2, PI3K, β-catenin, and VE-cadherin. Immunoreactive bands were visualized after 1 h incubation using horseradish-peroxidase-conjugated anti-goat antibodies, followed by a Western Blotting Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Determination of Cell Adhesion Molecules by ELISA
Measurements of cell surface adhesion molecules were carried out by enzyme-linked immunosorbant assay (ELISA). Confluent HUVECs in 96-well plates were cultured in the absence or presence of PLM-f74 for 20 h at 37°C. After washing, 5 ng/ml recombinant human IL-1β (Endogen, Woburn, MA, U.S.A.) was added to stimulate the cells (37°C for 6 h). The medium was removed and the cells were fixed with 1% paraformaldehyde at room temperature for 30 min. The plates were then washed two times with PBS +0.5% Tween and blocked with 10% FBS in PBS for 1 h, and washed again two more times with PBS +0.5% Tween. Monoclonal antibodies to human CD106 (ICAM-1),

CD54 (VCAM-1), and 62E (E-selectin) (BD Biosciences, Bedford, MA, U.S.A.) in 10% FBS in PBS were added at 2, 5, and 5 Mg/ml, respectively, and incubated at 37°C for 2 h. The secondary antibody (horseradish-peroxidase-conjugated anti-mouse IgG G) (Bio-Rad, Hercules, CA, U.S.A.) was added at 1:1,000 dilution and incubated at room temperature for 1 h. The visualization agent, horseradish-peroxidase substrate (Bio-Rad, Hercules, CA, U.S.A.), was then added for 1 h. The plates were read at OD 405 nm in a plate reader (UVM-340, ASYS, Austria). Background (OD reading of wells without adding antibodies) was subtracted from all other wells.

Components for PLM-f74

PLM-f74 was refluxed with 6 N HCl for 3 h and neutralized with NaOH. For detecting carbohydrates, carbohydrates were analyzed using ion chromatography (DIONEX 600IC System, Sunnyvale, CA, U.S.A.) with a CarboPac PA10 column, 200 mM NaOH (50% w/w)/1 l+18 mM NaOH (50% w/w)/1 l as eluents, a flow rate of 1.0 ml/min, and Pulsed Amperometry, ED50, AgCl electrode for detection. To analyze amino acids, an amino acid analyzer (Biochrom 20, Pharmacia Biotech, Cambridge, U.K.) was used.

Statistical Analysis

Data are expressed as mean±SD of three experiments. Statistical analysis was carried out using the Student's t-test following an ANOVA. P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Selection of λ-Bacteria from Fusiform Fish of *Trachurus japonicus*

Selected λ-bacteria were cultured in 250-ml flasks with a marine medium for 50 h at 25°C until their antioxidative capacities had increased, as shown in Fig. 1. As the culture time increased, the ORP values decreased to a reduced
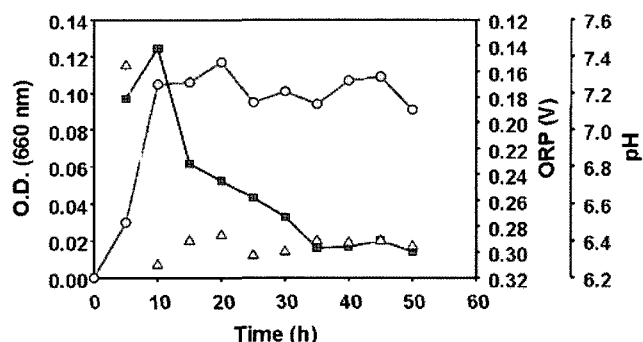

Fig. 1. Time course of biomass, pH, and ORP values of λ28 bacteria.
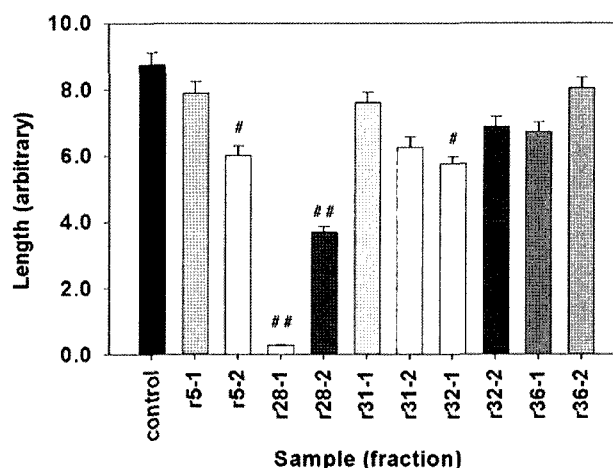●: Optical density at 660 nm; ■: ORP values; ▲: pH.

Fig. 2. Screening of strains bearing anti-angiogenesis effect from fusiform fish.
Data are the mean±SD of three experiments. $^{\#\#}P<0.01$, $^{\#}P<0.05$ compared with control.

category of -0.229 Volts, and thus, were designated as having higher antioxidative capacity. Similarly, five types of λ-bacteria were screened as λ5, λ28, λ31, λ32, and λ36 bacteria, according to their increasing antioxidative capacities. ORP values decreased quickly to the reduced category shown in Fig. 1; it seemed to be synthesized by some material carrying higher antioxidative capacity, such as protein-like materials.

Anti-angiogenic Effect of λ-Bacteria

Protein-like material (PLM) was taken from the λ5, λ28, λ31, λ32, and λ36 bacteria for their anti-angiogenic effects, and 30 μl samples from two different fractions of each strain were used. Of these λ-bacteria, the PLM from the λ28 strain (designated as PLM-28) had excellent anti-angiogenic effects (96.8% and 57.6%), as indicated from Fig. 2. All these λ-bacteria exhibited higher antioxidative capacities, but only the λ28 strain showed a higher anti-angiogenesis effect, which suggested that higher antioxidative capacity materials did not always indicate an anti-angiogenesis property.

Size Exclusion Chromatography (SEC) for PLM

The lyophilized PLM-28 was subjected to SEC using a 2 cm×150 cm column (D×H) packed with Sephadex G-100, with a flowing phosphate buffer (pH 7.0). The protein-like material was measured at 280 nm with a spectrophotometer, as shown in Fig. 3.

Antioxidative Capacity of PLM-28

The antioxidative capacity for the fractions numbered 27, 62, 66, 71, 74, 78, 82, and 86 from PLM-28 was measured by the FRAP method. Fractions numbered 62, 71, 74, and 82 showed the highest antioxidative capacities; 25 μM
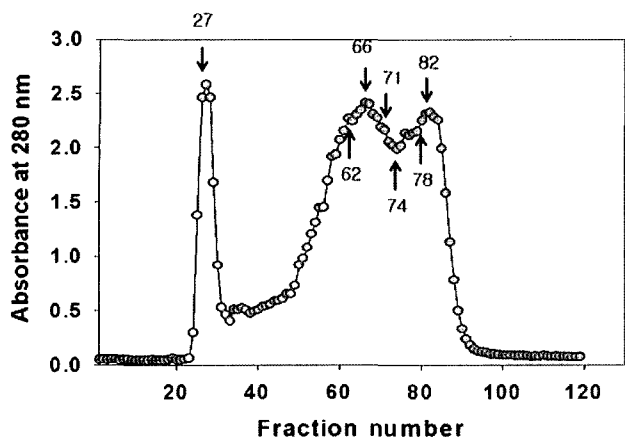
Fig. 3. A280 vs. fraction number of PLM from strain λ-28 with size exclusion chromatography.

EGCG was included as a positive control, as shown in Fig. 4.

Anti-angiogenic Effect of PLM-28

Fractions numbered 74 and 82 shown in Figs. 3 and 4 expressed higher anti-angiogenesis effects because of their antioxidative capacities.

Fig. 5 indicates the results for when these effective fractions were tested for anti-angiogenesis effects with different concentrations of PLM. The PLM-28 from fraction number 74 (PLM-f74) showed strong inhibition ratios of angiogenesis as 82.8%, 65.9%, 30.2%, and 22.3%; fraction number 82 exhibited 67.3%, 48.0%, 15.6%, and 3.3% at the concentration of 18.5 μg/ml, 7.4 μg/ml, 3.7 μg/ml, and 0.74 μg/ml, respectively. Low molecular weight PLM (No. 74) had a higher anti-angiogenesis effect than that of the high molecular weight PLM (No. 27). It was assumed that the low molecular weight PLM would inhibit the interaction
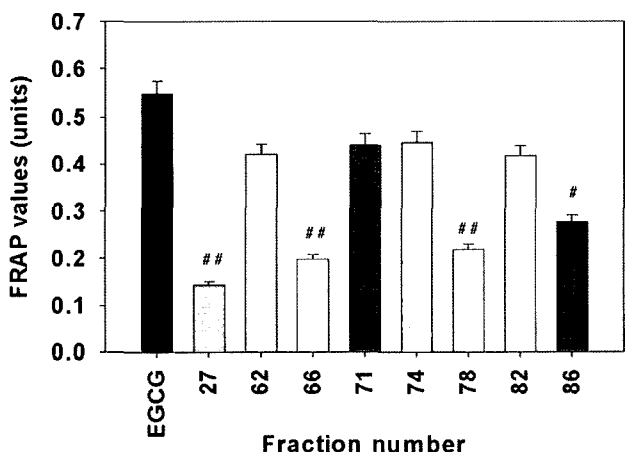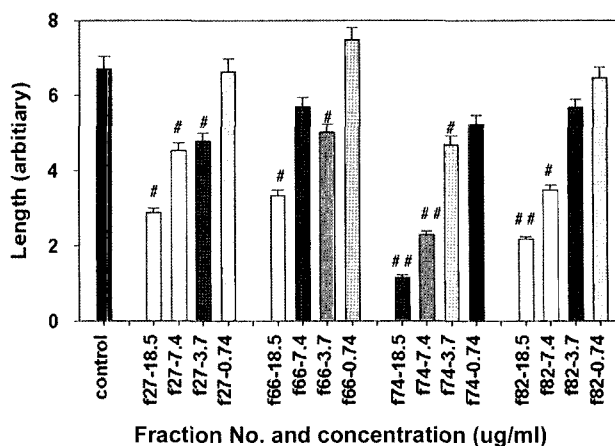

Fig. 5. Anti-angiogenic effect of PLM-28 with effective fraction on their concentration.
Data are the mean±SD of three experiments. [##]P<0.01, [#]P<0.05 compared with control.

of immune and endothelial cells, which is mediated through the endothelial expression of cell surface adhesion molecules and ligands, thereby acting as receptor molecules [13].

Although PLM-f82 exhibited a lower anti-angiogenic effect than PLM-f74, and despite that PLM-f82 was a lower molecular than PLM-f74, it would seem to be a suitable structure for interaction between PLM and receptor molecules [11].

Toxicity of PLM-28

When toxicity for the four types of effective fractions numbered 27, 66, 74, and 82 was examined of different concentrations of PLM-28, fraction numbers 74 and 82 expressed lower toxicities, as shown in Fig. 6. The toxicity of a compound occurs by the uptake of the compound by
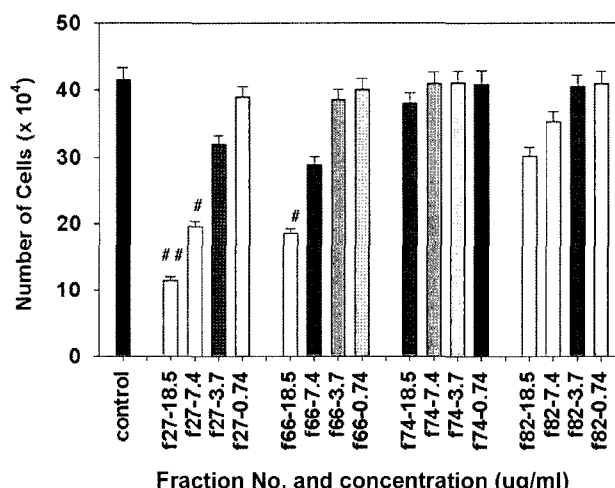

Fig. 4. Antioxidative capacity of fractions from PLM-28, with 25 μM EGCG as a positive control.
Data are the mean±SD of three experiments. [##]P<0.01, [#]P<0.05.

Fig. 6. Cytotoxicity with PLM-28 fraction against HUVECs.
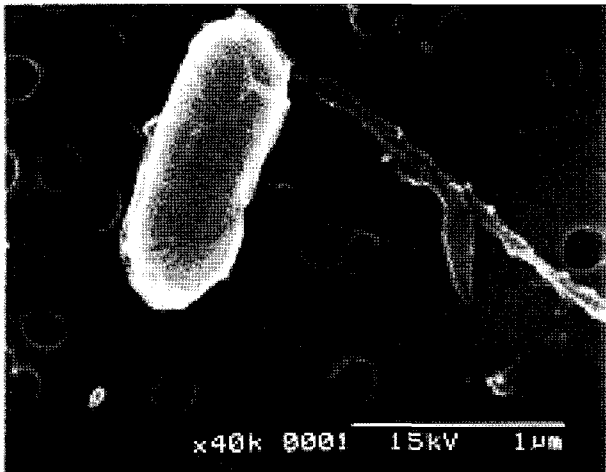Data are the mean±SD of three experiments. [##]P<0.01, [#]P<0.05 compared with control.

Fig. 7. SEM photograph of strain λ-28 with unidentified material like polysaccharide.

stimulated with IL-1β, however, adhesion increased significantly. This stimulated adhesion was clearly inhibited by PLM-f74, and in a dose-dependent manner. PLM-f74 at 0.74, 3.7, 7.4, and 18.5 μg/ml suppressed U937 cell adhesion by 12.1, 21.2, 50.9, and 78.2%, respectively.

the cell or through interaction with the cell membrane and associated molecules, so that a higher molecular weight fraction (27th or 66th fraction) would less likely be taken up than the lower molecular weight fractions (74th or 82nd).

Identification of λ-28 Strains

By morphology, this bacterial strain was a rod-shaped bacterium (Fig. 7), Gram (−), and measuring 1.5 μm in length by 0.5 μm in width that grew rapidly on MRS medium at 25°C with a 2% NaCl concentration. From the results of Fig. 8, the λ-28 strain was identified as *Enterobacteria cancerogenus* having 99% homology according to GenBank data.

Cell Adhesion

U937 cell adhesion to IL-1β-stimulated HUVEC. The monocyte-like U937 cells did not adhere significantly to unstimulated HUVECs (Fig. 9A). When HUVEC was
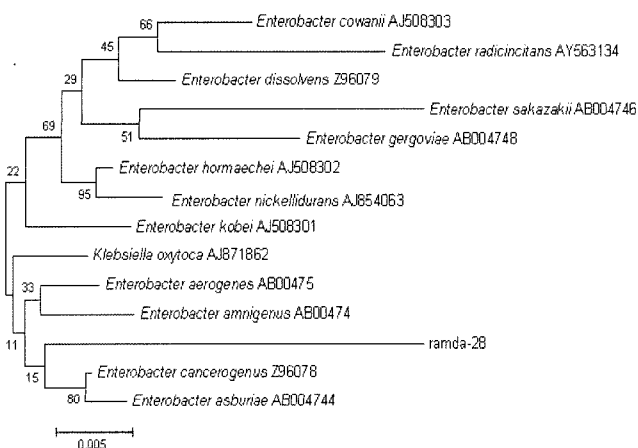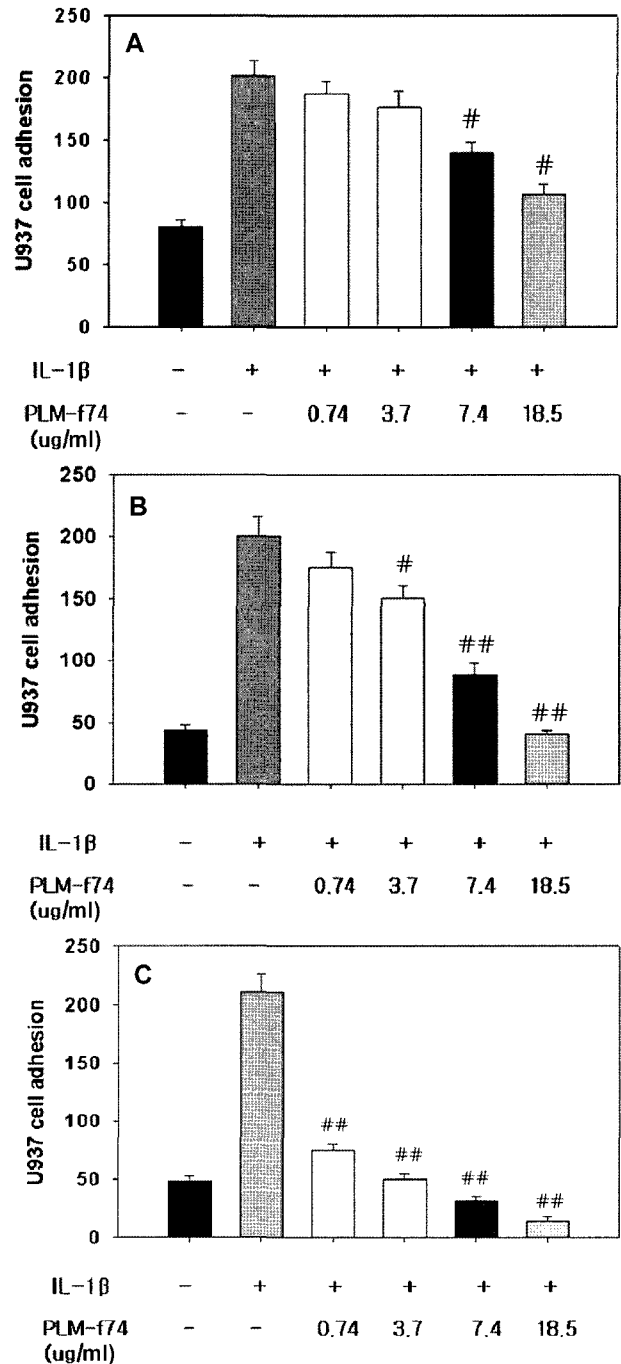

Fig. 8. Identification of strain λ-28 with 16S rDNA sequencing.

Fig. 9. The effect of PLM-f74 on inhibition of U937 cell adhesion to HUVEC with or without IL-1β. **A.** U937 stimulated with IL-1β; **B.** Cell adhesion to HUVEC; **C.** Both cells incubated with PLM-f74 and HUVECs stimulated with IL-1β. Data are the mean±SD of three experiments.

IL-1β-Stimulated U937 cell adhesion to HUVECs.

U937 cells were treated with the PLM-f74 at different doses and stimulated with *IL-1β* for 2 h, and then tested for their adherence to untreated and unstimulated HUVECs (Fig. 9B). A dose-dependent decrease in adhesion (by 15.8, 31.9, 70.8, and 102%) was observed in U937 cells treated with the same concentrations as above, respectively. The inhibitory effect of PLM-f74 on the reduction of U937 cell adhesion to HUVECs was more pronounced when U937 cells were treated with PLM-f74 compared with when HUVECs were treated with these PLM-f74.

U937 Cell adhesion to HUVECs when both cells were treated with PLM-f74.

The adhesion of U937 cells to IL-1β-stimulated HUVECs was markedly decreased when both cell types were pretreated with PLM-f74 (Fig. 9C). PLM-f74 dose dependently (p<0.01) inhibited the adhesion of U937 cells to HUVECs by 83.7, 99.2, 110, and 120.8% at the same concentrations as above, respectively.

Cell adhesion is very important for cell morphogenesis, cell maintenance, and tumor metastasis. Because PLM-f74 significantly suppressed cell adhesion induced by IL-1β, which regulates cell surface adhesion molecules and the adhesion of circulating monocytes to the arterial endothelial lining, PLM-f74 could be very useful in preventing metastasis and atherosclerosis.

Western Blot for Signal Molecules for PLM-f74

To determine whether the PLM-f74 inhibition of tubular formation in HUVEC is through suppression of the signal pathways of VEGFR-2, PI3K, β-catenin, and VE-cadherin, the expression of signal molecules was assessed by use of anti-signal molecule antibodies such as anti-VEGFR-2(Flk-1), anti-PI3K, anti-β-catenin, and anti-VE-cadherin, respectively. As shown in Fig. 10, VEGF significantly increased the signal molecules, and with pre-supplementation of HUVECs with dose-dependent dosages of 0.74 and 18.5 µg/ml, PLM-f74 inhibited expression of all four types of signal molecules. Finally, because PLM-f74 suppressed the pathways from four kinds of signal molecules to NF-kB, angiogenesis would be decreased. Specifically, VE-cadherin is an important molecule in cell-cell recognition and also to strengthen cell contact during vascular morphogenesis [32].

Recently, Carmelit *et al.* [7] reported that VE-cadherin molecules are also involved in VEGF signaling for vascular endothelial cell survival and proliferation in angiogenesis. VE-cadherin and its associated molecule, β-catenin, chemically cross-link with VEGFR2-PI3 kinase and supports the survival of endothelial cells and the development of new capillaries. VEGF, which has been shown to be a major factor in tumor growth and other growth factors produced from tumor cells and blood vessel cells, plays an important role in expansion of the microvascular network needed to supply oxygen and nutrients for the rapid growth of tumor masses [15].

Expression of Adhesion Molecule by HUVEC with ELISA

PLM-f74 reduced IL-1β-stimulated HUVEC expression with dose dependence, and showed a significant inhibition with ICAM-1 by 40%, VCAM-1 by 60%, and E-selectin by 73% at the concentration of 18.5 µg/ml.

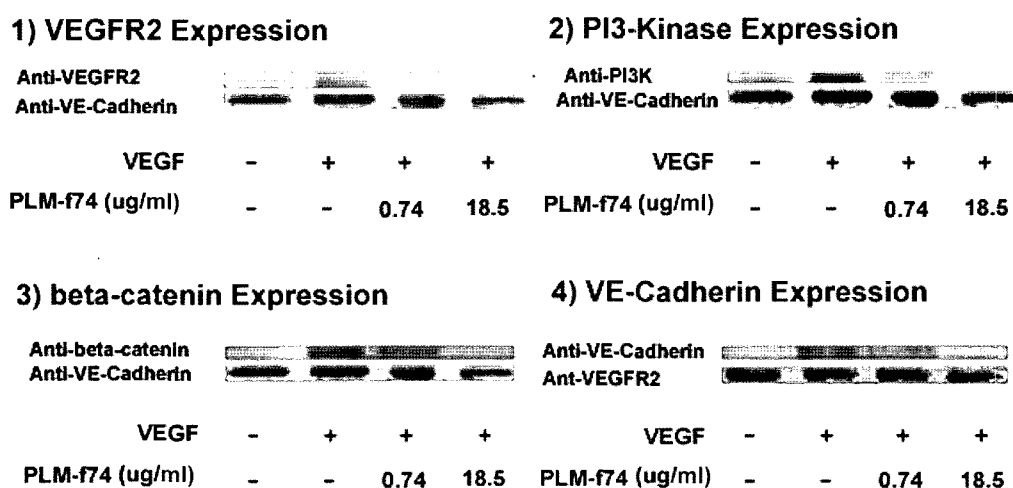Our data demonstrating a reduction of IL-1β monocyte adhesion by PLM-f74 and a decrease of ICAM-1 production

Fig. 10. Effect of PLM-f74 on interaction of VE-cadherin with VEGFR-2, PI3-kinase, and β-catenin upon cell activation with VEGF. HUVEC cell extracts were immunoprecipitated with VE-cadherin antibodies (1–3) and immunoblotted with antibodies to VEGFR-2, PI3-kinase, and β-catenin, IP with VEGFR2 (4) and IB, VE-cadherin.
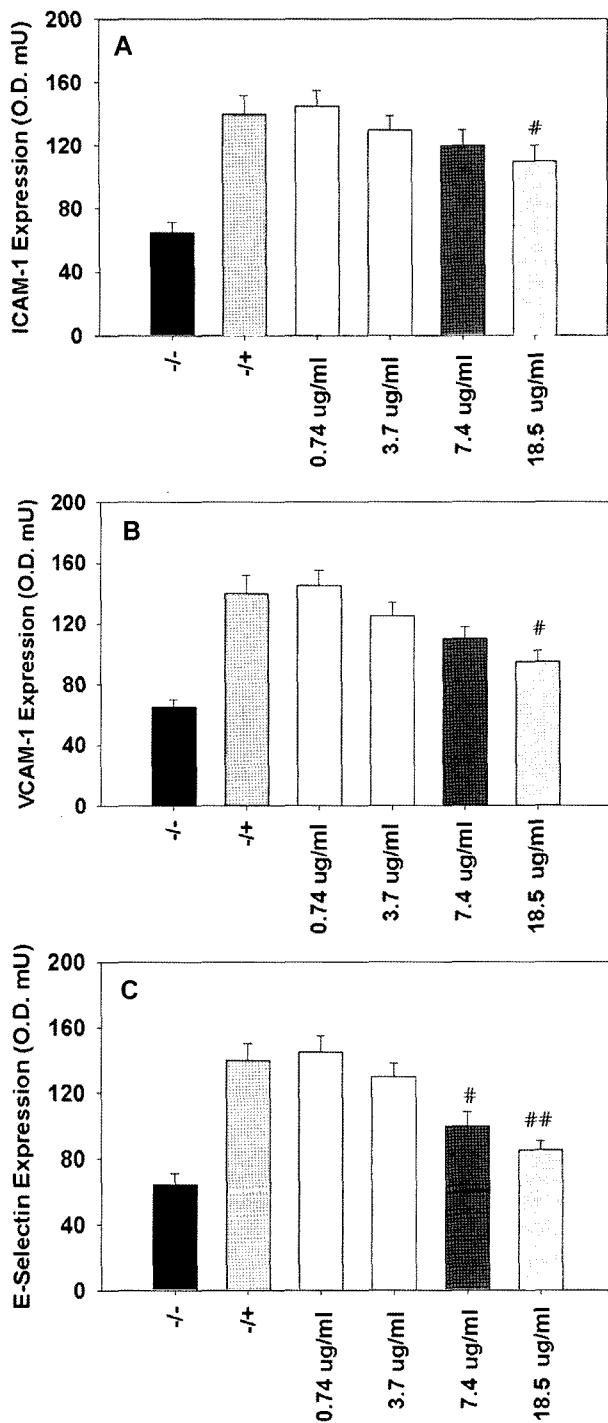Cells were starved during supplementation for 24 h before stimulation with VEGF (50 ng/ml).

Fig. 11. Inhibition of endothelial cell adhesion molecule expression by PLM-f74 at different doses of ICAM-1 (**A**), VCAM-1 (**B**), and E-selectin (**C**).
Data are the mean±SD. of three experiments. $^{##}P<0.01$, $^#P<0.05$ compared with control.

reported to be reduced by antioxidants such as N-acetyl cystein [22]. α-Tocopherol has also been reported to inhibit adhesion of the U937 monocyte cell to HUVECs when stimulated with agonists such as IL-1β [14]. In this study, PLM-f74 had higher antioxidative and capacity and also inhibited IL-1β-induced ICAM-1, VCAM-1, and E-selectin. Similar results were identified by Faruqui et al. [14], who reported that IL-1β-induced monocyte adhesion was correlated with reduced expression of E-selectin by HUVECs. Because IL-1β regulates the expression of specific proteins involved in the adhesion and subsequent migration of leukocytes into tissues [9], PLM-f74 could have an important role in the inhibition of angiogenesis and atherosclerosis based on IL-1β actions.

Components of PLM-f74
The photograph of this strain showed polysaccharide-like material on its cell wall (Fig. 7), and PLM-f74 was measured at A280 nm and some bands were obtained in SDS-PAGE (data not shown). From an analysis by ion chromatography, three types of saccharides were detected: D-glucose, D-arabinose, and lactose. Amino acid analysis indicated 18 components: glu, amm, met, arg, leu, his, Ile, ser, ala, lys, gly, tyr, thr, val, phe, asp, cys, and pro.

PLM-f74 is assumed to be a glycoprotein, because of the identification of saccharides and amino acids, and no lipids by hexane extraction.

Characteristics of PLM-f74
A λ28 strain bearing higher antioxidative capacity was selected as a potential anti-angiogenic material identified with Enterobacteria cancerogenus. PLM-f74 (protein-like material from the 74th fraction of SEC), comprised of saccharides and amino acids, showed strong inhibition ratios of angiogenesis (82.8%, 65.9%, 30.2%, and 22.3% at the concentrations of 18.5 μg/ml, 7.4 μg/ml, 3.7 μg/ml, and 0.74 μg/ml, respectively). PLM-f74 also controlled non-activated U937 monocyte cell adhesion to HUVECs activated with IL-1β by 78.0% at the concentration of 18.5 μg/ml. Adherence of U937 cells treated with PLM-f74 and stimulated with IL-1β to unstimulated HUVECs decreased by 102% at the same concentrations. When both cell types were pretreated with PLM-f74, the adhesion of U937 cells to IL-1β-stimulated HUVECs was completely suppressed, i.e., 98.0%, 99.3%, 110%, and 121% at the concentrations of 0.74 μg/ml, 3.7 μg/ml, 7.4 μg/ml, and 18.5 μg/ml, respectively. PLM-f74 suppresses angiogenesis because the signal pathways from the signal molecules VEGFR-2, PI3K, β-catenin, and VE-cadherin to NF-kB that activate angiogenesis were blocked significantly, as indicated by western blot analysis. The expression of cell adhesion molecules of ICAM-1, VCAM-1, and E-selectin was inhibited by PLM-f74, as determined by ELISA. New anti-angiogenic and anti-cell adhesion materials suggesting

by HUVECs can be interpreted to support important roles of use for early events in atherosclerosis. Furthermore, IL-1β-induced expression of VCAM-1 by HUVECs has been

high antioxidative capacities and reduced toxicities may be expected from these results.

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