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

MATERIALS AND METHODS

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/Tyr204) 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...
used, as described previously.\textsuperscript{11) 

**Human Hair Follicle Organ Culture** Human scalp hair follicles were isolated and cultured in vitro, as described previously.\textsuperscript{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\textsubscript{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.\textsuperscript{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 antmycotic 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\textsubscript{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\textsubscript{2}.

**Thiazolyl 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\textsuperscript{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/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 μM W for 2 h and then were as above treated with the indicated concentration of minoxidil 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\textsuperscript{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 μs 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 polycyclic antibody, 1:500; anti-phosphorylated Erk polycyclic antibody, 1:500; anti-Akt polycyclic antibody, 1:500; anti-phosphorylated Akt polycyclic 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 antigen-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
combinations 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. The difference was not statistically significant (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 nm) for 1 h compared with vehicle control at 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 levels 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 ±46% (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**
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
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. 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 anti-proliferative, pro-differentiative and partially cytotoxic effects at millimolar concentrations. 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. Retinol is a known antioxidant, 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 regrowth from regressing follicles than either compound alone.

The role of the ERK signaling pathway is critical in mitogenesis and cell growth. Previous reports also suggested that Akt plays a critical role in mediating survival signals. 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-

![Graphs and images](image-url)
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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