Effect of Several Growth Factors on Hair Follicle Growth in Hair Follicle Organ Culture

Jeong Aee Kim, M.D., Hee Chul Eun, M.D.

Department of Dermatology, Seoul National University, College of Medicine, Seoul, Korea

***Background*: Factors that regulate hair matrix cell division within the hair follicles and control hair growth cycle have been poorly understood untill now. One of the main causes seems to be lack of good in vitro models.**

***Objective*: This study was performed to investigate the hair growth promoting potencies of several agents including individual components of keratinocyte growth media.**

Methods: Several agents such as epidermal growth factor, insulin, bovine pituitary extract which were contained in keratinocyte growth media as well as minoxidil and transforming growth factor-α were added to the isolated anagen hair follicles. Measuring the length of hair follicle, thymidine and leucine uptake were used for hair growth parameter.

***Results*: Isolated anagen hair follicles in keratinocyte growth media showed a significant increase in length over 48 hours. [Methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake were sustained at basal state as well as over 48 hours and [methyl-^{3}H] thymidine uptake increased in the matrix cells under autoradiography. Insulin with a concentration above 0.5μg/ml and transforming growth factor-α with a concentration above 10ng/ml showed a promoting effect on hair growth. However, other agents did not promote hair growth at all.**

***Conclusion*: Our in vitro model resembles the in vivo status of hair growth for a limited period of time and we think that normal human hair organ culture may be a useful model for developing hair growth promoting agents in vitro.**

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Key Words: Hair follicle, Insulin, Organ culture, Transforming growth factor-α

Hair performs no vital function in humans, but its psychologic functions are inestimable[1]. Hair loss can be observed in various diseases such as alopecia areata and in physiologic conditions such as male type baldness. However, the mechanism that regulates the hair growth cycle is poorly understood and developement of drugs that promote hair growth are still in a primitive stage. One of the main causes seems to be a lack of good in vitro models[2-4].

Cell culture technique has been recently being tried in various fields of dermatology including hair research. Outer root sheath cells and papilla cells have been cultured[5-9]. However, dispersed cell culture techniques have limitations in respect of integrated hair growth biology because hair growth is the final result of intergral part of each hair component[10].

Short term hair organ culture model has been developed by several authors and its usefulness has been already suggested[2-4, 11-13]. One advantage of the organ culture system is that the relationship between hair matrix cells and dermal papillae is maintained. For hair organ culture, other authors have used different media such as Eagle's minimal essential media[3], Williams' E media[11,12] and Ham's F12 media[13]. In our preliminary experiment, we have found that keratinocyte growth media(KGM) which has been used for growing keratinocytes in low calcium monolayer culture system[14] has a good

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Reprint request to: Hee Chul Eun, M.D., Department of Dermatology, Seoul National University Hospital, 28 Yunkun-Dong Chongno-Ku, Seoul 110-744, Korea

growth promoting effect on hairs grown by human hair organ culture.

The aims of this study were to see whether keratinocyte growth media was superior to other media for hair growth in vitro and to verify which components of keratinocyte growh media have growth promoting effects. We also wanted to know the growth promoting effect of some other agents such as transforming growth factor-α (TGF-α) and minoxidil. For this purpose we have cultured isolated human hair follicles and measured their length under the microsope. We also measured DNA and protein synthesis using radioisotopes after in vitro pulse labelling.

MATERIALS AND METHODS

Materials

Keratinocyte growth media(KGM), keratinocyte basal media(KBM, unsupplemented MCDB 153), epidermal growth factor (EGF), insulin, and bovine pituitary extract (BPE) were supplied by Clonetics (San Diego, USA). Williams' E media was supplied by Gibco (Grand Island, NY, USA) and transforming growth factor-α (TGF-α) were purchased from Collaborative research (Grand Island, NY, USA). Minoxidil was a kind gift from Pacific Co.(Seoul, Korea). Soluene was supplied from Packard (Downers Grove, Illinois, USA). [Methyl-^{3}H] thymidine (specific activity 25 Ci/mmol), [U-^{14}C] leucine (specific activity > 300mCi/mmol) and scintillation cocktail solution were from Amersham (Burkingamshire, UK). NTB-2 nuclear emulsion, D-19 solution and Kodak fixer were from Eastman Kodak(Rochester, NY, USA).

Linbro tissue culture multiwell (24 flat bottom well) was supplied by Falcone (New Jersey, USA) and counting vial (standard size) was from Costar (Meriden, Illinois, USA).

KBM is the basal nutrient component of MCDB 153 and in KGM some other important factors such as EGF, insulin, and BPE are included. Williams'E media is composed of nutrient components and vitamins and supplemented with 1% fetal calf serum, glutamine, insulin etc.

Isolation and culture of human hair follicles

Human hair follicles were isolated by microsection from human scalp obtained from facelift surgery or neurosurgical operations as Philpott.et al suggested[11]. Briefly, human scalp samples were placed in a petri dish containing PBS with antibiotics, and they were cut through the dermosubcutaneous interspace. The intact hair follicles were removed from the subcutaneous tissue by gentle pulling using small forceps. Hair roots that are of equal diameter throughout or broadened at base and are firmly encased by root sheaths are considered as anagen hairs[1], and we used only anagen hairs. Isolated hair follicles were maintained in 500μl of media with or without growth factors. The follicles were maintained free-floating in each well at 37°C in an atmosphere of 5% CO_2/95% air.

Measurement of hair follicle length

Hair follicle length was measured by using an inverted binocular microscope with eye-piece measuring graticule.

Measurement of the amount of radioisotope incorporation

The rate of DNA and protein synthesis in isolated hair follicles was measured by counting the amount of uptake of [methyl-^{3}H] thymidine and [U-^{14}C] leucine respectively. Four μCi/ml of [methyl-^{3}H] thymidine and 1μCi/ml [U-^{14}C] leucine were added to the media at a given time. After 4 hours, hair follicles were rinsed in ice-cold PBS, were put in each counting vial and solubilized with soluene. The amount of radiolabel uptake was measured by scintillation counter.

Autoradiography

The hair follicles were incubated for 6 hours in media containing 5 μCi of [methyl-^{3}H] thymidine. After incubation, the follicles were washed in ice-cold PBS and then fixed overnight in 10% buffered formalin and processed for paraffin embedding. Sections were dipped in NTB-2 nuclear track emulsion and stored in a dark container with dessicant for 3 weeks at 4°C. The emulsion was developed at room temperature for 2 min in D-19, rinsed twice in water, then fixed in Kodak fixer. Slides were counterstained with hematoxylin and eosin.

Statistics

Wilcoxon signed rank sum test was performed for the evaluation of DNA and protein synthesis,

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and hair length measured to compare whether there was any difference between the treated groups and the control groups.

RESULTS

In vitro growth behavior of isolated hair follicles

We could isolate about 100 intact hair follicles within 1 h, and selected anagen hairs by their morphology under an inverted microscope. When maintained free floating in each well of 24-well plates, they showed a significant increase in length over 48h in KGM(0.58mm/48h, Fig. 1). However, after 48h this rate of increase in length became reduced and growth was negligible. In KBM and Williams' E media, we could observe hair growth within the first 24 h (0.23 mm/24 h and 0.29mm/24h respectively) and there was no significant growth thereafter(Fig. 1).

The amount of [methyl-^{3}H] thymidine uptake was sustained at high level until 72h in KGM however the amount decreased significantly in

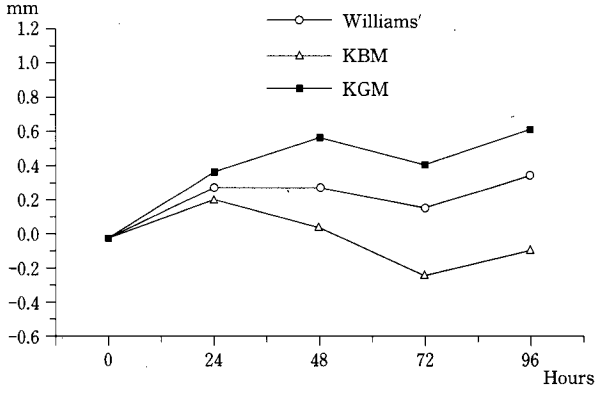

Fig. 1. Increase in hair follicle length(mm) according to culture duration and cultrue media (n=6). Isolated hair follicles sustained in vivo rate (0. 3mm/day) of hair growth in KGM over 48 hours.

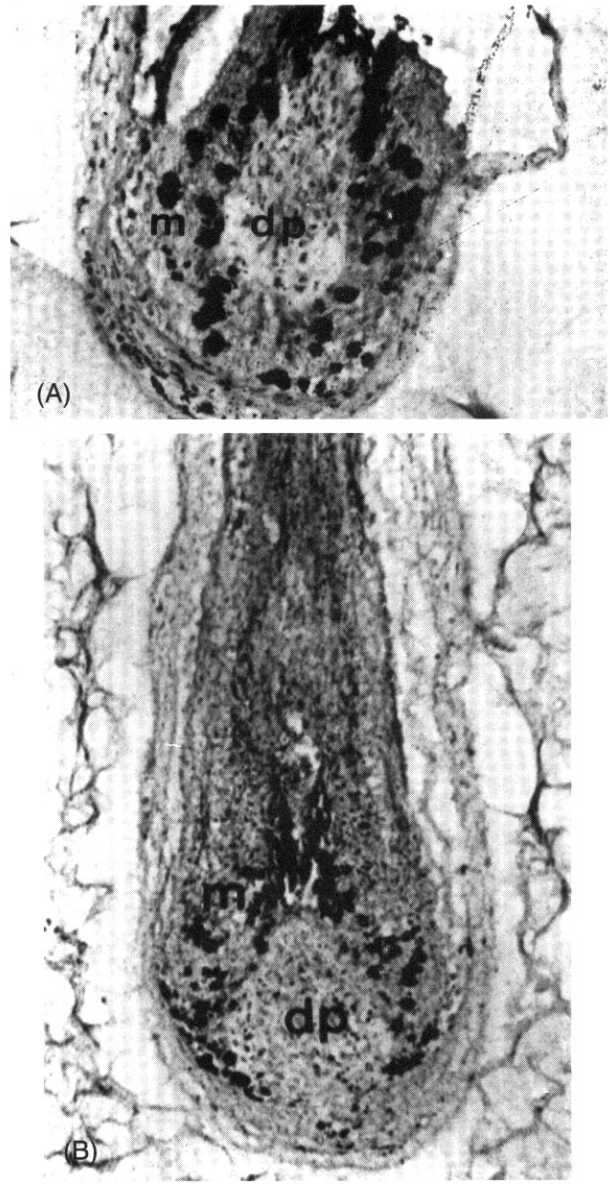

Fig. 2. Tritiated thymidine autoradiographs of isolated human hair follicles showing : (A) freshly isolated (H&E, x800) ; and (B) maintained for 48 hours in KGM (H&E, x500). The majority of thymidine uptake occurs in the matrix cells (m) of the hair follicle bulb, adjacent to the dermal papilla (dp).

Table 1. [methl-^{3}H] thymidine incorporation(DPM) according to cul-

Media	Duration	0 hr	24 hr	48 hr	72 hr
Williams' media	Mean	6794.1	708.2	494.8	398.8
	±SD	3650.1	401.7	172.9	190.0
KGM	Mean	3820.5	6260.5	3638.8	3409.4
	±SD	1397.5	4729.8	3114.6	1486.0

Table 2. [U-^{14}C] leucine incorporation(CPM) according to culture media and culture duration(n=6)

Media	Duration	0 hr	24 hr	48 hr
Williams' media	Mean	5103.2	623.1	365.6
	±SD	2843.0	307.0	180.3
KGM	Mean	3038.8	7784.0	3002.8
	±SD	1258.1	5021.1	1027.1

Table 3. The effects of several agents on isolated human hair follicles sustained in vitro over 48 hours (n=8)

Treatment	Follicle growth (mm)	[^{3}H] thymidine uptake(DPM)	[^{14}C] leucine uptake(CPM)
KBM	0.24±0.32	1081.6±486.8	1043.4±711.3
EGF(0.1ng/ml)	0.27±0.25	1486.9±708.7	1590.1±980.0
Insulin(5.0μg/ml)	1.07±0.68	2108.8±1031.1*	2566.3±1286.0*
BPE(0.4% v/v)	0.46±0.40	1122.5±803.7	1251.6±1027.7

* The mean value in insulin treated group was significantly higher than that in KBM group by Wilcoxon signed rank-sum test (P<0.05).

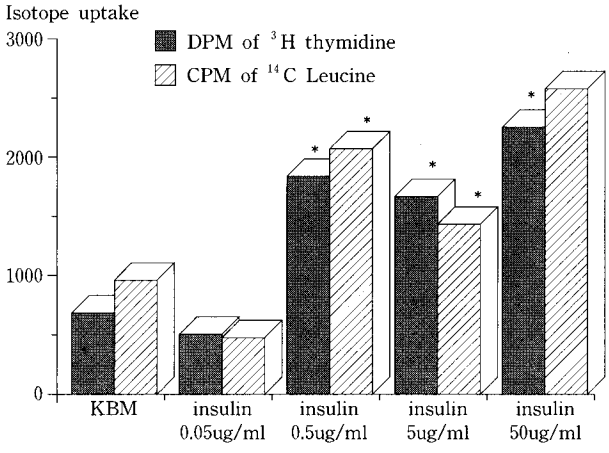

Fig. 3. The effects of insulin on isolated human hair follicles sustained in vitro over 48 hours. Asterisks indicate a significant difference from control(KBM) group. p<0.05 (n=6)

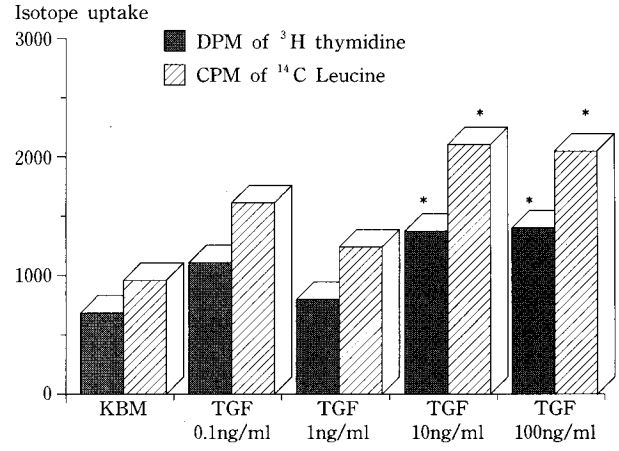

Fig. 4. The effects of TGF-alpha on isolated human hair follicles sustained in vitro over 48 hours. Asterisks indicate a significant difference from control(KBM) group, p<0.05.(n=5 or 6)

KBM and in Williams' Emedia after 24h (Table 1). The uptake pattern of [U-^{14}C] leucine was the same as [methyl-^{3}H] thymidine (Table 2).

[Methyl-^{3}H] thymidine autoradiography showed that in freshly isolated hair follicles (Fig. 2A) [methyl-^{3}H] thymidine uptake was located mainly in the matrix cells adjacent to the dermal papillae. After 48h in KGM (Fig. 2B), the uptake was still in the matrix cells, and there were no significant morphologic changes in the hair follicles.

The effects of several growth factors on hair follicle growth in vitro and the amount of isotope uptake

Hair follicles were cultured for 48hr in KBM supplemented with 0.1ng/ml EGF, or 5.0 μg/ml insulin, or 0.4% v/v BPE which are additional ingredients of KGM. The results of this study are shown in Table 3. With 5.0μg/ml insulin, the uptake of [methyl-^{3}H] thymidine and [U-^{14}C] leucine was significantly more than in the control group (p<0.05). When hair follicles were cultured at

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various concentrations of insulin, the isotope uptake was significantly increased when insulin concentrations were above 0.5μg/ml($p<0.05$). However, we did not observe a dose-response relationship(Fig. 3).

As well as insulin, we could observe that isotope uptake increased when hair follicles were cultured in KBM supplemented with TGF-α above a concentration of 10ng/ml (Fig. 4, $P<0.05$).

When hair follicles were maintained in media supplemented with EGF(0.001ng/ml, 0.01ng/ml, 0.1ng/ml, 1ng/ml) or BPE (0.004% v/v, 0.04% v/v 0.4% v/v, 4% v/v) or minoxidil (10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M), no significant changes were observed.

DISCUSSION

The rate of increase of hair follicle length is known to be about 0.35mm/day[15]. We have observed that the hair follicles isolated by microdissection and maintained free-floating showed a significant increase in length for 2 days in KGM and for 1 day in KBM or in Williams' E media. Isotope tagging studies also suggested that the increase in length may be due to DNA and protein synthesis. These changes were marked in KGM and the increase sustained over 72 or 96 hours. The majority of [methyl-^{3}H] thymidine uptake in the autoradiography study were observed mainly in the matrix cells adjacent to dermal papillae which is the same area as in vivo hair growth[11-13, 16, 17]. In short, we could demonstrate successful maintenance and growth of hair follicles over 48 hours by using KGM.

We tried to select only anagen hair follicles and wanted to use them for our experiment. However, on average, one out of six anagen hair follicles did not grow at all and their isotope tagging was negligible. This is partially explained by the fact that there are six stages in anagen hairs and some difference in protein and DNA synthesis rate according to the stage[15]. In our experiment, some follicles had more broader bases and their dermal papillae were surrounded incompletely by matrix cells. We think these follicles may be metanagen follicles and there may be some variations in growth rate and isotope tagging from follicle to follicle even within the anagen stage. To get more reliable and significant data, a method to obtain hair follicles in the same stage should be developed.

As hair follicles grow well in KGM, we have suspected that some growth factors supplemented in KGM-such as insulin(5.0μg/ml), EGF(0.1ng/ml), or BPE(0.4% v/v) might have growth promoting effect on the hairs. We have investigated these components at various concentrations and found that insulin could promote hair growth.

Insulin is one of the well-characterized growth factors affecting skin that stimulate keratinocyte proliferation[18,19]. However supra-physiologic concentrations of insulin (greater than 500ng/ml) are required to activate DNA synthesis and there is a high degree of structural homology between insulin and the IGF-I(insulin like growth factor-I) receptors, so insulin may exert its growth promoting effect by a cross-reaction with the IGF-I receptor[20]. We think it is possible that hair growth promoting effect of insulin in our experiment might be due to cross-reaction with insulin and IGF-I. To clarify this, it seems necessary to observe insulin's effect after blocking the IGF-I receptor by using a monoclonal antibody directed against IGF-I receptor.

In EGF-treated sheep, EGF induced a catagen-like stage in the hair follicles[21], however the effects of EGF on human hair growth are not well known[22]. Philpott et al[11] reported that human hair follicles maintained with 10ng/ml EGF showed a significant decrease in the rate of [methyl-^{3}H) thymidine uptake. However, recently they reported that there was a significant increase in the [methyl-^{3}H] thymidine uptake rate in rat hair follicles maintained in EGF[23]. It is possible that they observed inconsistent results because EGF receptors can be demonstrated only in outer root sheath(ORS) cells of hair follicle[19,21,24,25]. It is more probable that EGF has no effect on hair growth as shown in our experiment because EGF receptors can be demonstrated only in ORS cells of hair follicles.

The effect of TGF-α on hair follicles is not well investigated. TGF-α is antigenically related to EGF and has a sufficient structural homology to bind to a common receptor, the EGF/TGF-α receptors[19,26,27]. However, their effects are not always the same quantitativly or qualitatively[19,26]. Therefore, separate TFG-α receptor in some cell types cannot be excluded[28]. We have observed that

TGF-α at concentrations over 10ng/ml stimulated [methyl-^{3}H] thymidine and [U-^{14}C] leucine uptake in hair follicles but we could not see any effect with EGF. There are two explanations for this result. First, we have added too low a concentration of EGF compared to TGF-α and secondly, TGF-α may have some different effect on hair follicles from EGF.

Minoxidil is the only drug proved effective in treating androgenic alopecia[29], and is widely used[30,31]. However, little is known about its mechanism of action on hair follicles[29]. A unifying theme in minoxidil's action is that the drug appears to work only on suboptimal follicles-miniaturized follicles in a balding scalp, vellus body hairs and alopecia areata follicles[29]. As we have used normal human hairs, this might be one of reasons why we could not observe any effect.

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