Korean J. Chem. Eng., **25**(2), 295-301 (2008)

Effect of mesenchymal cells on human hair growth and death

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(*Received 9 June 2007 • accepted 11 July 2007*)

Abstract−Animals have typically been used in efficacy tests, but there are a number of dissimilarities between humans and animals. To overcome the problems associated with animal testing, a model which is reproduced *in vitro* with long-term culture with cell growth with *in vivo* activity must be developed. We made a gel-type dermal equivalent (DE) that contained dermal papilla cells (DPCs) or dermal sheath cells (DSCs) isolated from human hair bulbs in order to mimic human scalp tissue. Hair follicles were organ-cultured on DE containing DPCs or DSCs. The DE used for organ culture was a reconstructed 3-dimensional contraction of collagen gel, and the cell density of the DE did not affect the increase in hair length. We tested the effects of cell types in DE on increases in hair length, and the results showed a large increase in hair length and long-term viability in the air-liquid interface culture on DE containing DSCs. We compared the submerged culture with the hair air-liquid interface culture on DE using immunohistochemical staining, and found that the hair follicles that were air-liquid interface cultured on DE maintained the growth phase (anagen) for a longer period of time than the hair follicles that were submerged. Since the hair follicles were cultured under an air-liquid interface condition, the increase in hair length was a reflection of the epithelial cell growth that resulted from the improved oxygen supply and paracrine factors secreted from hair origin cells.

Key words: Hair Growth, Mesenchymal Cell, Organ Culture

INTRODUCTION

The hair follicle is a complex structure composed of both epithelial (the matrix and outer root sheath) and dermal components (the dermal papilla and dermal sheath). Human hair grows in a continuous cyclic pattern. It is the only organ in the mammalian organism to continuously undergo life-long cycles of rapid growth (anagen), regression (catagen), and resting periods (telogen) [1,2]. Hair cycle is maintained by epidermal-mesenchymal interaction (EMI), a signaling cascade between epidermal and mesenchymal cell populations [3]. DP cells play a crucial role in the EMI, and they form just once every 9-12 weeks [4]. DP cells tend to regress between cycles 15 and 20 due to heredity, hormones, stress, etc. Their activity is abnormal and they fall out at immature state. Androgenetic alopecia, or hair loss mediated by the presence of the androgen dihydrotestosterone (DHT), is the most common form of alopecia in both men and women [5]. Hair follicles contain androgen receptors, and DHT combines with these androgen receptors. In the presence of DHT, the genes that shorten the anagen phase are activated, and hair follicles shrink or become miniaturized [6].

Alopecia is not essential for survival; however, it has a great effect on a number of human social activities. Many medicines have been developed in an effort to cure alopecia, including a hair forming medicine and a hair growth drug. Animal test methods have typically been used to test the efficacy of these hair loss remedies. However, there are sometimes discrepancies in assessments of the efficacy of these treatments due to the dissimilarity of biological characteristics among rodents [7-9]. The stumptailed macaque can provide a good model for human hair loss because their hair loss pattern is similar to that of humans; however, efficacy tests employing these animals are very expensive [10]. Animal testing has recently received a great deal of criticism from animal lovers and society, but the EU had already placed legal limitations on the use of animals for product testing.

To overcome the problems associated with animal testing, we need an organ culture model which is reproduced *in vitro* and that has its own long-term growth activity similar *in vivo*.

Therefore, many researchers have attempted to use various methods to maintain the hair follicle for a long period of time. Most of these research efforts have sought to investigate the physical effects, biological effects, and their combined effects on hair growth. Initially, the physical effects on hair growth were investigated in hair follicles placed in 1.5 ml of incubation medium in a closed 5-ml glass tube that was rolled at 15 rpm at 36 °C. Hair root sheaths also grew along with the hair shafts. Remarkable hair growth was noted for 7 to 8 days. The structure of the hair bulbs was well maintained for at least 6 days, but then the hair matrix cells started to degenerate [11]. When hair follicles were cultured in serum-free media at 31 °C, the length of the cultured hair increased in a time-dependent manner for 96 h. The histological findings demonstrated that the hair germinative cells maintained their normal morphology and that there was an increase in DNA synthesis in the hair bulb, as compared with hair follicles cultured at 37 °C [12]. Waldon et al. cultured follicles at the air-liquid interface on a modified gelatin matrix (Gelfoam; Upjohn, Michigan, USA). This study showed that ultra-high sulfur keratin (UHSK) expression was significantly higher in the air-liquid interface cultures compared to the submerged culture [13].

And some researchers investigated the effects of biological fac-

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tors on hair. Philpott found that EGF induced catagen in cultured human hair follicles [7]. When hair follicles were cultured without insulin, they showed rapid transformation from anagen to catagen [8]. However, upon the addition of insulin-like factor-1 (IGF-1) to the culture medium, the hair follicles recovered their natural growth activities. Ogawa observed higher expression of hepatocyte growth factor (HGF) in DPCs isolated from cultured hair follicles. He demonstrated an increase in hair length and DNA synthesis by adding 10 ng/ml HGF to the culture medium. These are the studies that observed a complex interaction of physical and biological effects [14,15]. Hair follicles cultured at the air-liquid interface produced a 2.7-fold increase in hair growth and maintained an anagen-like morphology. Substrates such as nylon mesh seeded with skin fibroblasts, full thickness skin supported hair growth. Ultra-high sulfur keratin (UHSK) was expressed at a significantly higher level in the air-liquid interface cultures than in the submerged culture [16].

At monolayer cell culture, both physical and biological factors affected human hair cell growth [15,17]. Therefore, we reconstructed gel-type dermal equivalent (DE) that contained dermal papilla cells (DPCs) or dermal sheath cells (DSCs) isolated from human hair bulbs in order to mimic human scalp tissue. Hair follicles were organ-cultured on DE containing DPCs or DSCs. DE for organ culture was reconstructed in a 3-dimensional contraction of collagen solution in order to reconstruct a bioartificial scalp dermis. Mesenchymal cells, such as dermal papilla cells (DPCs) and dermal sheath cells (DSCs), were isolated and used to reconstruct the gel-type dermal equivalent. Since the hair follicles were cultured under an air-liquid interface condition, the increase in hair length was a reflection of epithelial cell growth that resulted from the improved oxygen supply and paracrine factors secreted from hair origin cells. We believe that the paracrine factors secreted from the bioartificial scalp (DE)-containing cells affected the growth of the hair cells. Thus, we compared the effects of submerged culture and air-liquid interface culture on DE. And we investigated the effect of DPCs or DSCs increase in the hair length and altered the structure of the hair follicle, and apoptosis in hair follicle was observed by using immunohistochemistry.

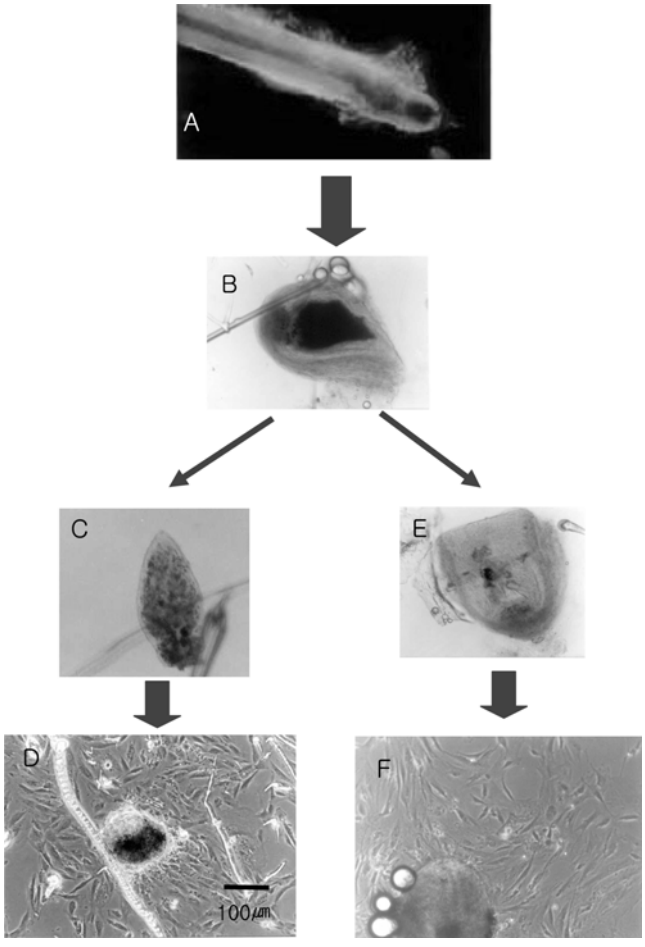

Fig. 1. Schematic diagram of primary cultures DPCs and DSCs from hair follicles. The first step was to cut the bulb (B) from the isolated hair follicle (A). The matrix cells were then removed from the bulb and DP was picked out (C) from the lower bulb by using a 26G syringe needle. Each of DP, DS were delivered to an FBS-coated tissue culture dish in advance to attach the tissue (original magnification=×100).

MATERIALS AND METHODS

1. Isolation of Hair Follicles and Primary Cultures of DPCs and DSCs

Human scalp skin tissue was obtained from autobiopsy or facelift surgery with the consent of the patients. The tissue was prewashed in Dulbecco's phosphate balanced solution (D-PBS) containing penicillin G, streptomycin, and amphotericin B. Scalp skin was delivered to the laboratory in Williams' E medium containing penicillin G, streptomycin, and amphotericin B.

Intact scalp skin was cut with a surgical blade along the direction of hair growth. The additional subcutaneous fat was carefully removed with a scalpel and forceps without causing injury to the hair follicle bulbs. Intact human anagen hair follicles were isolated with a scalpel. The isolated follicles were dissected at the infrainfundibular level without epidermis or sebaceous gland tissue, but with a narrow band of perifollicular connective tissue.

All steps were observed with a stereoscope at a magnification of 40× (KSZ, Korea). The bulbs were cut off, and a 26 G syringe needle was used to isolate the DP and DS by gently teasing out the papilla, cutting off its basal stalk and isolating the dermal sheath cells. Finally, the papilla were transferred into a 35-mm culture dish coated with 400 μl of FBS on the surface. To isolate the DSCs, the bulb-removed DP (Fig. 1E) was reversed in order to expose the DP to the surrounding tissue, and it was then transferred into a 35-mm culture dish coated with 400 μl of FBS on the surface. 2 ml of DMEM (Dulbecco's Minimal Essential Medium) was added until reaching a final FBS concentration of 20%. The media was not changed until the cells grew from the attached tissue. When we observed cell growth, the concentration of FBS was reduced by 10%. The media was then changed twice a week. Confluent cells (Fig. 1D, F) were subcultured using 0.05% trypsin/0.02% EDTA.

2. Reconstruction of Gel-type Dermal Equivalent

The dermal equivalent cultures were prepared by a method modification from Bell et al. [18].

Mesenchymal cells were suspended at a concentration of 1×10^5 cells/ml of DMEM containing 10% FBS and harvested at 800 rpm by a centrifuge. This cell pellet was gently stirred with a solution

containing 7 volumes of 5 mg/ml collagen solution, 2 volumes of 5× DMEM, and one volume of 0.05 N NaOH containing 2.2% sodium bicarbonate and 200 mM HEPES buffer solution. Aliquots of the cell/collagen mixtures were placed on culture plates. Temperatures were raised to 37 ºC, and polymerized collagen gels were incubated. After polymerization, mesenchymal cells were dispersed throughout the gels, and the mixture became the attached gel. To obtain cultures of floating collagen gels, the attached gels were gently lifted off the bottom of the wells with the collagen gel into a fibrillar connective tissue-like dermal equivalent by 15 days of incubation within the culture medium at 37 ºC, 5% CO_2 in air [19].

3. Effects of Concentration and Type of Mesenchymal Cells on Increases in Hair Length

We reconstructed DS gel which consisted of collagen gel with DSCs at a concentration of 1×10^5 cells/ml or at a concentration of 5×10^5 cells/ml in order to observe the effect on the increase in hair length by cell density in DE. The hair follicle was cultured on contracted gel in Williams' E medium supplemented with ITS 2× and hydrocortisone for 15 days and observed under a stereomicroscope. We reconstructed DP gel which consisted of collagen gel with DPCs at a concentration of 1×10^5 cells/ml or DSCs at a concentration of 1×10^5 cells/ml in order to observe the effects of cell type on the increases in hair length in DE. DE without cells was used as a control.

4. Effects of Culture Methods: Submerged Culture vs. Air-liquid Interface Culture

Intact anagen hair follicles were used for organ culture. The hair follicles were cultured in Williams' E medium supplemented with ITS 2× (Sigma, USA), 10 ng/ml hydrocortisone, 50 μg/ml penicillin, and 50 unit/ml streptomycin. Hair follicles were cultured in 500 μl of media per well in a 24 multi-well dishes (Nunc, USA), and the media was changed every three days. DE for organ culture was contracted with DMEM/10% FBS for 2 weeks and then washed by using PBS to remove the remaining FBS before organ culture. Contracted DE was delivered in a Transwell (12 multi-well dish; Corning, USA) in which the pore size was 3.0 μm, and three hair follicles were delivered on each DE. Medium (1.5 ml) was supplied to each well and changed every other day. Increases in hair length and changes in the structure of the bulb were observed with a CCD camera connected to a stereomicroscope (KSZ, Korea). To eliminate phase difference, hair follicles were observed without culture medium, and they were cultured for 15 days. Hair follicles were rapidly measured from the lower bulb to the hair shaft to protect the hair from dryness. Pictures of each hair follicle were magnified and measured with a ruler (error range: ±5 μm). Only intact anagen hair follicles were used for organ culture, and hair follicles with little growth were excluded. In anagen, the hair showed continuous growth and a connection between DP and matrix tissue was observed. In catagen, the connection was cut off from the DP and matrix tissue and an epithelial strand appeared. The DP and matrix tissue was cut off and club hair fell out of the scalp in telogen [2].

5. Immunohistochemistry

We investigated the expression of cytokeratin 19, an active proliferative epithelial cell marker and a marker for putative stem cell-containing epithelial compartments [20,21]. The samples were cut into small pieces containing approximately six intact follicles. Unfixed tissue samples were then embedded in Tissue-Tek OCT compound (Miles, USA), quick frozen over dry ice (−80 ºC), and stored at −80 ºC until used for immunohistochemistry. Due to their low frequency (<5%), catagen follicles were dissected out before being individually embedded in Tissue-Tek OCT compound. Longitudinal frozen sections (5 μm) of hair follicles were prepared on a cryostat HM 500 M (Microm, France) in which the chamber temperature was set at −40 ºC. The sections were then air-dried and stored at 4 ºC overnight before being processed.

Monoclonal antibodies were chosen according to their IgG subclasses in order to avoid cross-reaction during multilabeling experiments. Biotinylated or fluorescent species-specific secondary antibody (Ks 19.1, Progen, Germany) was applied for 30 min or 1 h at room temperature. Biotinylated secondary antibody was revealed by using avidin-biotin-peroxidase complex (ABC; Dako, France) and 3-amino-9-ethylcarbazol (AEC; Sigma, France) as a substrate. The slides were finally mounted under coverslips using glycerol gelatin (Sigma, U.S.A). Hematoxylin was used for counterstaining, and the ABC stainings were analyzed with a microscope (Nikon, Japan).

To evaluate the apoptotic cells, we used an established, commercially available terminal deoxy-nucleotidyl transferase mediated dUTP nick end-labeling kit (TUNEL kit; ApopTag, Oncor, U.S.A.) according to the manufacturer's protocol. Briefly, a dideoxynucleotide labeled with exogenous digoxigenin was bound to the 3'-OH DNA ends by a TdT enzyme on the cryosections. The sections were then incubated with an anti-digoxigenin antibody conjugated with horseradish peroxidase. The peroxidase activity was analyzed by exposing the sections to a solution containing 0.05% 3,3'-diaminobenzidine tetrahydroxychloride and 0.01% H_2O_2 in Tris-HCl buffer (DAB solution) at pH 7.6 for 5 min. Finally, the sections were counterstained with 1% hematoxylin. In the negative controls, distilled water was used instead of the TdT enzyme solution.

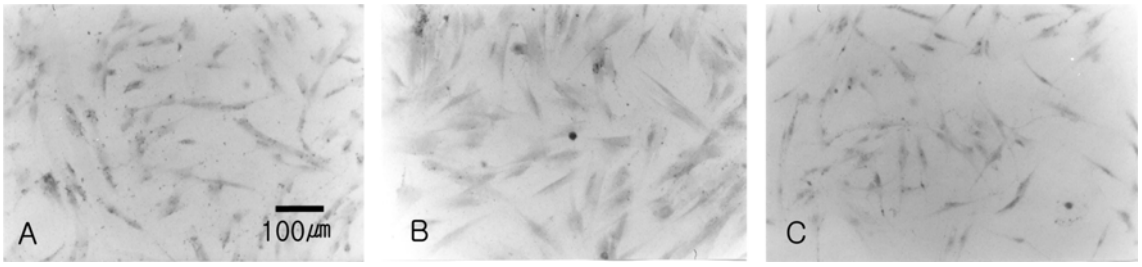

Fig. 2. Expression of α-smooth muscle actin. The dermal sheath cells showed more α-smooth muscle actin (brown) expression than the dermal papilla and dermal fibroblast cells. The data were expressed as dermal papilla cells (A), dermal sheath cells (B), and dermal fibroblasts (C) (original magnification=×100).

RESULTS AND DISCUSSION

1. Isolation of Hair Follicles and Culture of DSCs and DPCs

To observe the bulb morphology under a stereomicroscope, we chose intact anagen hair follicles and hair follicles that were organ cultured (Fig. 1A). DPCs and DSCs were isolated by the explantation method. We scratched at the DP tissue using a syringe needle to cause rapid cell immigration. The DSCs and DPCs showed fibroblast-like morphologies and were spindle shaped. Cultured cells were confirmed by α-smooth muscle actin (α-SMA) immunostaining. α-SMA is expressed more intensively in DSCs than in skin dermal fibroblasts [22]. In addition, DPCs do not express α-SMA *in vivo* but cultured cells expressed *in vitro* [23]. All of them expressed α-SMA (Fig. 2), but the DSCs expressed α-SMA more intensively than DPCs and fibroblasts, indicating that the isolated cells originated from DP and DS.

2. Reconstruction of Gel Type DE

Since DE was detached under mechanical stimuli from the well plate, DE was contracted as in Fig. 3A. After 15 days DE was contracted as in Fig. 3B. We reconstructed and cultured DS gel which consisted of collagen gel with DSCs at densities of 1×10^5 cells/ml or 5×10^5 cells/ml in order to observe the effects of cell density in DE on increases in hair length.

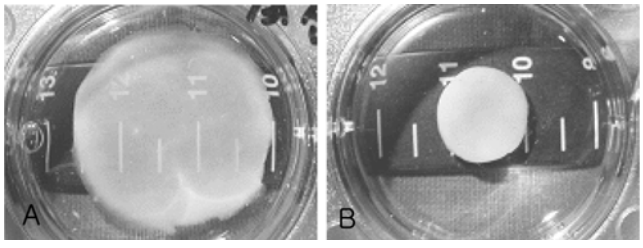

Fig. 3. Photograph of the dermal equivalent (DE) during contraction. The data were expressed as 5 days contracted DE with cell (A) and 15 days contracted DE with mesenchymal cells (B) (original magnification ×10).

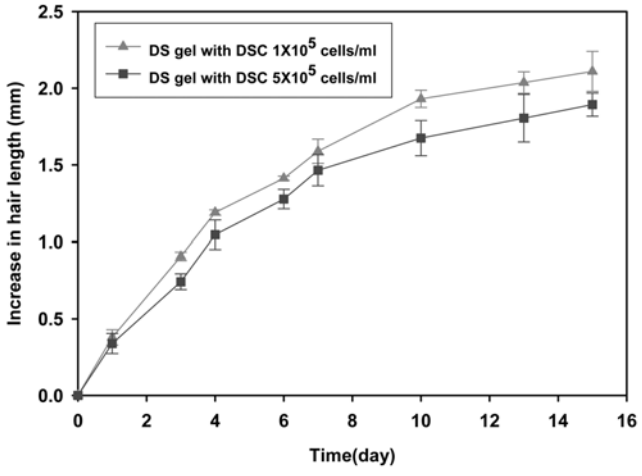

Fig. 4. Increase in human hair length on DS gel at various densities. Hair follicles were measured as described in the Methods section. The data were expressed as the cumulative growth of the hair follicle on the DS gel at a density of 1×10^5 cells/ml (triangles) on the DS gel at a density of 5×10^5 cells/ml (squares). Values represent the mean±SD (n=7). Two additional experiments yielded similar results.

The mean hair growth on DS gel at a density of 1×10^5 cells/ml was 2.11±0.09 mm, and the mean hair growth on DS gel at a density of 5×10^5 cells/ml was 2.00±0.07 mm. We could not observe any significant differences for the increase in hair length between the high density DE and the low density DE according to culture time (Fig. 4, Table 1).

3. Effects on the Increase in Hair Length by Concentration and Type of Mesenchymal Cells

The density of the cells in DE did not affect the increase in hair length; therefore, we reconstructed DP gel or DS gel. The cell density in the solution was 1×10^5 cell/ml of DPCs or DSCs, and 2.5 ml of solution was poured into each well of a 6-well plate. Although there were some differences among the donors, the trend in hair growth was similar. The amount of hair growth on DE with cells was relatively higher (75.0%) than the hair growth on collagen gel;

Table 1. Increase in hair length on the DS gel

	DAY 3 (mm)	DAY 13 (mm)
DS gel with DSC 1×10^5 cells/ml	0.91±0.02	2.11±0.09
DS gel with DSC 5×10^5 cells/ml	0.74±0.04	2.00±0.07

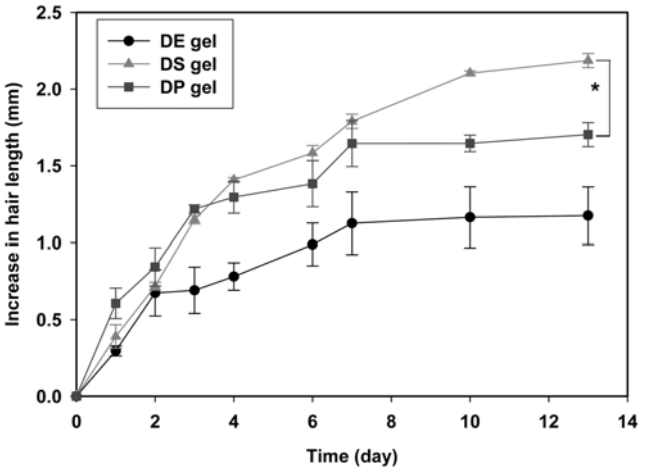

Fig. 5. Increase in human hair follicles on collagen gel, DS gel, and DP gel. Human hair growth on DS gel (collagen gel with DSC (triangles)) was higher than the amount of hair growth on DP gel (collagen gel with DPC (square) and collagen gel (collagen gel without cell (circles)). Hair follicles were measured as described in the Methods section. The data were expressed as the cumulative growth of the hair follicle on the collagen gel (circles), on the DS gel at a density of 1×10^5 cells/ml (triangles), on the DP gel at 1×10^5 cells/ml (squares). Values represent the mean±SD (n=7). Two additional experiments yielded similar results (*p<0.001).

Table 2. Increase in hair length on the DS gel or DP gel

	DAY 3 (mm)	DAY 13 (mm)
Collagen gel	0.69±0.15	1.13±0.02
DS gel 1×10^5 cells/ml	0.72±0.02	2.10±0.02
DP gel 1×10^5 cells/ml	1.10±0.04	1.68±0.08

thus the DSCs had a greater effect on hair growth than the DPCs. This finding is summarized in Fig. 5 and Table 2. Immunohistochemical analysis showed a similar trend for both types of cells. As shown in Fig. 6, an increase in hair follicle was observed under a stereomicroscope after 6 days of culture (B, D), as compared to the size of the follicle before culture (A, C). After 6 days, hair growth over the ORS was observed in both sets of DE containing DPCs or DSCs (Fig. 6B, D).

4. Effects on Culture Methods: Submerged Culture vs. Air-liquid Interface Culture

A higher viability of hair cells was observed in the DE than in the submerged culture. The partially blue spot (nucleus) was slightly

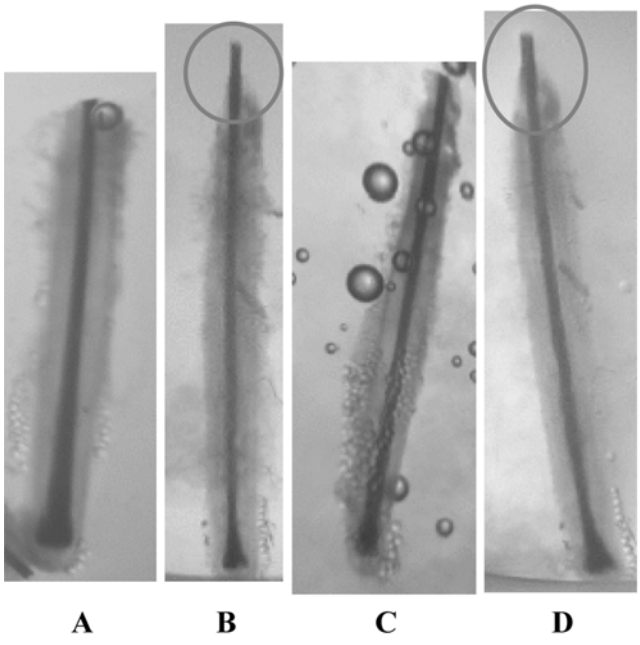

Fig. 6. Morphology of the organ-cultured human hair follicles cultured for 6 days on DE containing different cell types. The hair follicles were photographed (original magnification ×40). Red lines represent hair growth during the culture period. The data were expressed as the initial hair follicle on DS gel (A), hair follicle on DS gel for 6 days (B), initial hair follicle on D gel (C), hair follicle on DS gel for 6 days (D).

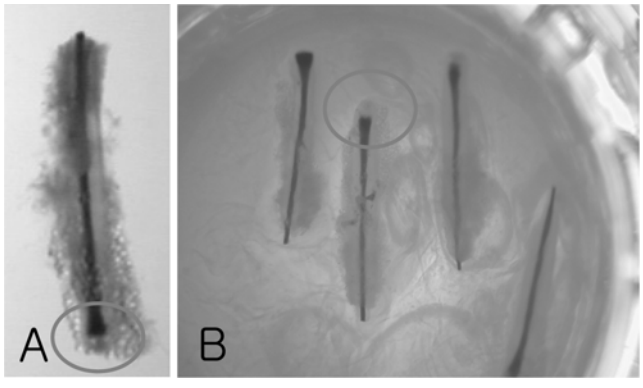

Fig. 7. The environment of the hair follicle organ culture: Submerged culture (A), Co-culture on DE (B).

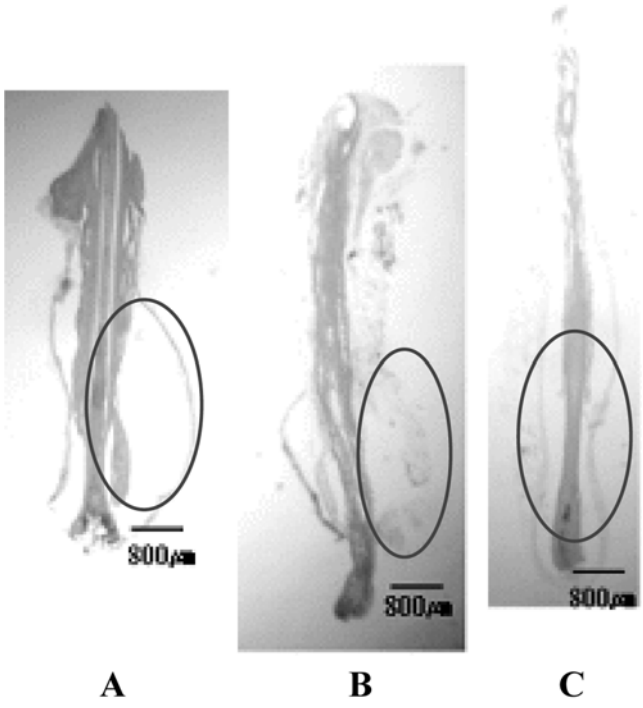

Fig. 8. Histology of organ-cultured hair follicles in different culture environments. The hair follicles were photographed (original magnification ×40). The data were expressed as 9 days of submerged culture (A), 9 days of overlay cultured on DS gel (B), 18 days of overlay cultured on DS gel (C).

more expressed in the hair follicle cultured on DE than in the submerged hair follicle (Fig. 8A, B). In the hair growth mechanism, matrix cells were differentiated into IRS and non-keratinized hair shaft, and ORS brought the non-keratinized hair shaft and IRS to the epidermis. Their function was so well controlled that the hair follicle did not degenerate during anagen. However, in catagen, the matrix cells degenerated and reduced the synthesis of hair; thus the hair should become thinner. Finally, the connection between the matrix cells and DP was cut in telogen. The hair on DE was thinner after day 18, and this phenomenon resulted from a reduction in the synthesis of hair keratin synthesis by matrix cells (Fig. 8C). Cytokeratin 19 is known to be expressed in epithelial precursor cells or putative stem cell-containing epithelial compartments. CK19 was co-expressed at the upper bulb and in the bulge area in anagen. As the hair degenerated, precursor cells in the bulge area migrated to the bulb, and the expression of CK19 was scattered all over the ORS layer [20,21]. The submerged hair showed weak CK19 expression all over the hair without bulb after 9 days (Fig. 9A). In contrast, the hair on DE showed strong expression in the upper bulb and bulge areas after 9 days (Fig. 9B, arrows), which suggests that the hair was in active growth. Hair enters the catagen phase at day 18. There is a hypothesis that when epithelial cells are injured, stem cells in the bulge area migrate and cure the epithelial cells. Consistent with this hypothesis, we observed that ORS cells in the bulge area moved into the bulb to remove club hair (Fig. 9C, arrows). The hair matrix and proximal outer root sheath (ORS) are degenerated by apoptosis during catagen, resulting in the cessation of hair formation in telogen. At the onset of anagen, bulge stem cells transiently pro-

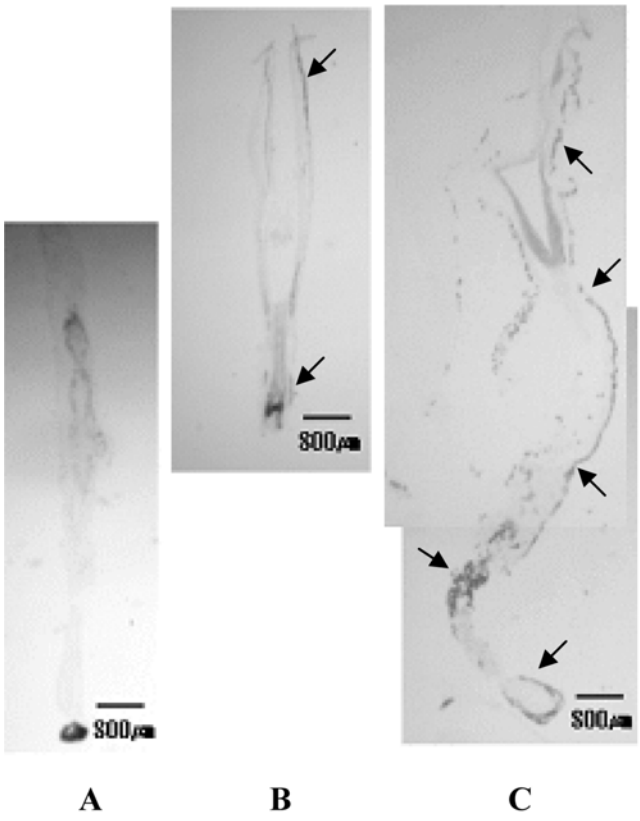

Fig. 9. Immunohistochemical staining of organ-cultured hair follicles against CK19. The hair follicles were photographed (original magnification ×40). The data were expressed as 9 days of submerged culture (A), 9 days of culture on DS gel (B), 18 days of overlay cultured on DS gel (C).

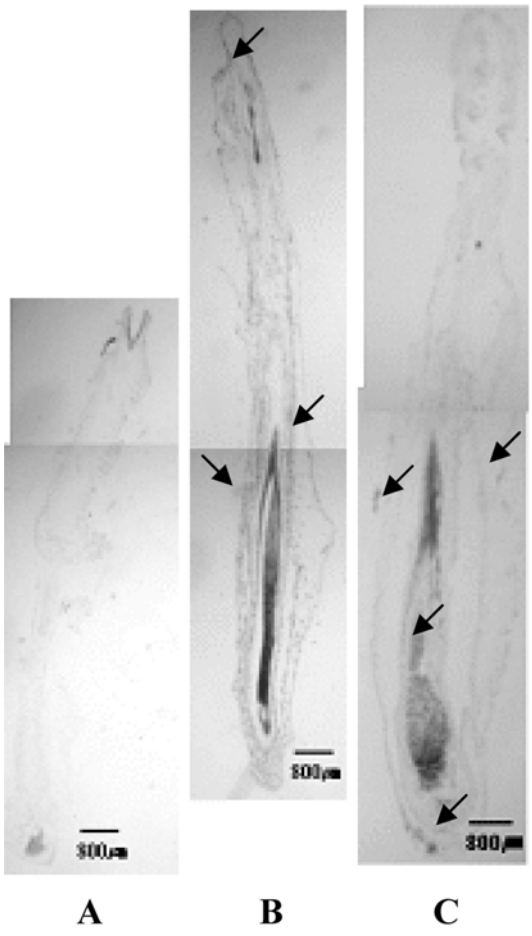

Fig. 10. Immunohistochemical staining of organ-cultured hair follicles against TUNEL. The hair follicles were photographed (original magnification ×40). The data were expressed as 9 days of submerged culture (A), 9 days of overlay cultured on DS gel (B), 18 days of overlay cultured on DS gel (C).

duce their descendants that actively proliferate and migrate to the lower follicles to regenerate hair matrix cells [24].

Hair cells continuously proliferate and undergo cell death. TUNEL assay is an effective method for examining cell apoptosis in tissue because the expression regions vary throughout the hair cycle. TUNEL-positive cells were detected in matrix cells, IRS and ORS, which are the TUNEL positive regions in anagen [24,25]. Submerged hair was not detected all over the hair after 9 days (Fig. 10A). However, the hair on DE was strongly expressed in the outermost ORS after 9 days (Fig. 10B) and in the matrix and bulge after 18 days (Fig. 10C, arrows).

CONCLUSIONS

Hair follicles were supplied with sufficient nutrients and oxygen with vessels *in vivo*, and they were transferred growth factors and cytokines from neighboring cells. However, they lost nutrients and oxygen supply from the vessels *in vitro*. We reconstructed a gel-type dermal equivalent (DE) that contained dermal papilla cells (DPCs) or dermal sheath cells (DSCs) isolated from human hair bulbs in order to mimic human scalp tissue. Hair follicles were organ-cultured on DP gel and DS gels. The optimal condition of DE for hair growth was 1×10^5 cell/ml of DSCs.

We tested the effects of cell type in DE on the increase in hair length, and the results showed a large increase in hair length and long-term viability in air-liquid interface culture on DE containing DSCs.

Immunohistochemical analysis revealed that the hair follicles that were air-liquid interface cultured on DE maintained the growth phase (anagen) for a longer period of time than the hair follicles that were submerged. Since the hair follicles were cultured in an air-liquid interface condition, the increase in hair length was a reflection of epithelial cell growth (ORSC, matrix cells) that resulted from the improved oxygen supply and paracrine factors secreted by hair origin cells.

We applied a hair follicle organ culture to a bioartificial scalp dermis using an air-liquid interface culture method, improved the dissimilarity of activity among *in vivo* environments, which is a limitation of the monolayer cell culture test. The effects on proliferation and differentiation of hair follicle and the rate of apoptosis in hair cells differed depending on the cell type in DE.

ACKNOWLEDGMENTS

This work is financially supported by the MOCIE through the

Basic Technology Development Program and MOE, MOCIE, MOLAB though the fostering project of the Lab of Excellency in Korea.

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