The Additive Effects of Minoxidil and Retinol on Human Hair Growth *in Vitro*

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Minoxidil enhances hair growth by prolonging the anagen phase and induces new hair growth in androgenetic alopecia (AGA), whereas retinol significantly improves scalp skin condition and promotes hair growth. We investigated the combined effects of minoxidil and retinol on human hair growth *in vitro* and on cultured human dermal papilla cells (DPCs) and epidermal keratinocytes (HaCaT). The combination of minoxidil and retinol additively promoted hair growth in hair follicle organ cultures. In addition, minoxidil plus retinol more effectively elevated phosphorylated Erk, phosphorylated Akt levels, and the Bcl-2/Bax ratio than minoxidil alone in DPCs and HaCaT. We found that the significant hair shaft elongation demonstrated after minoxidil plus retinol treatment would depend on the dual kinetics associated with the activations of Erk- and Akt-dependent pathways and the prevention of apoptosis by increasing the Bcl-2/Bax ratio.

Key words minoxidil; retinol; hair growth; hair follicle organ culture; dermal papilla cell; keratinocyte

Minoxidil, a pyrimidine derivative (2,4-diamino-6-piperidino-pyrimidine-3-oxide) is an adenosine triphosphate (ATP) sensitive potassium chennel (K_{ATP} chennel) opener, and is the most commonly used drug for the treatment of androgenetic alopecia.[1] Topical minoxidil shortens the telogen phase by induction of the entry of resting hair follicles into the anagen phase, prolongs anagen growth phase and increases the volume of hair follicles. Although the mechanism through which minoxidil promotes hair growth is still speculative, a number of *in vitro* studies using minoxidil have been described in various skin and hair follicle cell types including the stimulation of cell proliferation, and the induction of vascular endothelial growth factor[2,3] and prostaglandin synthesis.[4] In addition, minoxidil has been shown to increase blood circulation around hair follicles[5,6] and to markedly elevate 17β-hydroxysteroid dehydrogenase activity, which accelerated the conversion of testosterone to weaker androgens.[7]

Retinol significantly improves the scalp skin condition and promotes hair growth, in terms of the keratinization of skin and hair.[8] Retinol is ingested in precursor forms; animal sources contain retinyl esters whereas plants contain carotenoids. Tissue cells convert these organic precursor forms to retinol, and this to either retinal or retinoic acid. These two species exert profound effects on the growth, maturation, and differentiation of many cell types, both *in vivo* and *in vitro*.[8] Retinoic acid, the active metabolite of vitamin A, has been reported to play an important role in the growth, differentiation and maintenance of hair follicles.[9] Moreover, cellular retinoic acid-binding proteins (CRBPs) are known to be important for the mediations of some of the effects of retinoids. These effects are mediated by two classes of nuclear retinoic acid receptors, termed retinoic acid receptor (RAR) and retinoid x receptor (RXR), and each of these classes is composed of subtypes designated alpha, beta, and gamma.

Recently, we showed that minoxidil prolongs the anagen stage and that the enhanced hair growth may result from two mechanisms: (i) the activations of Erk and Akt signaling which enhance the survival of cultured DPCs, and (ii) an increase in the Bcl-2/Bax ratio which protects cells against cell death.[10] The aim of the present study was to evaluate possible additive interaction between minoxidil and retinol on human hair growth and to investigate the mechanism of action of minoxidil plus retinol using cultured human DPCs and keratinocytes.

MATERIALS AND METHODS

Materials Minoxidil and retinol were purchased from Sigma (St Louis, MO, U.S.A.). Minoxidil was dissolved in 0.12 mM HCl and a 1.0 mM minoxidil stock solution was stored at −20 °C. Retinol was dissolved in absolute ethanol and shielded from the light using aluminum foil until required. All inhibitors [U0126, an ERK kinase (MEK1 and 2)-inhibitor; LY 294002 (LY) and Wortmannin, selective PI3K inhibitor] were purchased from Calbiochem (San Diego, CA, U.S.A.). Anti-phosphorylated-Erk-1/2 (Thr202/Tyr 204) antibody, anti-total Erk-1/2 antibody, anti-phosphorylated-Akt (Ser473) antibody, and anti-total Akt antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.). Anti-Bax antibodies were obtained from Dako (Glostrup, Denmark). Anti-β-actin and anti-Bcl-2 antibodies were obtained from Santa Cruz Biotech Inc. (Santa Cruz, CA, U.S.A.).

Human Hair Follicle Samples Tissue samples of the occipital scalp region were obtained by excisional biopsy. Ten healthy male volunteers aged between 20 and 35 years were recruited. The subjects were free any current or prior relevant disease and had not been taking medication for at least 1 month prior to sampling. The Institutional Review Board at Seoul National University Hospital approved all of the procedures used in this study. Written informed consent was obtained from all volunteers. Tissue samples containing more than 100 hair follicles were cautiously dissected into single hair follicles. Hair follicles in the anagen stage were

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used, as described previously.[11)]

Human Hair Follicle Organ Culture Human scalp hair follicles were isolated and cultured *in vitro*, as described previously.[12)] Briefly, dissected hair follicles were cut individual into small pieces of approximately 2.5 mm in length from the bottom of the dermal papilla, and then cultured in Williams E medium (Gibco BRL, Gaithersburg, MD, U.S.A.) containing 10 ng/ml hydrocortisone, 10 μg/ml insulin, 2 mM L-glutamine, and 100 U/ml penicillin at 37 °C in a 5% CO_2 atmosphere. Minoxidil and retinol were added to this culture medium alone or in combination. Minoxidil was treated at final concentrations ranging from 1 to 10 μM, and retinol was used at concentrations from 17 nM to 1.7 μM. The individual inhibitors and the concentrations were used U0126 at 20 μM and LY294002 at 50 μM. A total of 30 anagen hair follicles (3 different volunteers and 10 follicles per individual) were used per each test condition, and this was performed in triplicate. Data are presented as the means of triplicate cultures±S.E.M. In all experiments, tissue culture medium containing minoxidil and/or retinol was changed every other day. After culturing for 12 d, hair follicle elongation was measured directly using an Olympus stereomicroscope with an eyepiece containing a graticule.

Culture of Human DPCs and HaCaT DPCs were cultured as described previously.[13)] Briefly, cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, U.S.A.) containing 2 mM L-glutamine, 1× antibiotic antimycotic solution (1000 μg/ml of streptomycin sulfate, 1000 units/ml of penicillin G sodium, and 2.5 μg/ml of amphotericin B) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT, U.S.A.) at 37 °C, in a 5% CO_2 incubator. Fourth-passage DPCs were used. On the other hand, HaCaT were incubated in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 1 mM sodium pyruvate, 50 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in 5% CO_2.

Thiozolyl Blue [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide] (MTT) Assay Cell viabilities were determined using MTT assays. Briefly, DPCs and HaCaT cells at 1.5×10^4 cells/well were seeded into 96-well plates, cultured for 24 h in serum-free DMEM, and then treated with vehicle (0.12 mM HCl or DMSO diluted 1 : 1000 in serum-free DMEM) as a control, minoxidil (0.01 μM), retinol (1.7 pM—1.7 nM), or minoxidil (0.01—1.0 μM) plus retinol (1.7 pM—1.7 nM) for 5 d. HaCaT cells were mock-treated (control), or pre-treated with 10 μM LY, 10 μM U, 200 nM W for 2 h and then were as above treated with the indicated concentration of monoxidil plus retinol. Twenty microliters of MTT solution (5 mg/ml) was added per well and incubated for 3 h at 37 °C in the dark. The supernatant was then removed, and the formazan crystals were dissolved in 200 μl of DMSO. The samples were incubated for 30 min at room temperature, and quantified by measuring optical density at 540 nm using an ELISA reader (TECAN, Salzburg, Austria). Results are expressed as percentages of control cells; experiments were performed 8 times.

Trypan Blue Exclusion Assays To evaluate the effects of minoxidil and retinol alone or in combination on the viability and proliferation of HaCaT, the percentages of viable cells were determined by trypan blue staining. HaCaT cells were seeded into 96-well plates at 1.5×10^4 cells/well, cultured for 24 h in serum-free DMEM, and treated with the vehicle (0.12 mM HCl/DMSO diluted 1 : 1000 in serum-free DMEM) as control, minoxidil (0.01 μM), retinol (1.7 pM—1.7 nM), or minoxidil (0.01—1.0 μM) plus retinol (1.7 pM—1.7 nM) for 5 d. Cell growths and viabilities were measured by adding 0.4% trypan blue in 0.9% saline to a 50% dilution, and cells were counted using a hemocytometer.

Western Blot Confluent DPCs and HaCaT were incubated for 24 h treated with vehicle, minoxidil (0.01 μM), retinol (1.7 pM), or 0.01 μM minoxidil plus 1.7 pM retinol for 1 h to evaluate Erk and Akt expression and for 24 h to evaluate Bcl-2/Bax ratios and β-actin expression. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then extracted with an extraction buffer (1% NP-40, 1 μg/ml aprotinin, and 400 mM NaCl in 20 mM HEPES, pH 7.2). Soluble extracts were prepared by centrifugation at 15000 rpm for 15 min at 4 °C, and protein levels were quantified using BCA protein assay kits (Pierce, IL, U.S.A.). Protein (50 μg) was loaded into 10% or 12% SDS-polyacrylamide gels, separated by electrophoresis, and blotted onto nitrocellulose membranes. Blotted membranes were incubated with primary antibody (anti-Erk polyclonal antibody, 1 : 500; anti-phosphorylated Erk polyclonal antibody, 1 : 500; anti-Akt polyclonal antibody, 1 : 500; anti-phosphorylated Akt polyclonal antibody, 1 : 500; anti-Bcl-2 monoclonal antibody, 1 : 1000; anti-Bax monoclonal antibody, 1 : 1000; anti-β-actin monoclonal antibody, 1 : 1000) at 4 °C overnight. Membranes were then incubated with anti-mouse IgG-HRP conjugate (1 : 2000) or anti-rabbit IgG-HRP conjugate (1 : 2000) for 1 h at room temperature. The antibody-antigen complex was detected using an ECL system (Amersham Pharmacia Biotech; Little Chalfont, U.K.) and analyzed using a Bio-Rad GS-700 imaging densitometer (Hercules, CA, U.S.A.).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) Assays To measure the number of apoptotic cells, we used a commercially available TUNEL kit (ApopTag plus peroxidase *in situ* apoptosis detection kit, Chemicon, U.S.A.). Cells were fixed in 1% paraformaldehyde (10 min at RT), washed with PBS, incubated with equilibration buffer (1 min at RT), then with TdT enzyme for 1 h at 37 °C. Cells were then washed using a stop wash solution for 10 min at RT, and incubated with digoxigenin-dUTP for 1 h at room temperature. AEC (3-amino-9-ethylcarbazole, Zymed, U.S.A.) was used as a color-developing reagent and cells were counterstained by Mayer's hematoxylin (Dako, U.S.A.) and mounted with aqueous mounting solution (Dako, U.S.A.). Cells were then examined under a light microscope at ×200.

Statistical Analysis Statistical analyses were performed using the Student's *t*-test or multivariate ANOVA for parametric analyses. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Minoxidil Plus Retinol Enhances Human DPCs and HaCaT Proliferations to a Greater Extent Than Minoxidil or Retinol Alone Minoxidil (0.01 μM) plus retinol (1.7 pM—1.7 nM) significantly increased the proliferation of human DPCs compared with the vehicle-treated control, and the cells proliferated more efficiently in 0.01 μM minoxidil in

combination with 1.7 pM or 17 pM retinol than minoxidil alone ($p<0.05$). The enhancement was greatest at 0.01 μM minoxidil plus 17 pM retinol (Fig. 1A). Proliferation of vehicle treatment for 5 d was slightly increased about 115% compared with vehicle control at 0 h (statistically not significant, data not shown). Minoxidil (0.01 μM) or the lower concentrations of retinol (1.7 pM or 17 pM) alone enhanced the proliferation of HaCaT significantly compared with the control. The effect of minoxidil (0.01 μM) plus retinol (1.7 pM) on the proliferation of HaCaT was significantly greater than that of minoxidil alone. The proliferation of HaCaT was suppressed significantly by the higher concentration of retinol (17 nM) alone or retinol (1.7 nM or 17 nM) plus minoxidil (0.01 μM) compared with the control (Fig. 1B). The trypan blue exclusion assays produced results that were very similar to those of the MTT assay (Fig. 1C). In cultured human HaCaT, the pretreatment of inhibitors (U0126, LY294002 and Wortmannin) demonstrated statistically significant decrease of cell proliferations compared with minoxidil plus retinol-treated condition (Fig. 1D).

Minoxidil Plus Retinol Increased the Phosphorylation of Erk More Effectively Than Minoxidil or Retinol Alone Phosphorylated Erk (p-Erk) levels were significantly increased in DPCs and HaCaT treated with minoxidil (0.01 mM) for 1 h compared with vehicle control to 241±63% ($p<0.01$) and 133±22% ($p<0.05$), respectively. Retinol also significantly increased p-Erk levels compared with vehicle control to 343±113% ($p<0.01$) and 149±27% ($p<0.01$) in DPCs and HaCaT, respectively. Moreover, treatment of minoxidil plus retinol significantly increased the p-Erk compared with vehicle control to 556±178% ($p<0.001$) and 204±30% ($p<0.01$) (Figs. 2A, B). However, total Erk levels in both cells were barely changed in response to the various treatments. The values shown in Fig. 2 are percentages of control values from five different trials. In the time course of the p-Erk activities, we confirmed that p-Erk level showed at the peak time of 1 h in HaCaT (Fig. 2C). To identify the relation between specific signaling pathways in HaCaT, we pretreated U0126 (10 μM) and found that it had significantly reduced p-Erk expression as compared with minoxidil plus retinol. Pretreatment of PI3-kinase inhibitor, LY294002 (10 μM) was unable to change the p-Erk level (Fig. 2D).

Minoxidil Plus Retinol Increased the Phosphorylation of Akt More Effectively Than Minoxidil or Retinol Alone In DPCs and HaCaT, minoxidil significantly elevated phosphorylated Akt (p-Akt) to 272±37% ($p<0.01$) and 146±37% ($p<0.05$) compared with the control, respectively. Minoxidil plus retinol significantly increased the p-Akt activities in these two cells to 481±146% ($p<0.01$) and 198±23% ($p<0.01$), respectively, whereas retinol alone was 394±88% ($p<0.01$) and 180±26% ($p<0.01$) (Figs. 3A, B). As a result of determination of time dependent activity of p-Akt, the peak activation was also shown at 1 h in HaCaT (Fig. 3C). Pretreatment of PI-3 kinase specific inhibitors, LY294002 (10 μM) and Wortmannin (200 nM) significantly reduced the activation of p-Akt *versus* co-treatment of minoxidil plus retinol in HaCaT (Fig. 3D). The effect of U0126 was not obvious. The values shown are percentages of controls for five different batches of DPCs and HaCaT.

Minoxidil Plus Retinol Increased Bcl-2 Expression, But

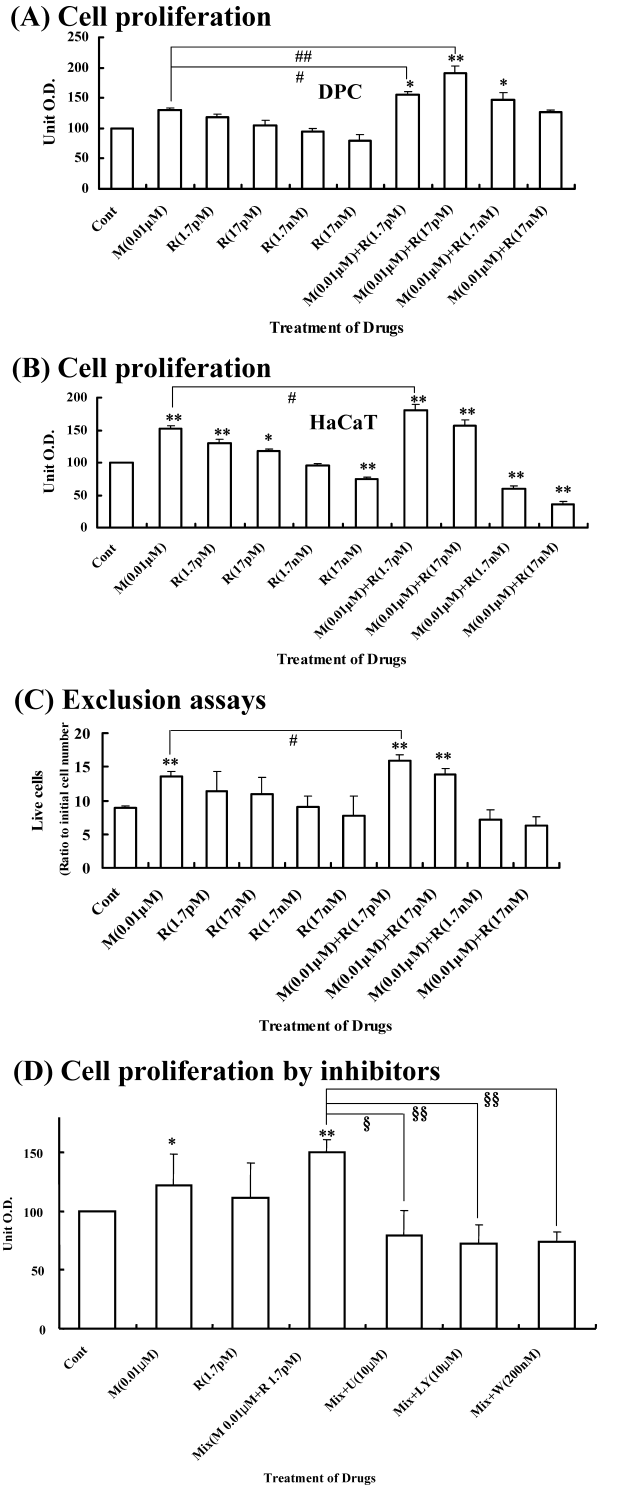

Fig. 1. Effects of Minoxidil and Retinol on Proliferation in Cultured Human DPCs and HaCaT

(A, B) Human DPCs and HaCaT cells were plated in 96 well plates at a density of 1×10^4 in 100 μl and then treated with the indicated doses of minoxidil and retinol for 5 d. The relative cell proliferation was evaluated by means of an MTT assay. (C) HaCaT cells were vehicle treated or treated with minoxidil (0.01 μM) plus retinol (1.7 pM—1.7 nM) for the indicated periods of time. Cells were harvested by trypsinization, and viable cells which excluded trypan blue were counted using a hemocytometer. (D) HaCaT cells were mock-treated (control), or pre-treated with 10 μM LY, 10 μM U, 200 nM W for 2 h and then were as above treated with the indicated concentration of monoxidil plus retinol. The relative cell survival was analyzed by an MTT assay. Each value represents the means±S.D. of triplicate measurements performed on separate experiments. Values are shown as percentages of controls. $*p<0.05$, $**p<0.01$, compared with vehicle control. $\#p<0.05$, $\#\#p<0.01$, compared with minoxidil alone. $\S p<0.05$, $\S\S p<0.01$, compared with minoxidil plus retinol. M: minoxidil, R: retinol, Mix: minoxidil plus retinol, LY: LY 294002, U: U0126, W: Wortmannin.

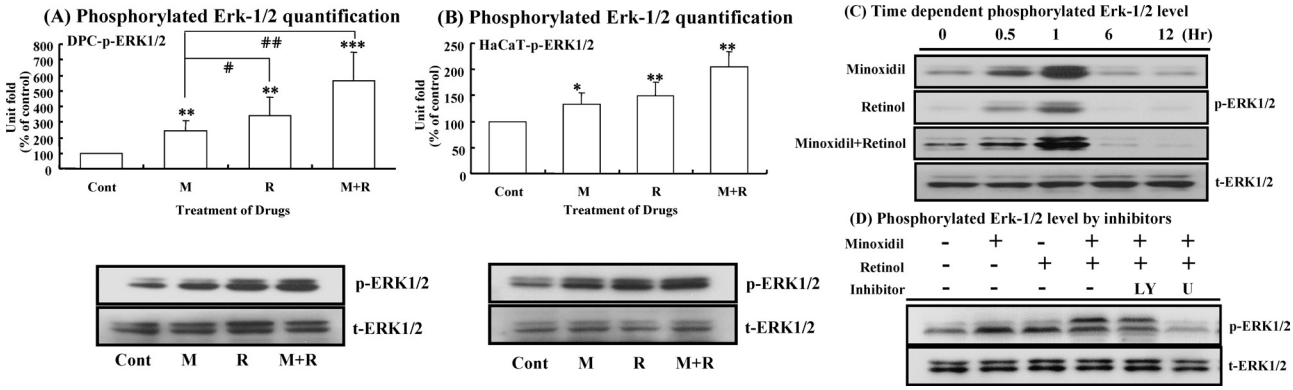

Fig. 2. Effect of Minoxidil and Retinol on Phosphorylation of Erk in Cultured Human DPCs and HaCaT

(A, B) Human DPCs and HaCaT cells were vehicle treated (control), or treated with the indicated concentrations of minoxidil and retinol for 1 h. Cell lysates (50 μg of protein) were subjected to immunoblot analysis using the indicated antibodies. The ratios of Erk activity in vehicle treated cell to minoxidil plus retinol treated cell were calculated. Ratios for the mock-treated (control) group were taken to be 100%. (C) To determine maximum activity time, phosphorylation of Erk was evaluated with the indicated concentrations of minoxidil and retinol by the time course exposure. (D) HaCaT cells were vehicle treated (control), or were pre-treated with 10 μM LY, 10 μM U for 2 h and then were as above treated with the indicated concentration of monoxidil plus retinol. Cell lysates (50 μg of protein) were subjected to immunoblot analysis using the indicated antibodies. Data shown were representatives of three separate experiments. Values are shown as percentages of controls. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with vehicle controls. $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with minoxidil alone. M: minoxidil, R: retinol, LY: LY 294002, U: U0126.

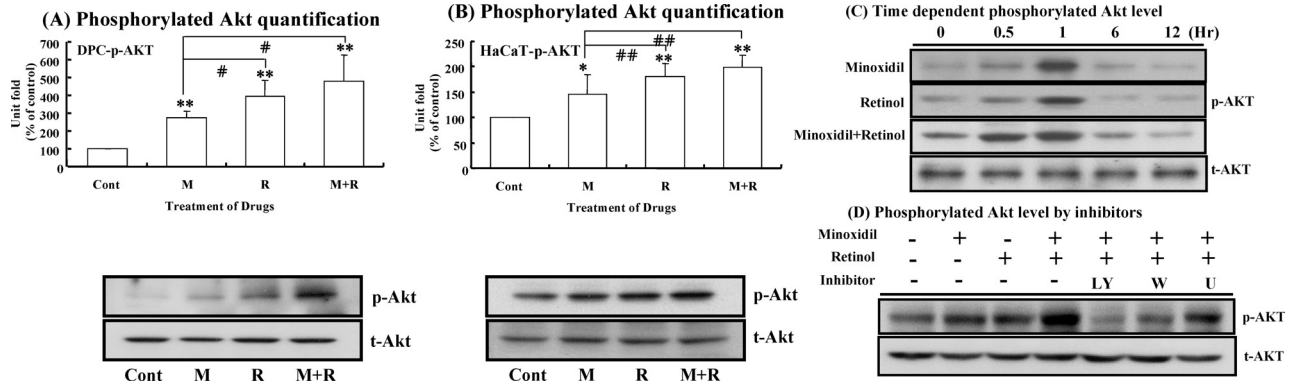

Fig. 3. Effect of Minoxidil and Retinol on Phosphorylation of Akt in Cultured Human DPCs and HaCaT

(A, B) Human DPCs and HaCaT cells were vehicle treated (control), or were treated with the indicated concentrations of minoxidil and retinol for 1 h. Cell lysates (50 μg of protein) were subjected to immunoblot analysis using the indicated antibodies. The ratios of Akt activity in vehicle treated cell to minoxidil plus retinol treated cell were calculated. Ratios for the mock-treated (control) group were taken to be 100%. (C) For determination of maximum activity time, phosphorylation of Akt was evaluated with the indicated concentrations of minoxidil and retinol by the time course exposure. (D) HaCaT cells were vehicle treated (control), or were pre-treated with 10 μM LY, 200 nM W, 10 μM U for 2 h and then treated with the indicated concentration of monoxidil plus retinol. Cell lysates (50 μg of protein) were subjected to immunoblot analysis using the indicated antibodies. Similar results were obtained in three independent experiments. The values shown are percentages of control. $^{*}p<0.05$, $^{**}p<0.01$, compared with vehicle controls. $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with minoxidil alone. M: minoxidil, R: retinol, LY: LY 294002, U: U0126, W: Wortmannin.

Decreased Bax Expression and TUNEL-Positive Cells More Than Minoxidil or Retinol Alone To evaluate the possible association between cellular apoptotic property and changes in the expression of Bcl-2 family, we investigated the effects of minoxidil and/or retinol on the expression of Bcl-2 and Bax. The Bcl-2 expression was increased after treatment with minoxidil or retinol for 24 h. Minoxidil increased the Bcl-2 expression to 228±61% ($p<0.01$) and 115±12% (statistically not significant) in human DPCs and HaCaT, respectively, and retinol increased Bcl-2 expressions to 333±95% ($p<0.01$) and 148±15% ($p<0.05$), respectively. In addition, treatment of minoxidil plus retinol significantly elevated Bcl-2 expression to 386±86% ($p<0.01$) and 177±11% ($p<0.01$) in these two cell types *versus* the vehicle controls (Figs. 4A, B).

In contrast, the expression of Bax was reduced after treatment with minoxidil alone to 70±14% ($p<0.05$) and 75±16% in human DPCs and HaCaT, respectively. Moreover, treatment of minoxidil plus retinol significantly reduced Bax expression to 28±3% ($p<0.001$) and 54±3% ($p<0.01$) *versus* vehicle controls, respectively (Figs. 4C, D). As shown in Figs. 4E and F, TUNEL assays were carried out to determine whether minoxidil plus retinol could prevent against apoptosis. The number of apoptotic cells was decreased significantly after minoxidil plus retinol treatment, *i.e.* it was reduced to 24% ($p<0.05$) and 43% ($p<0.01$) compared with the vehicle control and minoxidil alone, respectively in HaCaT.

Minoxidil Plus Retinol Enhanced Hair Growth More Efficiently Than Minoxidil or Retinol Alone in Human Hair Follicle Organ Culture Minoxidil significantly increased hair growth from 2.1±0.5 mm (vehicle control) to 3.2±0.4 mm at 1 μM ($p<0.001$) and to 3.0±0.4 mm at 10 μM ($p<0.01$), respectively. Retinol also tended to induce hair growth in a dose-dependent manner *versus* vehicle, but it was not statistically significant (Fig. 5A). Retinol tended to induce hair growth in a dose-dependent manner compared with the control, but this was not statistically significant. Minoxidil (1 μM) plus retinol (17 nM) significantly increased hair growth more than minoxidil alone (1 μM) ($p<0.001$). However, the mixing effect of 1 μM minoxidil and 17 nM retinol

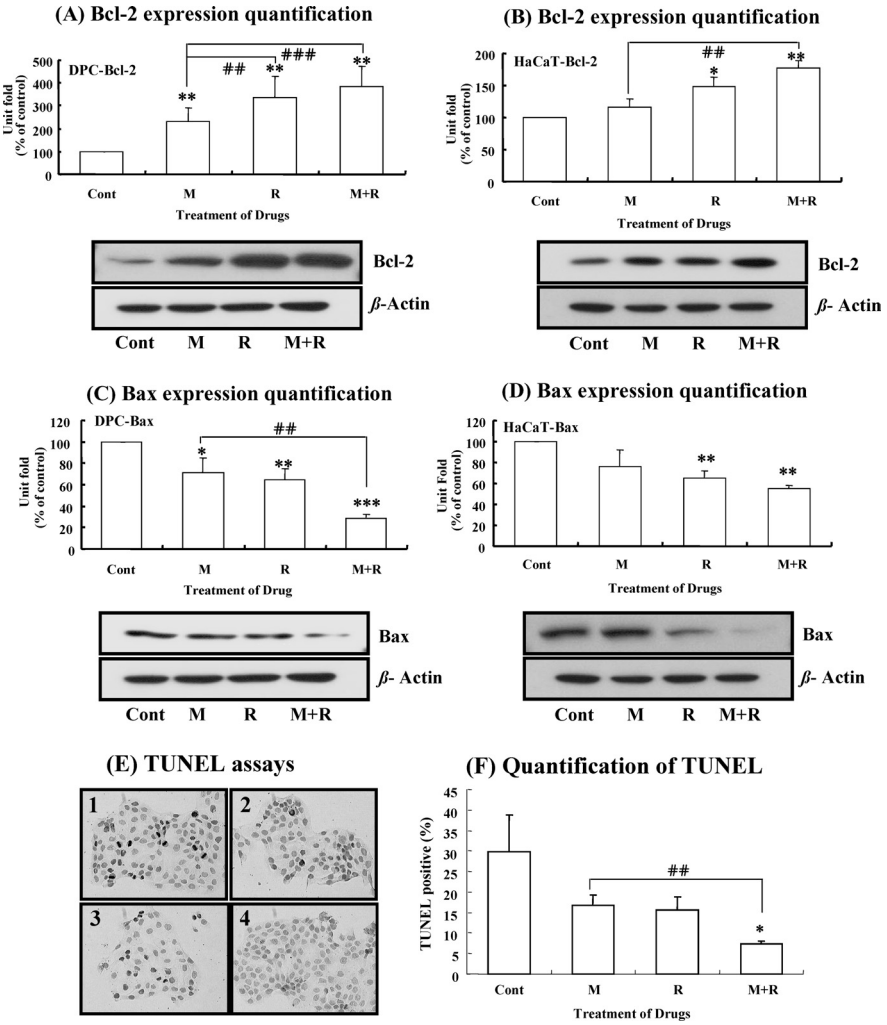

Fig. 4. Effect of Minoxidil and Retinol on Apoptosis in Cultured Human DPCs and HaCaT

Human DPCs (A, C) and HaCaT (B, D) cells were vehicle treated (control), or were treated with indicated concentrations of minoxidil and retinol for 24 h. Cell lysates (50 μg of protein) were subjected to immunoblot analysis using the indicated antibodies. The ratios of Bcl-2 and Bax expression in vehicle treated cell to minoxidil plus retinol treated cell were calculated. Ratios for the mock-treated (control) group were taken to be 100%. (E) TUNEL assays in HaCaT [1, vehicle; 2, minoxidil (0.01 μM); 3, retinol (1.7 pM); 4, minoxidil (0.01 μM) plus retinol (1.7 pM); original magnification $\times$200]. (F) Quantitative assessment of TUNEL assay. Stained and non-stained cells were counted in 3 separate fields containing at least 100 cells per field for each treatment. The bands are representatives of experiments repeated five times. The values shown are percentages of controls. $*p<0.05$, $**p<0.01$, $***p<0.001$, compared with vehicle controls. $\#\#p<0.01$, $\#\#\#p<0.01$, compared with minoxidil alone). M: minoxidil, R: retinol.

treatment was more effective than minoxidil or retinol alone on human hair follicle growth, although the elongation of hair follicles by the higher combination of the two was not less than minoxidil or retinol alone. To clarify the relationship between hair growth and the activation of signaling proteins, we pretreated with U0126 (20 μM) or LY294002 (50 μM) before treatment of minoxidil plus retinol. It was demonstrated that hair growth induced by minoxidil plus retinol was completely suppressed by those inhibitors ($p<0.001$) (Fig. 5B).

DISCUSSION

We previously demonstrated that minoxidil significantly induced the proliferation of human DPCs at concentrations ranging from 0.01 to 1.0 μM.[10] Minoxidil was found to have a biphasic effect on the proliferation and differentiation of normal human keratinocytes (NHK), *i.e.* minoxidil stimulated NHK proliferation at micromolar doses, but had antiproliferativie, pro-differentiative and partially cytotoxic effects at millimolar concentrations.[14] In another study, ATRA (All-trans retinoic acid) at concentrations ranging from 0.01 to 1.0 μg/ml (34 pM—3.3 μM) stimulated the proliferations of fibroblasts and epithelial cells.[15] Retinol is a known anti-oxidant, and moreover, retinoids increase the rate of hair growth, prolong the anagen phase of the hair cycle, play a role in converting vellus to terminal hairs, and act in conjunction with minoxidil to produce denser hair re-growth from regressing follicles than either compound alone.[16]

The role of the ERK signaling pathway is critical in mitogenesis and cell growth.[17,18] Previous reports also suggested that Akt plays a critical role in mediating survival signals.[19—22] In this study, minoxidil plus retinol was demonstrated to elevate the phosphorylations of Erk, Akt in cultured human DPCs and HaCaT, which could result in the increase of growth and survival of DPCs and HaCaT. In particular, the expression of p-Akt decreased in these cells after administrating U0126, which suggested that U0126 would have stronger suppressive effect on cell growth than LY294002 or Wortmannin (Fig. 3D). A previous study re-

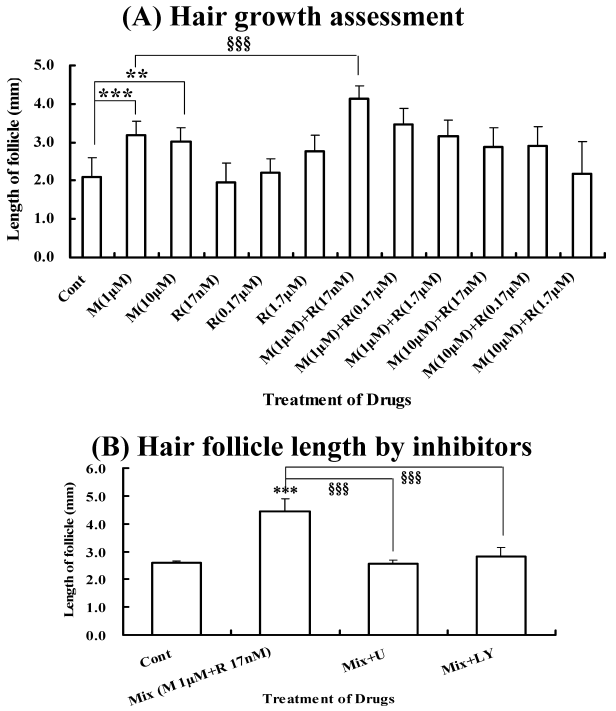

Fig. 5. Effect of Minoxidil and Retinol on Hair Growth in Hair Follicle Organ Culture

(A) Terminal hair follicles were incubated in the presence or absence of minoxidil, retinol and minoxidil plus retinol for 12 d. After incubation, hair follicle length was measured for quantitative analysis. Data are based on three male hair follicle donors (between 20 and 35 years) using >30 hair follicles per group. (B) Hair follicles were vehicle treated (control), or were pre-treated with 20 μM U, 50 μM LY for 2 h and then treated with the indicated concentration of monoxidil plus retinol. $^{**}p<0.01$, $^{***}p<0.001$, compared with vehicle control. $^{§§§}p<0.001$, compared with minoxidil plus retinol. M: minoxidil, R: retinol, Mix: minoxidil plus retinol, U: U0126, LY: LY 294002.

ported that U0126 was caused by their cytotoxicity on the late time (50—70 h) and then decreased the p-Akt activity.[23]

Minoxidil plus retinol was also shown to increase Bcl-2 and to decrease Bax expression in cultured DPCs and HaCaT. The Bcl-2 protein families constitute a critical intracellular checkpoint in the intrinsic pathway of apoptosis. The first proapoptotic homolog, Bax, was identified by its interaction with Bcl-2[24] and the Bcl-2/Bax ratio constitutes a measure of susceptibility to apoptosis *via* the intrinsic death pathway.[25]

Buhl *et al.* reported that minoxidil stimulates hair growth of mouse vibrissae follicles in organ culture.[26] In a whole skin piece culture system from 4-week-old mice,[27] it also demonstrated that minoxidil promoted hair follicle elongation. Minoxidil causes telogen hair follicles to enter the anagen phase, and prolongs the anagen period, and thus increases hair follicle size.[1] In human hair follicle organ culture, minoxidil plus retinol induced more hair growth than minoxidil or retinol alone. These results suggest that minoxidil plus retinol could have an additive effect on human hair growth, and to the best of our knowledge this study is the first to evaluate the effects of the combination of minoxidil and retinol on hair growth using organ culture of human scalp hair follicles.

In summary, we have demonstrated in this study that hair growth was significantly enhanced by the combination of minoxidil plus retinol compared with minoxidil or retinol alone. We suggest that the actual hair growth modulatory effects of the combination of minoxidil and retinol would be derived from different mechanisms, *i.e.* (i) *via* the activation of Erk- and Akt-dependent pathways and (ii) *via* the prevention of apoptosis by increasing the Bcl-2/Bax ratio and the number of TUNEL negative cells. Thus, the combination of minoxidil and retinol provides as an efficient hair growth modulatory strategy for the administration on hair loss (alopecia) patients, which supports the possibility more effectively than existing minoxidil alone materials.

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