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Apicularen A, a Macrolide from *Chondromyces* sp., Inhibits Growth Factor Induced *In Vitro* Angiogenesis

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Abstract Apicularen A (Api A) was recently isolated from *Chondromyces* sp. as a potent antitumor agent. Because of its unique chemical structure, a macrolide with a highly unsaturated amide side chain, and potent growth inhibitory effect in various cancer cell lines, Api A is currently in clinical trial for cancer therapy. In the present study, the effect of Api A on *in vitro* angiogenesis of bovine aortic endothelial cells (BAECs) was investigated. Api A potently inhibited the proliferation of BAECs in a dose-dependent manner. Treatment of the endothelial cells with up to 10 ng/ml of the compound did not show any cytotoxicity. In addition, it inhibited basic fibroblast growth factor (bFGF)-induced invasion and capillary tube formation of BAECs at concentrations of 2–5 ng/ml. These results, therefore, demonstrate that Api A is a novel antiangiogenic agent and may suppress the growth of tumors, at least in part, by the inhibition of neovascularization.

Key words: Apicularen A, angiogenesis, macrolide, *Chondromyces* sp., *Myxobacteria*

Angiogenesis is a multistep process of new blood vessel formation in endothelial cells. The process is controlled by oppositely regulating factors that induce or suppress the angiogenic differentiation of the cells, and disruption of this balance results in pathologic diseases such as outgrowth and spreading of tumor cells [5]. Accordingly, the specific inhibition of this process would appear to be a promising way to cure angiogenesis-related diseases, including cancer. Several angiogenesis inhibitors have been developed for this purpose. These include angiostatin, endostatin, and canstatin as endogenous peptide inhibitors and TNP-470, eponemycin, radicicol, FK288, and acalycixenolide E as low molecular weight compounds from natural products [3, 6, 7, 8, 9, 10, 13, 15, 16]. Recently, *Myxobacteria*, a fruiting body forming microorganism, was reported to produce several antitumor agents with unique chemical structures, such as apicularens, salicylihalamides, and KR-025 [1, 4, 11, 16]. Through our continuing efforts in screening new antiangiogenic agents from natural products, apicularen A (Api A) has been identified as a potent antiangiogenic agent. Api A isolated from *Chondromyces* sp., one of *Myxobacteria* species, is a novel macrolide with a unique unsaturated amide side chain [11]. Api A shows a remarkable structural similarity to salicylihalamide (Fig. 1), a compound isolated from marine organism, but has much less cytotoxicity. Potent growth inhibitory activities of Api A against several cancer cell lines have been reported [11]. However, very little studies on other biological activities of the compound have been carried out. In this study, Api A was shown to be a potent inhibitor of proliferation, invasion, and tube formation of BAECs at concentration which showed no cytotoxicity. These results demonstrate that Api A is a novel antiangiogenic agent

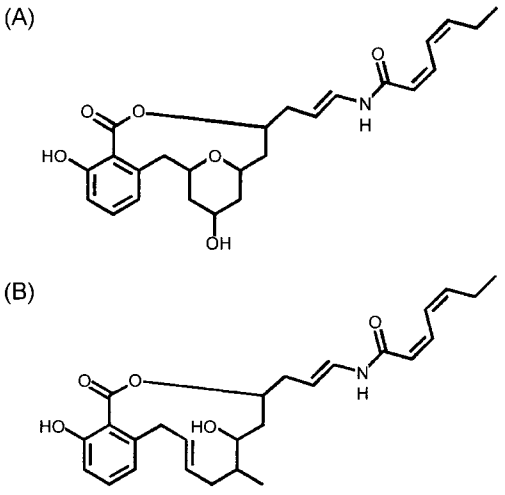

Fig. 1. Chemical structures of Api A and salicylihalamide.
The structural relationship between Api A (A) and salicylihalamide (B).

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with a unique chemical structure and can be developed as a new candidate for therapeutic agent of angiogenesis-related diseases.

The Api A was prepared as described previously [11]. The basic fibroblast growth factor (bFGF) was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma (St. Louis, MO, U.S.A.), cell culture media from Life Technology (Grand Island, NY, U.S.A.), the Matrigel from Collaborative Biomedical Products (Bedford, MA, U.S.A.), and the Transwell plate from Corning Costar (Cambridge, MA, U.S.A.). All chemicals used in this study were of the highest grade commercially available. The early passages (5-7 passage) of bovine aortic endothelial cells (BAECs) were kindly provided by Dr. Jo at the NIH of Korea. BAECs were grown in MEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2.

First, the effect of Api A on the growth of various cell lines, including B16/BL6 (murine melanoma cell), HT29 (human colorectal carcinoma cell), CHANG (human normal liver cell), and BAECs, was investigated. The cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. Various concentrations of Api A were applied to each well

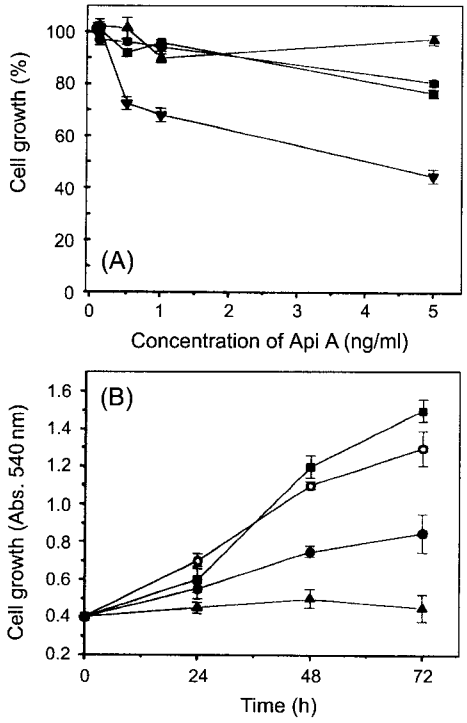

Fig. 2. Effect of Api A on the proliferation of BAECs.
The proliferation of BAECs was measured by MTT. A; Effect of Api A on the growth of various cell lines. Api A more potently inhibited the growth of BAECs. (▲), B16/BL6; (●), CHANG; (■) HT29; (▼), BAECs. B; Api A inhibited the proliferation of BAECs in a time- and dose-dependent manner. (■), control; (○), 2 ng/ml Api A; (●), 5 ng/ml Api A; (▲), 10 ng/ml Api A.

and cell growth was measured using MTT assay [9, 10]. Among the cell lines tested, BAECs were the most sensitive toward Api A compared to other cell lines (Fig. 2A). These data indicated that Api A inhibited the growth of cells with the specificity for endothelial cell. Next, the effect of Api A on the proliferation of BAECs was investigated at various time points. Api A potently inhibited the proliferation of BAECs in a dose-dependent manner (Fig. 2B). In addition, treatment with up to 10 ng/ml did not affect the endothelial cell viability, as confirmed by trypan blue staining (data not shown). Thus, all angiogenesis assays performed in this study were conducted at a concentration of 2–5 ng/ml of Api A. To explore the antiangiogenic effect of Api A, *in vitro* endothelial cell invasion assay was conducted as described previously by Kwon *et al.* [9]. Since endothelial cell invasion is a crucial step for spreading and migration of cells, the inhibition of this step has been considered to be a critical factor for anti-angiogenic agents [9]. Thus, the invasiveness of endothelial cells 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 mg/ml), whereas the upper side was coated with 10 μl of the Matrigel. BAECs (1×10^5 cells) were then placed in the upper part of the filter, and the chamber was 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 total number of cells in the lower side of the filter using optical microscopy at ×100 magnification. As shown in Fig. 3, bFGF effectively induced cell invasion through the filter, however, Api A potently inhibited bFGF-induced invasion of BAECs at the concentration of 2 ng/ml, and the invasiveness was completely inhibited at 5 ng/ml of Api A.

Next, the inhibitory effect of Api A on capillary tube formation, another key phenotype for endothelial cell differentiation of angiogenesis, was examined. Thus, 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 BAECs (1×10^5 cells) were then seeded on the surface of the Matrigel and treated with bFGF (30 ng/ml). Subsequently, the morphological changes of the cells and tube formation were observed under a microscope and photographed at ×100 magnification using a JVC digital camera (VICTOR, Japan). In the presence of bFGF, cultured BAECs on the Matrigel formed an extensive network of thick tubes (Fig. 4A), and treatment of BAECs with Api A resulted in a dose-dependent inhibition of tube formation (Figs. 4B, 4C). Trypan blue staining of formed tubes showed no stained cells, implying that the inhibition of tube formation by Api A was not merely due to the cytotoxic effect of the compound.

Interestingly, Api A showed a selective growth inhibitory pattern against BAECs compared to those of several cell

lines such as B16/BL, HT29, and CHANG cell at the concentration of 0.05–5 ng/ml (Fig. 2A). These results suggested that the antiangiogenic activity of Api A was due to specific inhibition of endothelial cell proliferation. In addition, Kunze *et al.* [11] reported that Api A induced morphological change in several cell lines which resulted in the formation of mitotic spindles with multiple spindle poles and thick stress fiber bundles in the actin cytoskeleton. In agreement with the above, we also observed that Api A induced the morphology of BAECs into a more elongated shape (data not shown). Microscopic observation of the cells suggested that Api A affected the cytoskeletal organization in the endothelial cells. These cytoskeletal changes may attribute to the inhibition of a certain step of angiogenesis such as the invasiveness of BAECs. Meanwhile salicylihalamide, a structural homologue of Api A, is known to inhibit mammalian V-type ATPase activity [2]. Notably, the expression level of ATPase in endothelial cells is high at plasma membrane, and angiostatin, a peptide inhibitor of angiogenesis, is known to inhibit the

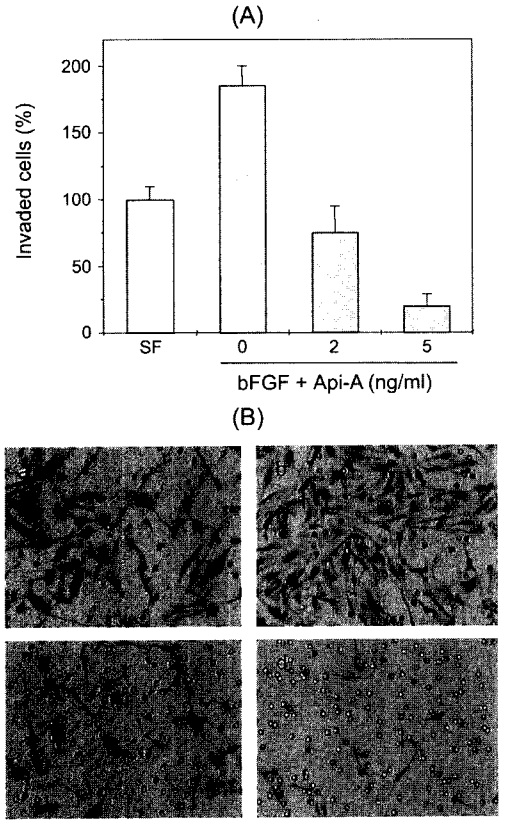

Fig. 3. Effect of Api A on bFGF-induced invasion of BAECs.
A; quantitive analysis of invasiveness of BAECs. B; microscopic observation of invaded cells. (a), serum free; (b), bFGF only; (c), bFGF+ 2 ng/ml of Api A; (d), bFGF+5 ng/ml of Api A. Figures were selected as representative scenes from two independent experiments. SF, serum free; bFGF, 30 ng/ml of basic fibroblast growth factor; Api A, Apicularen A. The experiment was repeated three times independently.

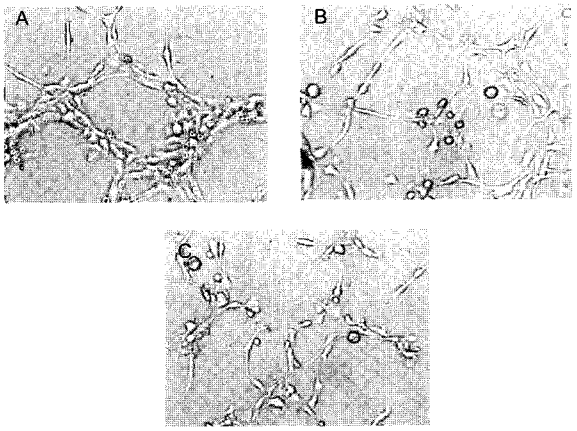

Fig. 4. Effect of Api A on capillary tube formation of BAECs.
Tube formation of BAECs was observed under microscope (×100 magnification). A, bFGF only; B, bFGF+2 ng/ml Api A; C, bFGF+5 ng/ml Api A. Figures were selected as representative scenes from two independent experiments.

enzyme activity [12]. Thus, ATPase-related enzymes in endothelial cells can also be taken into consideration for a biologically relevant target of Api A for its antiangiogenic activity.

In summary, while the exact mechanism for antiangiogenic activity of Api A remains to be investigated further, data presented in this study will provide new insight into the potent antitumor activity of Api A. Furthermore, this study demonstrated that Api A is a new antiangiogenic agent and can be used as a unique tool for the reverse chemical genetics study of angiogenesis.

Acknowledgments

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