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Anti-Angiogenic Activity of Acalycixenolide E, a Novel Marine Natural Product from *Acalycigorgia inermis*

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Abstract Angiogenesis is known as a crucial process in the growth and spreading of tumor cells. Accordingly, the effective inhibition of this process would appear to be a promising way to cure angiogenesis-related diseases, including cancer. This study demonstrates that acalycixenolide E (AX-E) from the marine organism *Acalycigorgia inermis* exhibits a potent anti-angiogenic activity both *in vitro* and *in vivo*. AX-E inhibits the bFGF-induced proliferation of HUVECs in a dose dependent manner, along with the bFGF-induced migration, invasion, and tube formation of HUVECs. Moreover, AX-E potently inhibits the *in vivo* neovascularization of the chorioallantoic membranes (CAMs) of growing chick embryos. Interestingly, AX-E suppresses the expression of metalloproteases 2 and 9, yet shows no effect on their activities. The novel chemical structure and potent anti-angiogenic activity of AX-E will be of great value in elucidating the molecular mechanism of angiogenesis as well as in the development of a novel anti-angiogenic drug.

Key words: Anti-angiogenic agents, acalycixenolide E, *Acalycigorgia inermis*

Angiogenesis is the process of new blood vessel formation by endothelial cells [7-10, 21]. The process is complex and involves several distinct steps such as membrane degradation by proteolytic enzymes secreted by the endothelial cells, chemotaxis toward the stimulus, proliferation of these cells, formation of vascular loops, maturation of neovessels following the perivascular apposition of pericytes and smooth muscle cells, and neosynthesis of basement membrane constituents [29]. Under normal conditions, all these steps are tightly regulated in order to avoid any undesired neovascularization, and several control mechanisms are known to be involved [10]. However, under pathological conditions, such as an outgrowing solid tumor, these tight regulatory mechanisms can be disordered mainly by a number of angiogenic factors constitutively secreted from either the tumor or accessory cells attracted to the site of neoplastic cells when their environment becomes hypoxic or inflammatory [1, 10, 16, 29].

Extensive studies have shown that angiogenesis is a crucial process for the out-growth of cancer cells and their spreading into other tissues. Therefore, the specific inhibition of angiogenesis is considered as a powerful way to suppress angiogenesis-related diseases, including cancer. Several angiogenesis inhibitors from natural products and chemical synthesis have been developed for this purpose. These include angiostatin [11, 30], endostatin [2, 6, 11, 30], and canstatin [15] as peptide inhibitors, and thalidomide [5], TNP470 [12, 27], radicicol [24, 25], and depudecin [18, 22, 23, 28] as low molecular weight compounds from microbial metabolites, and marimastat and sesquicillin as metalloproteinase inhibitors [13, 19]. Some of these compounds are currently undergoing clinical phase trials, yet novel angiogenesis inhibitors with different chemical and biological properties will still contribute to the development of new anti-angiogenic therapy and be used as powerful probes to decipher the complex mechanism of angiogenesis.

Marine organisms have provided several unique compounds with interesting biological activities [3]. However, very little attention has yet been paid to the anti-angiogenic activity of marine natural products. Nonetheless, it is possible that marine natural products may have a different chemical structure from that of known angiogenesis inhibitors since

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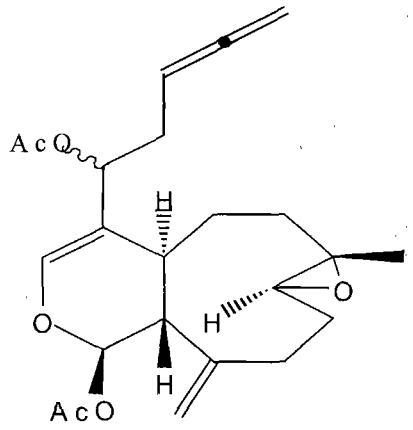

Fig. 1. Chemical structure of acalycixenolide E (AX-E).

most of the latter originate from terrestrial microorganisms or plants. This idea prompted a screening program for the anti-angiogenic activity of several bioactive compounds from marine organisms, and Acalycixenolide E (AX-E, Fig. 1) with a unique xenicane diterpenoid structure was found to have a potent anti-angiogenic activity both *in vitro* and *in vivo*. Accordingly, the present study demonstrates the anti-angiogenic activity of AX-E from the marine organism *Acalycigorgia inermis*.

MATERIALS AND METHODS

Materials

The AX-E was prepared as described previously [26]. The basic fibroblast growth factor (bFGF) was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.), the M199 and heparin from Life Technology (Grand Island, NY, U.S.A.), the Matrigel from Collaborative Biomedical Products (Bedford, MA, U.S.A.), the Transwell plate from Corning Costar (Cambridge, MA, U.S.A.), and the gelatin type B, triton X-100, and Coomassie Brilliant Blue G-250 from Sigma (St. Louis, MO, U.S.A.).

Cell Culture

The HUVECs were isolated from a human umbilical vein, as described previously [14]. The cells were grown in a gelatin-coated 25-cm^2 flask in M199 with 20% fetal bovine serum (FBS), 5.5 ml penicillin/streptomycin, 3 ng/ml bFGF, and 5 units/ml heparin at 37°C under 5% CO_2, 95% air. The cells used in this study were from passages 3 to 7. The CHANG cells (DMEM media) were grown with 10% FBS, 5.5 ml penicillin/streptomycin, and $NaHCO_3$.

Tube Formation Assay

Two hundred and fifty μl Matrigel (10 mg/ml) was placed in a 24-well culture plate and polymerized for 30 min at 37°C. The HUVECs (1×10^5 cells) were seeded on the surface of the Matrigel and treated with bFGF (30 ng/ml). Then, the marine natural product was added and incubated for 6–18 h. The morphological changes in the cells were observed under a microscope and photographed at a 40× magnification using the ImagePro Plus software (Media Cybernetics, Inc.). Each sample was assayed in duplicate and the experiment was repeated twice independently.

Viability Assay

The HUVE and CHANG cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. The cells were incubated in growth media and allowed to attach for 3 h. The cells were then washed with PBS and exchanged with fresh media. AX-E (1–20 μg/ml) was added to each well and incubated for 72 h. After the incubation, 50 μl of MTT (2 mg/ml stock solution, Sigma) was added and the plates incubated for an additional 4 h. After removal of the medium, 100 μl of DMSO was added. The plate was read at 540 nm using a microplate reader (Bio-Tek Instruments, Inc.). The experiment was repeated three times independently.

bFGF-Induced Proliferation of HUVECs

The HUVECs were starved for 6 h in an M199 medium containing 1% FBS and then harvested by trypsinization. The cells were inoculated at a density of 5×10^3 cells/well in a gelatin-coated 96-well plate supplemented with 150 μl of an M199 medium containing 1% FBS and incubated for 4 h. bFGF was added at a concentration of 30 ng/ml and various concentrations of AX-E were added. After 3 days, the cell proliferation was measured by an MTT assay. Each sample was assayed in duplicate and the experiment was repeated twice independently.

Chemomigration Assay

Chemomigration of the HUVECs was performed using 12-well transwell culture chambers with 8.0-μm-pore-polycarbonate filter inserts. The lower surface of the filter was coated with 10 μl gelatin (1 μg/ml), and bFGF prepared in 600 μl of M199 with 1% FBS was placed in the lower wells. The HUVECs (1×10^5 cells) were placed in the upper part of the filter and AX-E was applied to both sides of the filter. The chamber was incubated at 37°C for 4 h, then the cells were fixed with methanol and stained with hematoxylin/eosin [4]. The cells on the upper surface were carefully removed by rubbing with a cotton swab and mounted on slide glasses. The cell migration was determined by counting the whole cell numbers on a single filter using optical microscopy at 40× magnification. Each sample was assayed in duplicate, and the experiment was repeated three times independently.

Chemoinvasion Assay

The invasiveness of the HUVECs was performed *in vitro* using a transwell chamber system with 8.0-μm-pore-

polycarbonate filter inserts. The lower side of the filter was coated with 10 μl gelatin (1 μg/ml), whereas the upper side was coated with 10 μl of Matrigel. The HUVECs (1×10^5 cells) were placed in the upper part of the filter and AX-E was treated to both parts for 30 min at room temperature before seeding. The chamber was then incubated at 37°C for 18 h. The cells were fixed with methanol and stained with hematoxylin/eosin. The cell invasion was determined by counting the whole cell numbers in a single filter using optical microscopy at 40× magnification. Each sample was assayed in duplicate and the experiment was repeated three times independently.

Chorioallantoic Membrane (CAM) Assay

Fertilized chick eggs were kept in a humidified incubator at 37°C for 3 days. About 2 ml of egg albumin was then removed with a hypodermic needle allowing the CAM and yolk sac to drop away from the shell membrane. On day 3.5, the shell was punched out, removed, and the shell membrane peeled away. At the stage of a 4.5-day-old chick embryo, an AX-E-loaded thermanox coverslip was air-dried and applied to the CAM surface. Two days later, 2 ml of 10% fat emulsion was injected into the chorioallantois and the CAM was observed under a microscope. When the CAM-treated sample showed an avascular zone, the response was scored as positive and calculated based on the percentage of positive eggs to the total number of eggs tested. The experiment was repeated three times and more than 20 eggs were used each time.

Gelatin Zymogram Assay

The HT1080 cells were seeded at a density of 1×10^6 cells/well in a 12-well plate and incubated for 24 h. Before being treated with PMA (100 ng/ml), the cells were incubated in 1 ml of a serum-free medium containing various concentrations of AX-E. The conditioned media containing 100 μl of secreted proteins were analyzed by SDS-PAGE using 8% acrylamide copolymerized with gelatin (0.4 mg/ml). After electrophoresis, the gel was rinsed three times with 2.5% Triton X-100 for 30 min and incubated for 24 h at 37°C in a substrate buffer (0.05 M Tris · HCl, pH 7.5, 0.15 M NaCl, 0.01 M $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% NaN_3). Gelatinase was identified by staining the gel in 10% methanol, 10% acetic acid, and 0.1% Coomassie blue G-250 for 30 min and then destaining in 10% methanol and 10% acetic acid for 3 h. The gelatinase-digested area appeared as a clear band on a blue background, indicating the enzyme location. To investigate the effect of AX-E on the enzyme activity *in vitro*, cultured media of HT1080 cells were harvested and 100 μl of the media was incubated with AX-E, as described above. After a 1-h incubation, the reaction was stopped by the addition of an SDS sample buffer and the enzyme activity was analyzed as described above. The experiment was repeated three times independently.

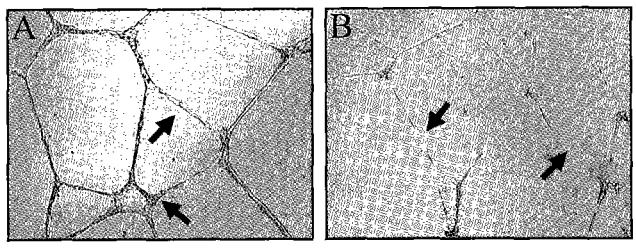

Fig. 2. AX-E inhibits tube formation in HUVECs.
HUVECs were seeded on Matrigel-coated wells at a density of 1×10^5 cells/well with bFGF (30 ng/ml) (A). The HUVECs were stimulated with bFGF and treated with 1 μg/ml AX-E (B). Photographs were taken 18 h after the drug treatment. Each sample was assayed in duplicate.

RESULTS AND DISCUSSION

AX-E Potently Inhibited Tube Formation of Endothelial Cells

Tube formation is one of the characteristic features of endothelial cells during angiogenic development. HUVECs cultured on Matrigel layers normally form incomplete and narrow tube-like structures in the absence of angiogenic factors, yet the capillary network formation is further stimulated by treatment with an angiogenic factor, such as bFGF (30 ng/ml), resulting in elongated and robust tube-like structures that are organized by a much larger number of cells (Fig 2A). Accordingly, a tube formation assay was previously used as the screening method to monitor whether or not marine natural products could affect angiogenesis. Among the 100 structurally different marine natural products tested, AX-E from *Acalycigorgia inermis* potently inhibited the tube formation of HUVECs (Fig. 2B). AX-E was recently isolated from orange-colored gorgonian *Acalycigorgia inermis* Hedlund from the sandy bottom offshore at Keomun Island, Korea. The crude organic extract of this animal exhibited considerable brine shrimp lethality (LC_{50} 127 ppm) and cytotoxicity against the P-388 (LC_{50} 37 μg/ml) and K562 (LC_{50} 4.7 μg/ml) cells [26]. Four novel diterpenoid derivatives with a unique xenicane moiety were isolated from the crude organic extract, and AX-E was the only compound that showed the anti-angiogenic activity in the study (data not shown). Notably, acalycixenolide D (AX-D), bearing an α, β-unsaturated lactone group, does not exhibit both anti-angiogenic activity and cytotocixity against K562 (LC_{50} 52 μg/ml) cells, suggesting that the lactone group of the compound is important for its biological activity [26]. Although several diterpenoids have been isolated from marine organisms, AX-E is the first compound that has exhibited anti-angiogenic activity. These unique chemical and biological properties of AX-E led to the current investigation of the effect of AX-E on the angiogenesis-related properties of HUVECs.

Dose Response of AX-E on Endothelial Cell Growth

To examine the anti-angiogenic activity of AX-E in detail, the optimum dose of AX-E on endothelial cell growth was

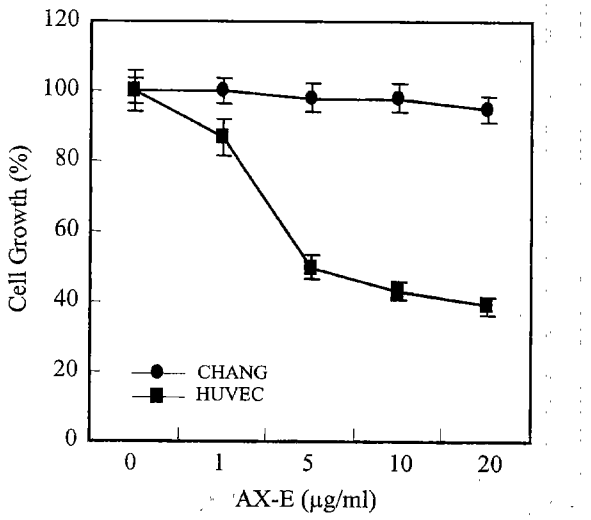

Fig. 3. Effect of AX-E on viability of HUVECs.
HUVE and CHANG cells were treated with various concentrations of AX-E (1–20 μg/ml) and incubated for 72 h. The cell viability was measured by an MTT assay. Data represent the means±SE of three independent experiments.

first determined. Various concentrations of AX-E (0–20 μg/ml) were applied to HUVE cells and the effect of AX-E on the cell growth was determined by an MTT assay. It was found that AX-E inhibited the growth of HUVE cells in a dose-dependent manner. Since 1 μg/ml AX-E inhibited the growth of HUVECs with no cytotoxicity within 24 h, the optimum dose of AX-E for HUVECs was determined as 1 μg/ml over 24 h. Interestingly, the growth inhibition activity of AX-E was not found in normal liver CHANG cells, thereby suggesting that AX-E has a selective growth inhibition activity on endothelial cells (Fig. 3).

AX-E Showed Potent Anti-Angiogenic Activities *In Vitro*

Angiogenic factors such as bFGF or VEGF are known to significantly enhance the angiogenic properties of HUVECs, such as proliferation, migration, and invasion [5]. Therefore, the effect of AX-E on the proliferation of HUVECs stimulated by bFGF was investigated. As shown in Fig. 4A, AX-E completely inhibited the proliferation of HUVECs induced by bFGF, thereby suggesting that AX-E can suppress a bFGF-induced proliferation signal. Next, the effect of AX-E on the motility of HUVECs stimulated by bFGF was investigated. AX-E efficiently inhibited the migration of HUVECs induced by bFGF, suggesting that AX-E can also suppress the bFGF-induced migration of endothelial cells (Fig. 4B). As another important property of angiogenesis, migrating endothelial cells must break and traverse through their own basement membrane to form new blood. Therefore, the effect of AX-E on endothelial cell invasion was studied using a polycarbonate filter of transwells coated with Matrigel, thereby preventing the migration of noninvasive cells. HUVECs were seeded on the filter and allowed to invade

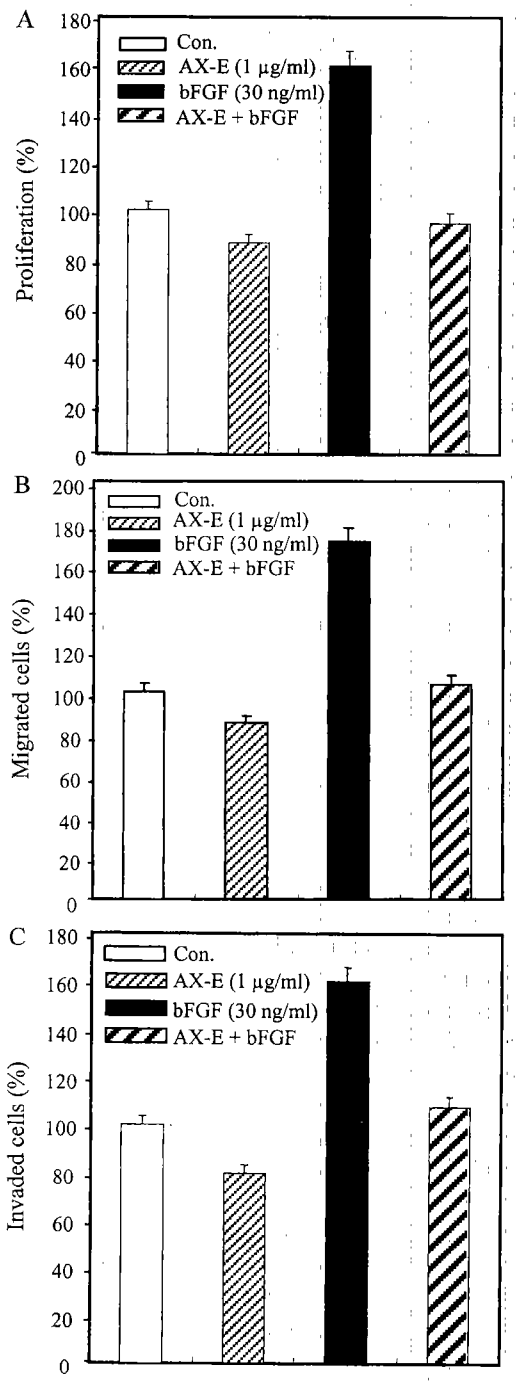

Fig. 4. Effects of AX-E on angiogenic properties of HUVECs *in vitro*.
(A) Effect of AX-E on the proliferation of HUVECs. The cells were treated with AX-E (1 μg/ml) with and without bFGF (30 ng/ml) and incubated for 72 h. AX-E completely suppressed any bFGF-induced proliferation of cells. (B) Effect of AX-E on the chemotatic motility of HUVECs. The inhibitory activity of AX-E on chemotatic motility of HUVECs was determined with and without bFGF (30 ng/ml). AX-E (1 μg/ml) significantly inhibited any bFGF-induced chemotatic motility. (C) Effect of AX-E on invasion of HUVECs. HUVECs were seeded at a density of 1×10^5 cells/well and treated with and without 30 ng/ml bFGF. The cells were then treated with the agent. AX-E significantly inhibited the bFGF-induced invasion compared to the control. All data represent the means ± SE of two different experiments.

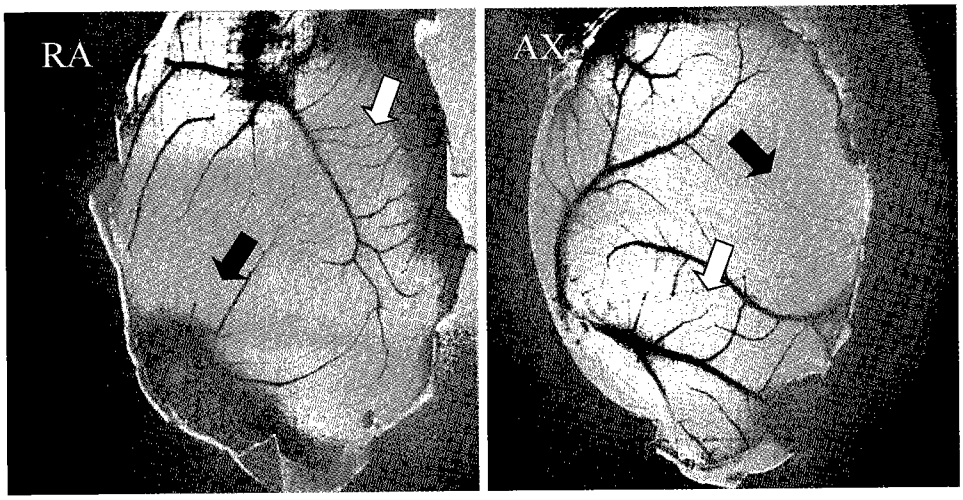

Fig. 5. Effects of AX-E on angiogenic properties of HUVECs *in vivo*.
Fertilized chick eggs were kept in a humidified incubator at 37°C. At the stage of a 4.5-day embryo, AX-E or a retinoic acid-loaded thermanox coverslip was applied to the CAM surface. Two days later, the chorioallantois was observed under a microscope. 1 μg/egg RA was used as the positive control for angiogenic inhibition. The filled arrow indicates the inhibition of angiogenesis, whereas the open arrow indicates neovascularization from the main vascular where it was not treated with the agent. Data are expressed as the mean±SE, and *$p<0.05$ compared to the control.

with or without bFGF treatment. As shown in Fig. 4C, AX-E significantly inhibited the bFGF-stimulated invasiveness of the HUVECs. All these data clearly show that AX-E potently inhibits the angiogenic properties of endothelial cells *in vitro*.

AX-E Showed Potent Anti-Angiogenic Activities *In Vivo*

The anti-angiogenic activity of AX-E was also demonstrated *in vivo*, utilizing a chorioallantoic membrane (CAM) assay. As shown in Fig. 5, AX-E produced a significant decrease in the development of angiogenesis in a chick embryo without any sign of thrombosis and hemorrhage. The inhibition of the angiogenesis of RA used as the positive control was 70.83±6.22% (n=16), yet AX-E (10 μg/egg) potently inhibited the neovascularization of the chick embryo (86±4.2%, n=20) without affecting any pre-existing vessels. The anti-angiogenic activity of AX-E *in vivo* was also confirmed using a Matrigel plug assay in an adult mouse (data not shown). Taken together, these results demonstrate that AX-E potently inhibits angiogenesis both *in vitro* and *in vivo* and is a novel inhibitor for angiogenesis with a different chemical structure from that of other known angiogenesis inhibitors.

AX-E Inhibited Expression of MMP-2 and -9, Yet Had No Effect on Enzyme Activities

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes, whose physiological functions include tissue remodeling and embryogenesis [4]. The importance of this group of enzymes in the process of tumor invasion and metastasis is widely recognized, and has led to an extensive search for MMP inhibitors for the development of novel type therapeutic drugs targeting cancer [13]. To determine whether the anti-angiogenic activity of AX-E originates from the inhibition of the activities of these proteins, a gelatin zymography assay was performed using HT1080 cells.

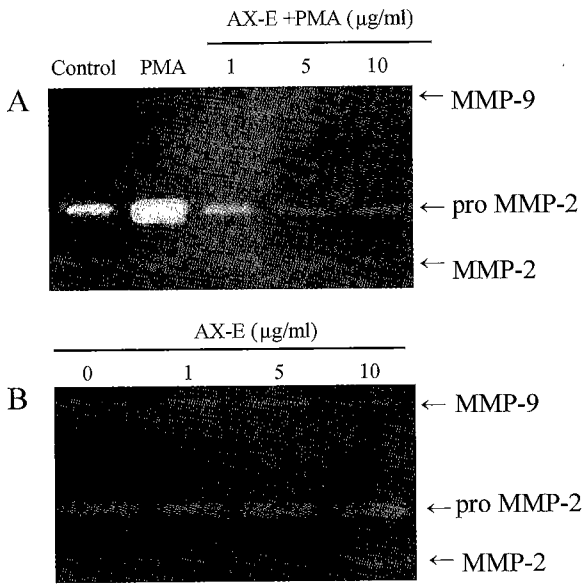

Fig. 6. Effect of AX-E on expression and activity of MMPs.
HT1080 cells treated with AX-E (1–10 μg/ml) were used in gelatin zymography assays. The culture media were used in gelatin-based electrophoresis and stained with Coomassie brilliant blue, as described in Materials and Methods. (A) Effect of AX-E on the expression of MMPs. The expression levels of MMP-2 and -9 were induced by PMA, whereas AX-E strongly suppressed the expression of the enzymes in a dose-dependent manner, thereby suggesting that AX-E may regulate the signal pathway for MMP expression. (B) Effect of AX-E on activity of MMPs. The MMP activity was not inhibited by AX-E, indicating that AX-E is not the catalytic activity inhibitor of MMP-2 and -9. The experiment was repeated three times.

The MMPs were observed as clear bands in a collagen copolymerized gel, and PMA (100 ng/ml) induced an expression level of MMP-2 and -9 of cells (Fig. 6A). Interestingly, AX-E strongly suppressed the PMA-induced expression of MMP-2 and -9 in a dose-dependent manner. However, AX-E showed no direct effect on the enzyme activity (Fig. 6B), thereby suggesting that the anti-angiogenic activity of AX-E may be related to the regulation of the metalloprotease-2 and -9 expression signal cascade, but not to the inhibition of enzymatic activities of MMPs like other MMP inhibitors [13]. Apicidin, a fungal metabolite that was known as a histone deacetylase (HDAC) inhibitor, showed the significant inhibition of H-ras-induced invasive phenotype of MCF10A human breast epithelial cells in parallel with a specific downregulation of MMP-2, but not MMP-9 [17]. Although AX-E did not show the selective downregulation of MMPs, AX-E may exhibit the anti-angiogenic activity by targeting the activity of HDAC, as shown in the case of apicidin. Since AX-E is a novel type angiogenesis inhibitor with a different chemical structure to that of other known inhibitors, mechanistic studies on its cellular target protein as well as its downstream effect on endothelial cells will provide new insights on both the molecular mechanism of angiogenesis and drug development related with angiogenesis therapy. A detailed study on the mechanism of the anti-angiogenic activity of AX-E is currently underway.

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