Action Mechanism of *Chamaecyparis obtusa* Oil on Hair Growth

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This study was carried out to examine the action mechanism of *Chamaecyparis obtusa* oil (CO) on hair growth in C57BL/6 mice. For alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT) activities in the skin tissue, at week 4, the 3% minoxidil (MXD) and 3% CO treatment groups showed an ALP activity that was significantly higher by 85% ($p < 0.001$) and 48% ($p < 0.05$) and an γ-GT activity that was significantly higher by 294% ($p < 0.01$) and 254% ($p < 0.05$) respectively, as compared to the saline (SA) treatment group. For insulin-like growth factor-1 (IGF-1) mRNA expression in the skin tissue, at week 4, the MXD and CO groups showed a significantly higher expression by 204% ($p < 0.05$) and 426% ($p < 0.01$) respectively, as compared to the SA group. At week 4, vascular endothelial growth factor (VEGF) expression in the MXD and CO groups showed a significantly higher expression by 74% and 96% ($p < 0.05$) respectively, however, epidermal growth factor (EGF) expression in the MXD and CO groups showed a significantly lower expression by 66% and 61% ($p < 0.05$) respectively, as compared to the SA group. Stem cell factor (SCF) expression in the MXD and CO groups was observed by immunohistochemistry as significant in a part of the bulge around the hair follicle and in a part of the basal layer of the epidermis. Taking all the results together, on the basis of effects on ALP and γ-GT activity, and the expression of IGF-1, VEGF and SCF, which are related to the promotion of hair growth, it can be concluded that CO induced a proliferation and division of hair follicle cells and maintained the anagen phase. Because EGF expression was decreased significantly, CO could delay the transition to the catagen phase.

Key words: Action mechanism, Anagen phase, C57BL/6 mice, *Chamaecyparis obtusa* oil, Hair growth

INTRODUCTION

Whereas in the past years people used to consider hair loss as a part of the aging process, it has now become one of the important contributions to stress in life, having negative effects with respect to personal appearance and interpersonal relations and it leads to a loss of confidence. Accordingly, research on hair loss and hair growth stimulators is being vigorously pursued with new drugs and cosmetics being developed following cytological, biochemical and molecular biological advances. While minoxidil (MXD) and finasteride are widely used in many countries, the

mechanisms of action of these agents against target cells remains unknown so that their uses have to be restricted due to multiple side effects and inconsistent efficacy.

The development of the hair cycle is profoundly influenced by signaling such as molecules, cytokines, hormones, neuropeptides in epithelial and mesenchymal cell components (1). However, the mechanisms underlying the switch between phases remain largely unknown (2) and no one factor appears to be predominant. Previous studies have reported hair loss is due to apoptosis of hair matrix cells caused by interleukin (IL)-1 and tumor necrosis factor (TNF)-α (3), and also a premature catagen phase, the anagen phase is becoming shorter by transforming growth factor (TGF)-β and androgen (4). While growth factors such as insulin-like growth factor (IGF)-1, keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) have been shown to prevent apoptosis of hair matrix cells and to promote hair growth (5). γ-Glutamyl transpeptidase (γ-GT) and alkaline phosphatase (ALP) are known as important factors in an increase of enzyme activity in the anagen phase (6).

Recently, we reported that *Chamaecyparis obtusa* oil

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(CO) had an excellent growth effect in C57BL/6 mice (7). The objective of this study was to examine the action mechanism of CO on hair growth in C57BL/6 mice by determining enzyme activities and cytokine expressions related to hair growth in the skin tissue.

MATERIALS AND METHODS

Reagents and apparatus. CO used for the experiment was purchased from Ecomist Korea (Korea) and jojoba oil was purchased from Desert Whale (USA). A 3% MXD was obtained from Hyundai Pharmaceutical Company (Korea). In order to make with the 3% experimental material, CO was mixed with the jojoba oil. ALP and γ-GT were obtained from Thermo (Finland) and Trizol was obtained from Invitrogen (New Zealand). Primers of GAPDH and IGF-1, cDNA synthesis PCR kit were purchased from BioNEER (Korea). Quantikine mouse VEGF and EGF immunoassay kits were obtained from R&D Systems (USA) and protein extraction and measurement solution were purchased from iNtRON (Korea). SCF antibody was purchased from Santacruz (USA). Auto biochemistry analyzer (Thermo, Konelab 20XT, Finland), homogenizer (IKA, T25 basic, Malaysia), PCR machine (Bio-RAD, Mycycler™ thermal cycler, USA), spectrophotometer (SHIMADZU, UV-1601, Japan), mini centrifuge (Hitachi, MIKRO 200R, Germany), white/UV transilluminator (KODAK, UXT-20M-8E, Germany), automatic immunostainer (Ventana Medical Systems, Bench-Mark XT, USA) and inverted microscope (Carl Zeiss, AXIOVERT 200, Germany) were used for the experiments.

Animals and treatments. Five-week-old female C57BL/6 mice were supplied by Daehan Biolink (Korea). The animals were housed in plastic cages at $22 \pm 1^{\circ}$C, with a relative humidity of $50 \pm 5\%$, an alternating 12 hr light/dark cycle, with food and water provided *ad libitum* and were allowed to adapt to the laboratory environment for one week. Both animal care and the protocol for this study were in accordance with IACUC (Institutional Animal Care and Use Committee) and OECD guidelines.

The animals were divided randomly into three groups (fifteen mice each), which consisted of a saline (SA) treatment group as the control group, a 3% MXD treatment group as the positive control group and 3% CO treatment group. The backs of C57BL/6 mice were shaved with an animal clipper at six weeks, by which time all of the HF (hair follicle) was synchronized in the telogen phase. One hundred μl of the test solutions were topically applied to the backs of mice in the respective groups once a day, 6 days a week, for 4 weeks. Under ether anesthesia, a extracted part of the dorsal skin was fixed with 10% neutral buffered formalin solution for histological analysis and the rest of the dorsal skin was stored in a freezer after freezing in liquid nitrogen for enzymatic and cytokine analyses.

Enzyme activities in the skin tissue. At weeks 1, 3 and 4, the dorsal skin samples from C57BL/6 mice were homogenized in 0.1 M phosphate buffered saline (PBS, pH 7.4) to obtain a 20% (w/v) homogenate. The homogenate was centrifuged at 12,000 rpm, 4°C, for 20 min. The supernatant was collected and stored in a freezer at -70°C until required. ALP and γ-GT activities were analyzed using an auto biochemistry analyzer (Thermo, Finland).

Determination of cytokine expression in the skin tissue. At weeks 1, 3 and 4, the dorsal skin samples from C57BL/6 mice were maintained at a low temperature and then were homogenized with 1 ml of Trizol per 50 mg of frozen dorsal skin samples. The homogenized samples were incubated at room temperature for 5 min, added with 200 μl chloroform and the samples were centrifuged at 15,000 rpm, 4°C, for 10 min. The supernatants were collected in a new tube, added with 500 μl isopropyl alcohol and the samples were centrifuged at 15,000 rpm, 4°C, for 15 min. The RNA pellet obtained was washed with 1 ml 70% ethanol, and centrifugation at 15,000 rpm, 4°C, for 2 min. The final RNA pellet obtained after removing the supernatant was diluted with diethylpyrocarbonate (DEPC), and quantified by $1.8 <$ optical density (OD) (A260/A280) < 2.0 using a white/UV transilluminator. cDNA was synthesized at $0.1~1$ μg/μl from the total RNA using a CycleScript RT PreMix (dT20) kit. cDNA synthesis was performed at 30°C for 1 min, and then 50°C for 4 min, with 12 cycles in 205 μl of DEPC, and the samples were heated at 95°C for 5 min.

The polymerase chain reaction (PCR) was used an Accu-power™ PCR PreMix kit and PCR product was amplified by the PCR reaction in 2 μl template, 15.2 μl sterile water with 10 pmol/L of specific primers. The reaction was performed over 35 cycles. The primers used for amplifying the respective fragments are listed in Table 1. The amplifica-

Table 1. Nucleotide sequence of the primers and expected size of PCR products

Items		Primers	Expected size (bp)[3]
IGF-1[1]	Forward (5' → 3')	AGAGACCCTTTGCGGGGCTGA	255
	Reverse (5' → 3')	CTTCTGAGTCTTGGGCATGT	
GAPDH[2]	Forward (5' → 3')	AACGGATTTGGYCGTATTGG	302
	Reverse (5' → 3')	AGCCTTCTCCATGGTGGTGAAGAC	

[1]IGF-1: Insulin-like growth factor-1. [2]GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. [3]bp: base pair.

tion products were separated by 1.5% agarose gel electrophoresis and visualized with using ethidium bromide. The DNA band densities were evaluated by using the KODAK GEL LOGIC 100 image analysis system (Table 1).

Immunoassay. At week 4, the dorsal skin samples from C57BL/6 mice were homogenized in 1.2 ml protein extraction solution (PRO-PREP^{TM}) using a homogenizer. The homogenized samples were incubated at -20°C for 30 min and centrifuged at 13,000 rpm, 4°C, for 5 min. The supernatants were collected in a new tube and stored in a freezer at -70°C. For experiment quantikine mouse VEGF or EGF immunoassay kit was used. To each of the samples in 96 well plates, 50 μl of assay diluent RD1N or RD1-21 was added and followed by incubation at room temperature for 2 hr. After aspirating each well and washing, 100 μl of mouse VEGF or EGF conjugate was added to each well, followed by incubation at room temperature for 2 hr, 100 μl of substrate solution was added to each well with a incubation at room temperature for 30 min. Finally, 100 μl of stop solution was added to each well, and the samples were quantified using a microplate reader set to 450 nm.

Immunohistochemistry. At week 4, the dorsal skin samples from C57BL/6 mice were fixed with 10% neutral buffered formalin solution for 12 hr and washed in running water. After dehydration (using 70, 80, 95 and 100% ethanol), clearance with xylene, and paraffin-infiltration using the standard techniques, the tissue samples were embedded in paraffin wax. The paraffin sections were cut in 4 μm thickness using a microtome and placed on slides, the paraffin was removed from the samples and an automated immunostainer (BenchMark XT, USA) was used for staining. Sections were washed for 3 min using reaction buffer and placed in 3% H$_2$O$_2$ for 3 min to suppress the activity of peroxidase. The primary antibody SCF (Dako, Japan) was diluted 1:50 before use. The sections were then washed using reaction buffer and allowed to react with biotin for 20 min and streptavidin for 25 min. After diamino-benzidine (DAB) (Ventana Detection kit, USA) staining, the sections were counter-stained and observed under an optical microscope.

Statistical analysis. Differences between the groups were evaluated statistically using one-way analysis of variance (ANOVA) followed by the Duncan's multiple range test for a *post hoc* comparison using SPSS 17.0 software. Statistical significance was set at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS

Enzyme activities in the skin tissue. ALP activity in the skin tissue was shown in Fig. 1. At week 1, there were
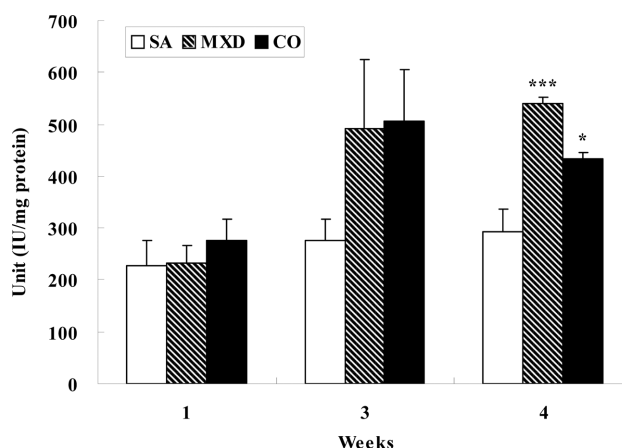

Fig. 1. Changes of alkaline phosphatase activity in the skin of mice. SA: saline, MXD: 3% minoxidil, CO: 3% *Chamaecyparis obtusa* oil. Test compounds were topically applied to the backs of C57BL/6 mice for 4 weeks. Values are mean ± SE of 5 mice. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to the SA group by ANOVA and Duncan's multiple range tests.

no significant differences among the 3 groups. At week 3, the MXD and CO groups showed high enzyme activities, as compared to the SA group, but there were no significant differences among the 3 groups. At week 4, the MXD and CO groups showed significantly higher activities by 85% ($p < 0.001$) and 48% ($p < 0.05$) respectively, as compared to the SA group. During the experimental period, the ALP activity in the MXD was increased gradually, whereas the CO group showed a decrease at week 4 (Fig. 1). γ-GT activity in the skin tissue was shown in Fig. 2. At weeks 1 and 3, there were no significant differences among the 3 groups.
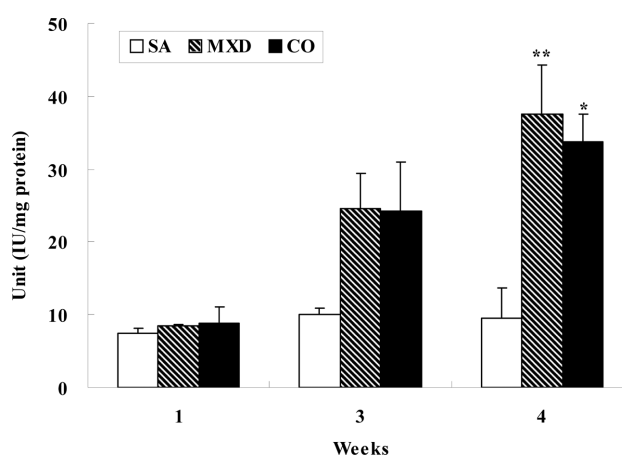

Fig. 2. Changes of γ-glutamyl transpeptidase activity in the skin of mice. SA: saline, MXD: 3% minoxidil, CO: 3% *Chamaecyparis obtusa* oil. Test compounds were topically applied to the backs of C57BL/6 mice for 4 weeks. Values are mean ± SE of 5 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the SA group by ANOVA and Duncan's multiple range tests.

At week 4, the MXD and CO groups showed significantly higher activities by 294% ($p < 0.01$) and 254% ($p < 0.05$) respectively, as compared to the SA group. The γ-GT activities in the MXD and CO groups were increased gradually during the experimental period (Fig. 2).

Cytokine expression in the skin tissue. IGF-1 mRNA expression in the skin tissue was shown in Fig. 3. The MXD and CO groups showed significantly higher expressions by 57% and 217% at week 1 and 199% and 282% at week 3, respectively, as compared to the SA group ($p < 0.05$, $p < 0.001$). At week 4, the MXD and CO groups also
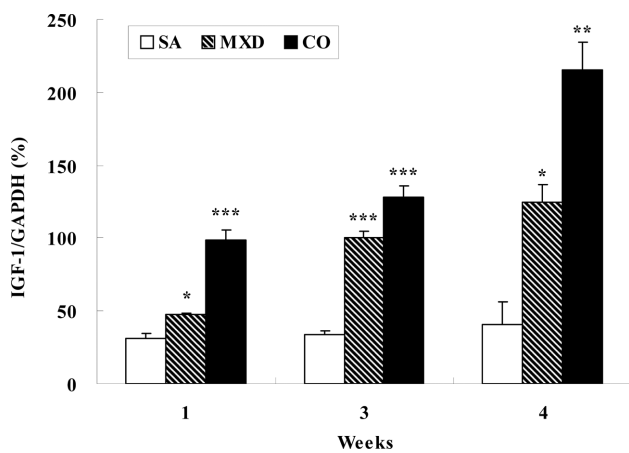

Fig. 3. Changes of insulin-like growth factor-1 mRNA expression in the skin of mice. SA: saline, MXD: 3% minoxidil, CO: 3% *Chamaecyparis obtusa* oil. Test compounds were topically applied to the backs of C57BL/6 mice for 4 weeks. Values are mean ± SE of 5 mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the SA group by ANOVA and Duncan's multiple range tests.
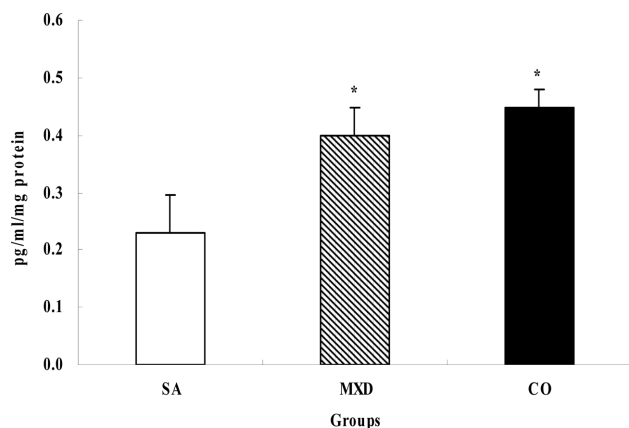

Fig. 4. Comparison of vascular endothelial growth factor expression in the skin of mice. SA: saline, MXD: 3% minoxidil, CO: 3% *Chamaecyparis obtusa* oil. Test compounds were topically applied to the backs of C57BL/6 mice for 4 weeks. Values are mean ± SE of 5 mice. *$p < 0.05$ compared to the SA group by ANOVA and Duncan's multiple range tests.
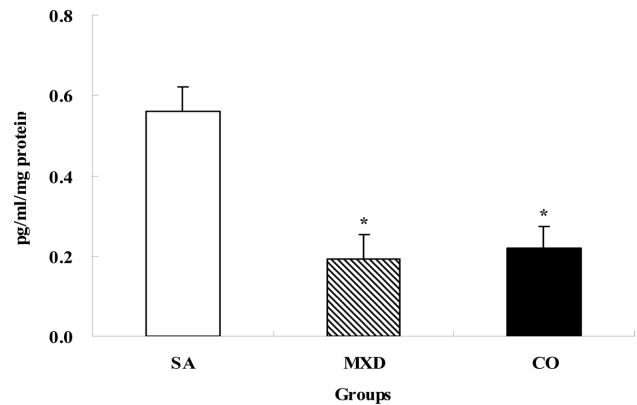

Fig. 5. Comparison of epidermal growth factor expression in the skin of mice. SA: saline, MXD: 3% minoxidil, CO: 3% *Chamaecyparis obtusa* oil. Test compounds were topically applied to the backs of C57BL/6 mice for 4 weeks. Values are mean ± SE of 5 mice. *$p < 0.05$ compared to the SA group by ANOVA and Duncan's multiple range tests.

showed significantly higher expressions by 204% ($p < 0.05$) and 426% ($p < 0.01$) respectively, as compared to the SA group. The CO group showed higher expression compared to the MXD group during the experimental period (Fig. 3).

VEGF expression in the skin tissue was shown in Fig. 4. At week 4, the MXD and CO groups showed significantly higher expressions by 74% and 96% ($p < 0.05$) respectively, as compared to the SA group, and the groups showed a similar effect (Fig. 4). EGF expression in the skin tissue was shown in Fig. 5. At week 4, the MXD and CO groups showed significantly lower expressions by 66% and 61% ($p < 0.05$) respectively, as compared to the SA group (Fig. 5). SCF expression in the skin tissue was shown in Fig. 6. The expression of SCF in the MXD and CO groups was observed to occur significantly in a part of the bulge around the HF region, as compared to the SA group. The SA group only showed a weak reaction in a part of the sebaceous gland. Also, significant SCF expression in the CO group was observed in a part of the basal layer of the epidermis compared to the SA and MXD groups (Fig. 6).

DISCUSSION

ALP is a zinc-metallo enzyme, which is mainly localized at the plasma membrane *via* a glycosyl-phosphatidylinositol anchor (8). Its activity is widely expressed in actively proliferating or remodeling tissues, in cells with a high metabolic rate (9). The ALP activity and its expression area correspond with the hair cycle and is expressed strongly in the DPC (dermal papilla cell) during all phases of the hair cycle (10). The activity in the DPC was moderate in the very early anagen phase, reaching a maximal level in early anagen phase, while decreasing in the proximal region of DPC after mid-anagen phase, and it was kept at a low level
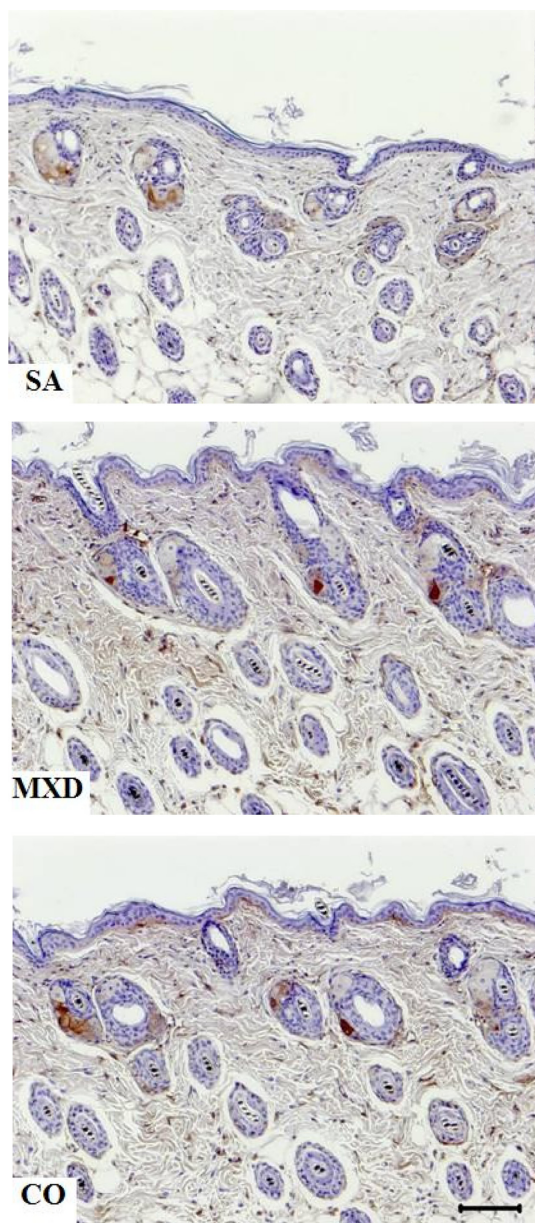
Fig. 6. Immunohistochemistry of stem cell factor in the skin of mice. SA: saline, MXD: 3% minoxidil, CO: 3% *Chamaecyparis obtusa* oil. ×200, scale bar 100 µm. Stem cell factor expressions in the MXD and CO groups were observed to be strong in a part of the bulge around the hair follicle and in a part of the basal layer.

during the catagen phase. The bulbar dermal sheath showed an intense ALP activity only in the early anagen phase (8). γ-GT is a membrane-bound enzyme that is believed to function in glutathione metabolism and in adsorption or secretion of amino acids or peptides through cell membranes and is reported to use cysteine for keratin synthesis in the HF (11). The enzyme is present exclusively in the outer and inner root sheaths of the HF and its expression is observed

mainly in cells which are active in proliferation and division. Also the γ-GT activity is more pronounced during the anagen phase and greatly diminished during the telogen phase (12). Therefore γ-GT and ALP are as useful markers of hair growth (6).

Observations of the ALP and γ-GT activities in the skin showed gradual increases in the MXD and CO groups during the experimental period. At week 4, the MXD and CO groups showed significantly higher activities compared to the SA group. These results are consistent with reports that the herbal extract mixture showed high activities of ALP and γ-GT with hair growth promotion in C3H mice (13), and that Hwanggumgung, a natural product, applied to C57BL/6 mice, promoted hair regrowth and increased the ALP and γ-GT activity (14). Therefore, the MXD and CO groups are in the anagen phase that is actively proliferating and dividing. Also, they promote keratin synthesis and improve blood circulation enabling the supply of nutritive substances necessary for hair growth.

The DPC are known to play a key role in the regulation of hair growth and they control the differentiation of each hair by transducing signals to the epithelial precursors or the surrounding undifferentiated matrix cells (15). It has been reported that growth factors, such as VEGF, FGF-5S, IGF-1 and KGF, induce the proliferation of cells in the matrix, the DPC and dermal papillary vascular system, increase the amount of extracellular matrix in DPC, and then maintain follicles in the anagen phase (16). IGF-1 is a paracrine growth factor in many organs and is expressed by mesenchymal cell of the dermis layer and the dermal papilla (17). It is reported that IGF-1 affects follicular proliferation, tissue remodeling and the hair growth cycle, as well as follicular differentiation (18). VEGF is expressed in outer root sheath keratinocytes during the anagen phase and has been implicated in creating new blood vessels around HF during the hair cycle (17). EGF expression in the outer root sheath of hair follicles is important for downward growth of the HF (2). However, there are contrasting reports about EGF. One is that it inhibits hair growth and the other is that it stimulates hair growth. SCF, which is expressed in the DPC of human HF (19), is one of the cytokines related to hair growth. SCF is important in embryonic melanocyte migration and maintaining adult rodent pigmentation (20).

In this study, IGF-1 and VEGF expression, which are well-known hair growth promoters, were analyzed using RT-PCR and immunoassay in the skin tissue. EGF and SCF expression were analyzed by immunoassay and immunohistochemistry in the skin tissue. The MXD and CO groups showed a significantly higher IGF-1 mRNA expression, as compared to the SA group. The result is consistent with recent reports that IGF-1 mRNA expression was increased in the region promoting hair growth (21) and, in the absence of IGF-1 protein, organ-cultured human HF undergoes a transformation reminiscent of the events at the beginning of

the catagen phase characterized by the lack of cells proliferation (22). In particular, the CO group showed a higher expression than the MXD group during the experimental period. The MXD and CO groups showed a significantly higher VEGF expression, as compared to the SA group. The result is consistent with recent report that when plant extracts were treated into cultured DPC, the expression of VEGF mRNA was dose-dependently increased (23). Therefore, it suggests that CO induces an activity of HF cells by stimulating VEGF which promotes protease production related to matrix proteolysis required in revascularization and increases vascular permeability (24). The physiological role of EGF on hair development and the signaling pathways involved remain unclear. In this study, the MXD and CO groups showed a significantly lower EGF expression, as compared to the SA group. Previous study has reported that in cultured human hair follicles, the stimulation of HF elongation and outer root sheath proliferation by EGF but inhibition on the proliferation of matrix cells could lead to an artificial catagen phase. The matrix cells remained connected to the dermal papilla by a thin strand of epithelial cells, and this epithelial strand is also seen in follicles at the catagen phase (25). However, our result is disaccord with report that EGF promotes hair growth (26). SCF expression at significant levels was observed in a part of the bulge around the HF and in a part of the basal layer of the MXD and CO groups. A previous study has reported that SCF/c-kit signaling was critically important for melanocytes proliferation, differentiation and pigment production, during the anagen phase (27). It was also reported that androgenetic alopecia cultured DPC secreted less SCF, measured by ELISA, than did normal cells (20). Therefore, CO promotes hair growth by increasing the synthesis of collagen and elastin around the HF and reduces the hair loss cycle (28).

The results of this study demonstrate that CO stimulated ALP and γ-GT activation related to hair growth, with the result that it can increase circulation that supplies nutrients required for hair growth. It also promoted the proliferation of HF cells and the increase in the expression of hair growth factors such as IGF-1 and VEGF.

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