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Gentisyl Alcohol, an Antioxidant from Microbial Metabolite, Induces Angiogenesis *In Vitro*

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Abstract Gentisyl alcohol isolated from *Penicillium* sp. has an antioxidative activity, protecting cells from oxidative stresses. From our *in vitro* angiogenesis assays with bovine aortic endothelial cells (BAECs), gentisyl alcohol was newly identified as a pro-angiogenic small molecule that induces new blood vessel formation of the cells. Gentisyl alcohol stimulated the proliferation of BAECs in a dose-dependent manner. Moreover, it induced *in vitro* angiogenesis of BAECs such as invasiveness, migration, and tube formation of the endothelial cells. Effects of gentisyl alcohol on invasion and tube formation were also dose-dependent. These results demonstrate that gentisyl alcohol could affect the angiogenic phenotypes of endothelial cells and be developed as a new small molecule with pro-angiogenic activity.

Key words: Angiogenesis, gentisyl alcohol, antioxidant, tube formation

Angiogenesis is a physiologically key process that is tightly regulated by numerous pro- or anti-angiogenic factors in the endothelial cells [6, 13, 18]. Therefore, abrogation of a tight balance of angiogenic regulation leads to several pathologic diseases that are associated with formation or inhibition of new vasculature [2–4]. Much attention has been drawn to identify the small molecules that inhibit angiogenesis, since several chronic diseases such as cancer, inflammation, and diabetes are associated with the formation of new vasculature [2–4, 7–9]. However, there has been comparatively little attention paid on the small molecules that induce angiogenesis as a potential therapy of ischemia-related diseases [22]. To discover such small molecules, we established an endothelial cell based assay and screened the chemical library of synthetic compounds and natural products. Consequently, gentisyl alcohol was identified as an active small molecule that induces the proliferation of endothelial cells.

Gentisyl alcohol was originally isolated from *Penicillium novae-zeelandiae* and known to exhibit an antioxidative activity preventing cells from oxidative stress [1]. Recently, Kim *et al.* [5] reported that the compound inhibits apoptosis

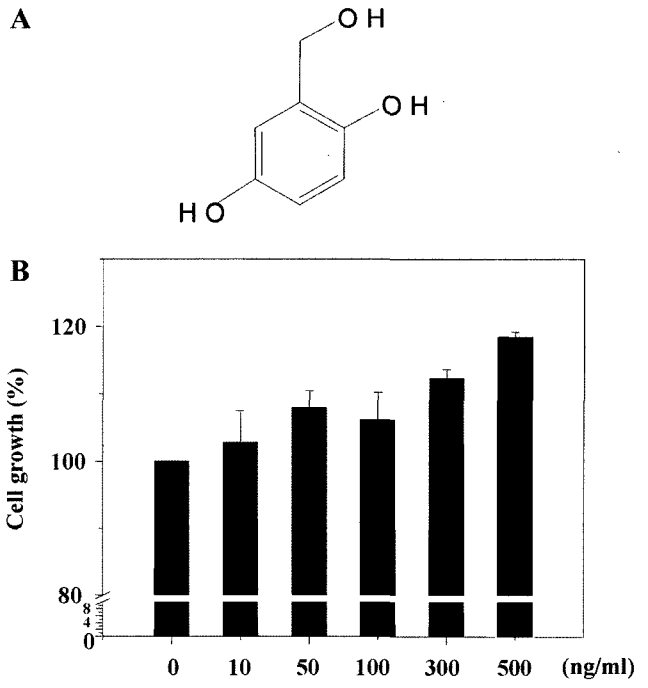

Fig. 1. Chemical structure of gentisyl alcohol ($C_7H_8O_3$) and its effect on the proliferation of BAECs.
A. Chemical structure of gentisyl alcohol. **B.** BAECs were treated with various concentrations of gentisyl alcohol (0.05–0.5 μg/ml) and incubated for 72 h. The cell proliferation was measured by MTT assay. The experiment was independently repeated twice.

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through the suppression of caspase-3 activity induced by etoposide. However, there has been no report so far on its pro-angiogenic activity. Consequently, this interesting biological activity of the compound led us to investigate in detail the effect of gentisyl alcohol on the angiogenic phenotypes of endothelial cells.

Gentisyl alcohol (Fig. 1A) was obtained from the culture extract of *Penicillium* sp. F020150 as described [5, 11, 15, 17, 21]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO, U.S.A.). Basic fibroblast growth factor (bFGF) was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.), cell culture media from Collaborative Biomedical Products (Bedford, MA, U.S.A.), and Transwell plates from Corning Costar (Cambridge, MA, U.S.A.). Early passage (4–8 passages) 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.

Using MTT assay, we first investigated the effect of gentisyl alcohol on the proliferation of BAECs. Thus, the BAECs were seeded at a density of 5×10^3 cells per well in a 96-well culture plate. Gentisyl alcohol (0.05–0.5 μg/ml) was added to each well and incubated for up to 72 h. After 72 h, MTT (50 μl, 2 mg/ml) was added to each well, and cells were incubated for an additional 4 h at 37°C. The supernatant was then removed, and dimethylsulfoxide (150 μl, DMSO) was added to dissolve formazan products. The absorbance of MTT-formazan was measured using the 540 nm filter-equipped microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, U.S.A.). As shown in Fig. 1B, gentisyl alcohol induced the proliferation of BAECs in a dose-dependent manner, showing that proliferation of BAECs reached a maximum (120% better growth than that of control) at the concentration of 0.5 μg/ml. At the higher concentration of 1 μg/ml, however, gentisyl alcohol did not stimulate the cell proliferation, but rather showed saturation of the activity. This suggests that the compound may induce the cell proliferation at a certain specific dose and

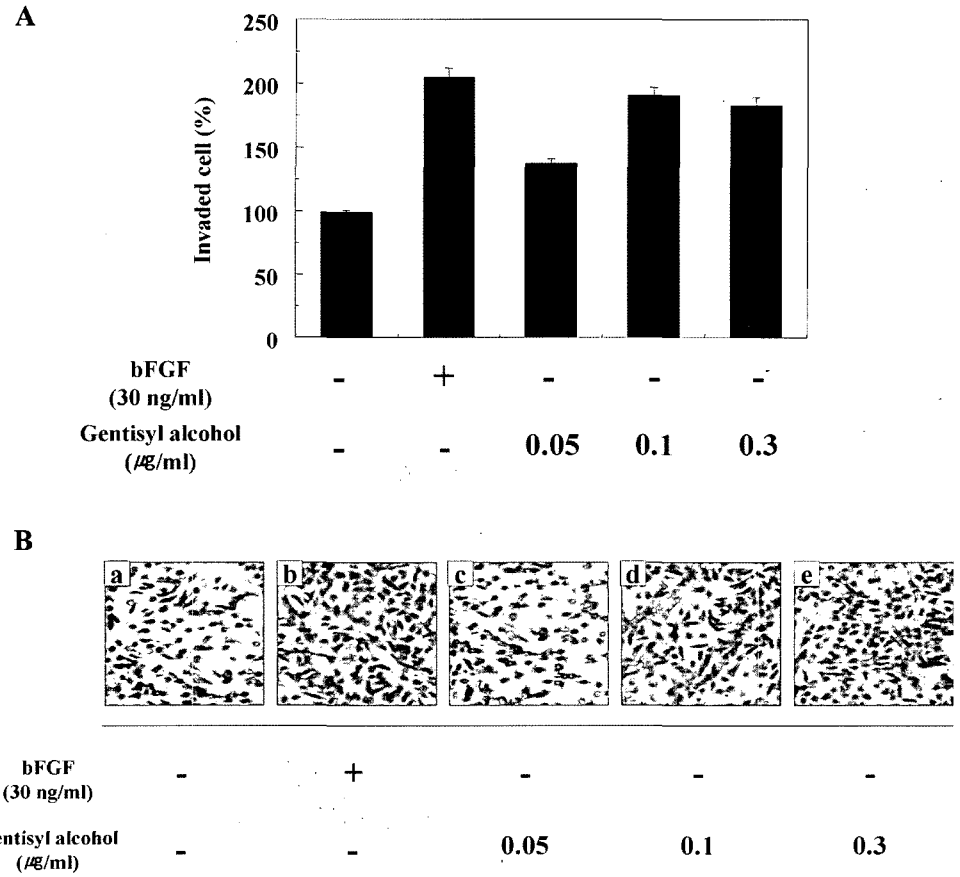

Fig. 2. Effect of gentisyl alcohol on the invasion of BAECs.

BAECs were seeded at a density of 1×10^5 cells/well and treated with or without bFGF (30 ng/ml). The cells were then treated with the compound. **A.** Enhanced activity of gentisyl alcohol on invasion of BAECs. Serum-starved cells in serum-free medium (Control) or treated with bFGF in the presence or absence of gentisyl alcohol were used for the invasion assay. The experiment was independently repeated twice. **B.** Microscopic observation of invaded cells (×100 magnification): (a), control; (b), bFGF alone; (c), gentisyl alcohol (0.05 μg/ml); (d), gentisyl alcohol (0.1 μg/ml); (e), gentisyl alcohol (0.3 μg/ml).

proliferation-stimulating activity of gentisyl alcohol may be attenuated by nonspecific activity of the compund higher than 1 μg/ml.

We next conducted an *in vitro* endothelial cell invasion assay to explore the pro-angiogenic activity of gentisyl alcohol in particular. Since endothelial cell invasion is a crucial process for the spreading and migration of cells, the activation of this process has been considered as an important property for pro-angiogenic agents [16]. The invasiveness of endothelial cells was performed *in vitro* using a transwell chamber system with 8.0-μm-pore-polycarbonate filter inserts, as described previously by Kwon *et al.* [7]. The lower side of the filter was coated with 10 μl of gelatin (1 mg/ml), whereas the upper side was coated with 10 μl of the Matrigel (10 mg/ml). BAECs (1×10^5 cells) were placed in the upper part of the filter. The chamber was then incubated at 37°C for 18 h. The cells were fixed with methanol and stained with hematoxylin/eosin. Finally, the cell invasion was determined by counting the total number of cells in the lower side of the filter using optical microscopy at ×40 magnification using a JVC digital camera (VICTOR, Yokohama, Japan). As shown in Fig. 2, the Matrigel prevented the migration of non-invasive cells whereas bFGF effectively induced cell invasion through the filter compared with that of the control (Fig. 2). Notably, 0.05 μg/ml gentisyl alcohol induced the invasion of cells and exhibited almost the same invasive activity at 0.1 μg/ml as that by bFGF, a pro-angiogenic protein.

As another important step in angiogenesis, degradation of basement membrane by matrix metalloproteinases (MMP) and migration of endothelial cells have been emphasized [10]. Accordingly, an *in vitro* migration assay was done to evaluate the effect of gentisyl alcohol on the migration of BAECs. A wound was made by gently scraping off the cells with confluence as described by Sato and Rifkin [20] with minor modifications. The cultures were rinsed with PBS to eliminate debris, and medium without supplements was added. Gentisyl alcohol (0.05–0.3 μg/ml) was added to each well and incubated for 9 h. The number of cells that had migrated across the wound line was counted in each

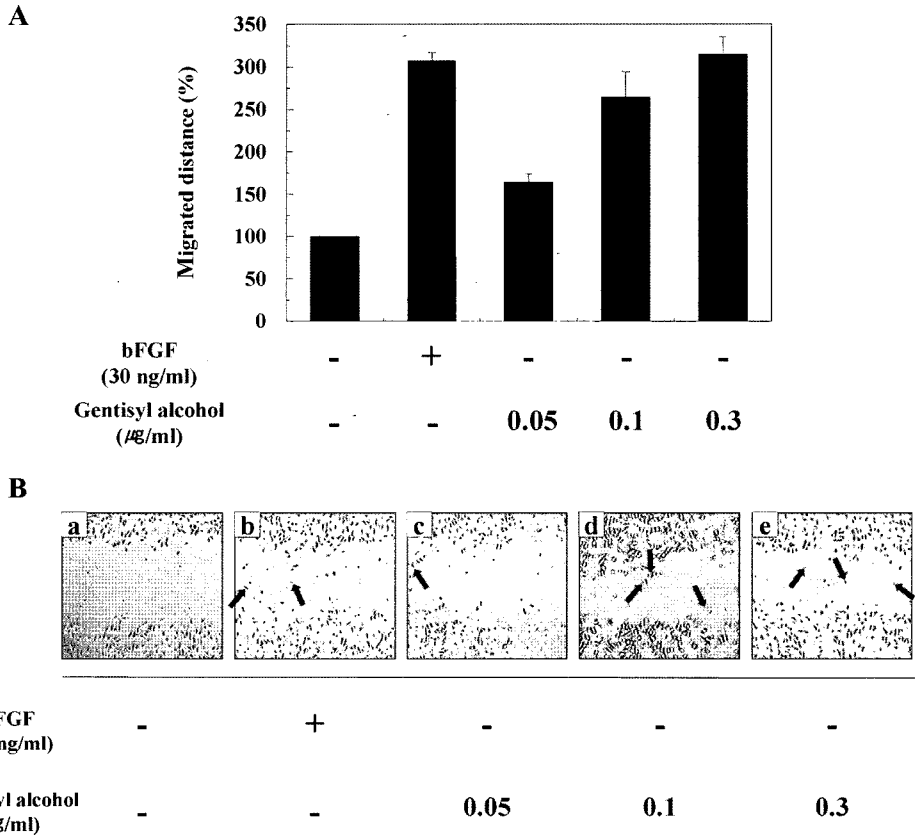

Fig. 3. Effect of gentisyl alcohol on the migration of BAECs.

BAECs were seeded at a density of 3×10^4 cells/well and incubated overnight. To initiate migration, the cell layer was scraped with a razor blade, and the medium was changed. Subsequently, the cells were treated with the compound and the dishes were returned to the incubator. After the desired period of time (3–24 h), the area of migration was photographed with a video camera system using microscopic observation. **A.** Enhanced activity of gentisyl alcohol on endothelial migration assay. Migrating distance of BAECs was measured and data were expressed as a percentage of control. **B.** Microscopic observation of migrated cells (×100 magnification): (a), control; (b), bFGF alone; (c), gentisyl alcohol (0.05 μg/ml); (d), gentisyl alcohol (0.1 μg/ml); (e), gentisyl alcohol (0.3 μg/ml). Arrows indicate the migrated BAECs.

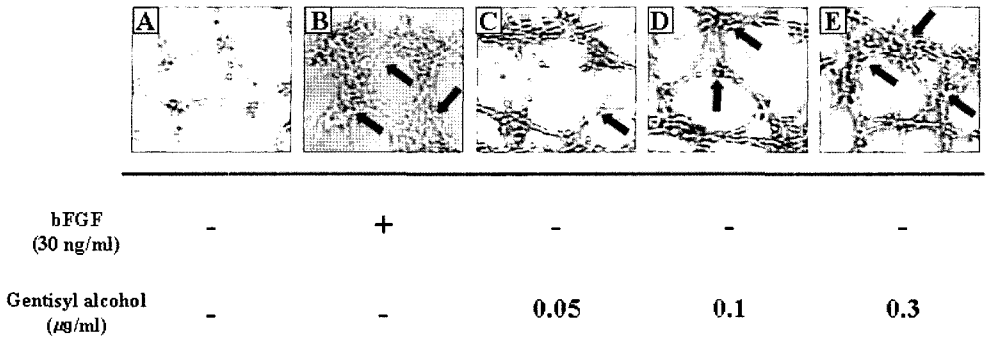

Fig. 4. Effect of gentisyl alcohol on the tube formation of BAECs.
BAECs were seeded on Matrigel-coated wells at a density of 1×10^5 cells/well without bFGF and the compound (**A**). **B**. The BAECs were stimulated with bFGF (30 ng/ml) alone. The BAECs were treated with (**C**) gentisyl alcohol (0.05 μg/ml); (**D**) gentisyl alcohol (0.1 μg/ml); (**E**) gentisyl alcohol (0.3 μg/ml). Photographs were taken 8 h after the compound treatment. The experiment was independently repeated twice. Arrows indicate the capillary tube formation of BAECs.

well, and the mean was taken as the result. Migration in bFGF-stimulated wells was subsequently calculated as a percentage of that in unstimulated wells of the same experiment. As shown in the invasion assay, bFGF stimulated the migration of cells about 3 times higher than that of non-treated control. Similarly, gentisyl alcohol up to the concentration of 0.3 μg/ml dose-dependently stimulated cell migration (Fig. 3).

Finally, the effect of gentisyl alcohol on capillary tube formation, another key phenotype of endothelial cells for angiogenesis, was examined. Matrigel (150 μl, 10 mg/ml) was placed in a 48-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). Then, the compound was added and incubated for 6–18 h. The morphological changes of the cells and tubes formed were observed under a microscope and photographed at ×40 magnification using a JVC digital camera. As shown in Fig. 4A, in the absence of bFGF, BAECs cultured on the Matrigel normally formed incomplete and narrow tube-like structures, whereas the capillary network formation was stimulated by the treatment of bFGF, resulting in elongated and robust tube-like structures (Fig. 4B). Gentisyl alcohol also induced the tube formation, similar to bFGF, in a dose-dependent manner (Figs. 4C–4E). Cytotoxicity was not observed at any concentration of the compound, as confirmed by trypan blue staining of the cells (data not shown).

Gentisyl alcohol has been shown to possess several biological activities, including antioxidant [16] and anti-mutagenic properties as well as being a potent inhibitor of apoptosis [19]. Besides these interesting biological activities, our study demonstrated a novel new biological activity (i.e., pro-angiogenic activity) of gentisyl alcohol and highlighted the compound as a new class of small molecule having pro-angiogenic activity. The mechanism of angiogenesis induction by the compound remains to be solved. As gentisyl alcohol is a phenolic compound, it is highly possible that the compound may produce reactive oxygen species such as superoxide radicals, hydrogen peroxide, or hydroxyl radicals to activate the angiogenic signal pathway [12, 14]. Further studies are in progress to discern whether the signaling regulation of VEGF or matrix metalloproteinase (MMP) is associated with the pro-angiogenic effect of gentisyl alcohol.

In conclusion, all results shown herein strongly demonstrate that gentisyl alcohol induces angiogenesis through the activation of proliferation, migration, and differentiation of BAECs *in vitro* and suggest that gentisyl alcohol can be developed as an agent for pro-angiogenic activity.

Acknowledgments

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