Growth Effect of Minoxidil and Minoxidil Sulfate on Cultured Human Keratinocytes and Outer Root Sheath Cells

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The evaluating method of treatment of hair loss have been mainly depended on clinical observation because lack of appropriate in vitro models. Hair cell culture method is a new approach in that sense, which is useful not only selecting new drugs curing hair loss but also verifying pathogenesis of alopecia.

We have tried to detect the growth promoting effect of minoxidil and minoxidil sulfate on cultured normal human epidermal keratinocytes and outer root sheath hair cells. MTT was used as an evaluating method for hair growth potency after incubation of cultured cells with varying dilutions of minoxidil and minoxidil sulfate.

In high concentrations, slight toxic effect were observed in minoxidil and minoxidil sulfate. Enhancing growth effect were observed in lower concentration of minoxidil sulfate in normal keratinocytes and outer root sheath hair cells. However, such enhancing effect were observed in minoxidil only in outer root sheath cell culture. (Ann Dermatol 4:(2) 72-76, 1992)

Key Words: Hair cell culture, Minoxidil, Minoxidil sulfate, MTT, Outer root sheath, Keratinocytes

Hair has several functions such as protecting skin from environment and having important cosmetic value. Hair loss can be observed in various causes such as various diseases as well as physiologic conditions.

Hair research is emerging as a dynamic field by knowing some pathogenesis of hair diseases and being discovered new drugs. In the past the evaluating method of treatment of hair loss has been mainly depended on clinical observation because lack of appropriate in vitro models. Hair cell culture method is a new approach in that sense, which is useful not only selecting new drugs curing hair loss but also verifying pathogenesis of alopecia[1-9].

Minoxidil, an antihypertensive drug has been known that has hair growing effect as a side reaction[10]. The mechanism of minoxdil is still unknown and there have been some suggestions that other mechanisms rather than vasodilating effect are related[10]. Minoxidil sulfate has more potent effect than minoxidil which also suggests that metabolite of minoxidil is important[10, 11]. Keratinocytes cultured from normal human skin have been widely used today and there have been also some recent reports of culturing outer root sheath hair cells[2-5]. We used outer root sheath hair cell culture method because it is relatively easier than hair matrix cell culture method which is very recently being tried[12].

3-[4, 5-dimethiazol-yl]-2, 5-diphenyltetrazolium bromide test (MTT) is a recently developed simple method for the detection of the number of cultured living cells by measuring density of formazan dyes[13].

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We have tried to investigate the growth promoting effect of minoxidil and minoxidil sulfate in addition to set up an in vitro model for selecting hair growth promoting agents. We used cultured normal human epidermal keratinocytes and outer root sheath hair cells. MTT was used as an evaluating method for hair growth potency after incubation of cultured cells with varying dilutions of minoxidil and minoxidil sulfate.

MATERIALS AND METHODS

1. Cell culture

We have used the culturing method of Boyce and Ham[14] and its detailed method has been described in our previous papers[15, 16]. Briefly, foreskin has been cut into small segments and treated with type I collagenase (Whortington) for 90 minutes. Peeled epidermal sheets were treated with trypsin-EDTA (0.05%/0.02%, Flow) and after inactivation by 10% fetal calf serum dispersed cells were inoculated and grown on the cultured dishes using keratinocyte growth media (Clonetics) for about 10 days and frozen until next experiment. Hair follicles were separated by scalpel blade and after cutting out upper 1/3 portion, remaining hair follicles were treated by the same method mentioned above. We have confirmed that liberated cells are mostly from outer root sheath cell origin because the internal root sheath and matrix portion were intact when we observed the residual hair segments under the inverted microscope after enzyme treatment. Cultured outer root sheath cells were preserved in the liquid nitrogen tank until next experiment. We have used third passage cells using 96 multiwells (Linbro).

2. MTT experiment

We performed MTT and its method which was already described in our previous papers[16].

To get a standard curve 100μl cells adjusted at a density of 2,500/cm^2-40,000/cm^2 were inoculated on the multiwells. Although the concentration of minoxidil for clinical use is usually 2.5%-5%, it is usually toxic in vitro cell culture stronger than 10^{-4}M concentrations. Therefore, minoxidil and minoxidil sulfate adjusted by 10^{-4}M-10^{-8}M were added to the multiwells of keratinocytes and outer root sheath hair cells which were inoculated one day before at a density of 10,000/cm^2. Six experiment were done and 100μl media were added to each well. 48 hours after adding drugs to the wells 20μl of DMSO were added after removing media and shaken for 10 minutes. Optical density data were obtained by ELISA reader (Behring, ELISA PROCESSOR II) using 570nm.

3. Statistics

MTT data with or without adding drugs were compared by using Mann Whitney test.

RESULTS

In normal human keratinocyte culture, there was a statistically significant decrease ($P<0.05$) in minoxidil treated group in the range of 10^{-6}M, 10^{-5}M and 10^{-4}M than control group without minoxidil. In minoxidil sulfate group there were slight increase in the range of 10^{-8}M, 10^{-7}M and 10^{-5}M, statistically not significant than normal control. However, there were statistically significant decrease in high concentration of minoxidil sulfate (10^{-4}M) (Table 1, Fig. 1)

In outer root sheath cell culture, there were

Table 1. Effect of minoxidil and minoxidil sulfate on cell proliferation of cultured human keratinocyte measured by MTT.

	Concentrations of drugs (moles)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Minoxidil	101±11	94±10	81±11*	58±11*	75±12*
Minoxidil sulfate	106±14	105±20	99±10	110±15	83±7*
Control	100±19				

Values represent mean±S.D. (% of control) of optical density in 6 experiments.
* The values are statistically different from the controls ($p<0.05$).

Table 2. Effect of minoxidil and minoxidil sulfate on cell proliferation of cultured human outer root sheath cells measured by MTT.

	Concentrations of drugs (moles)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Minoxidil	117±18	112±27	116±16	109±35	141±7*
Minoxidil sulfate	141±21*	127±31*	129±29*	85±34	83±26
Control	100±16				

Values represent mean±S.D. (% of control) of optical density in 6 experiments.
* The values are statistically different from the controls ($p<0.05$).

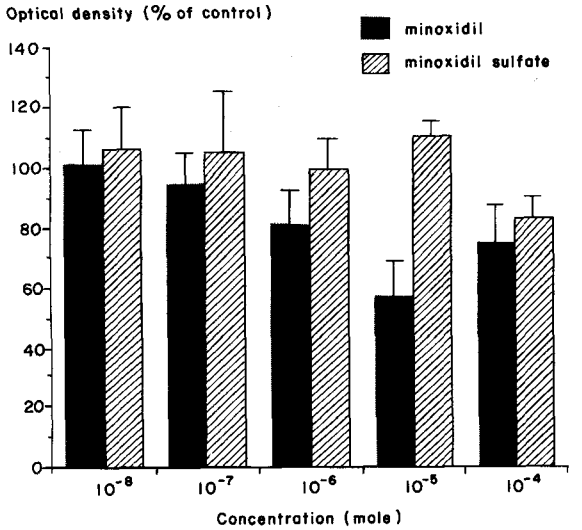

Fig. 1. Effect of minoxidil and minoxidil sulfate on cell proliferation of cultured human keratinocytes measured by MTT.

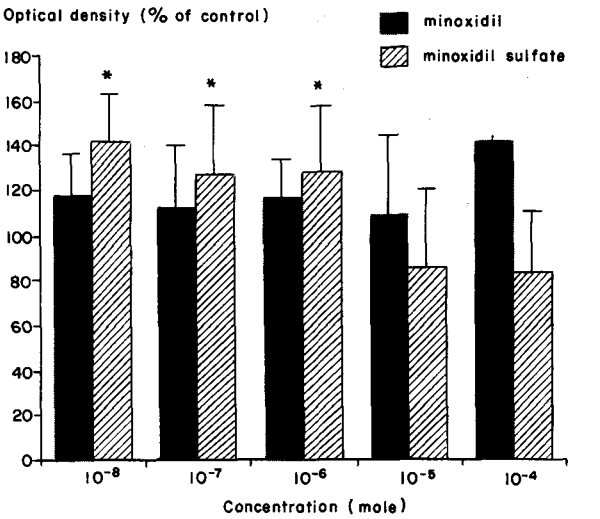

Fig. 2. Effect of minoxidil and minoxidil sulfate on cell proliferation of cultured human hair outer root sheath cells measured by MTT.
* Significantly different from control at $p<0.05$.

increase in all concentrations of minoxidil. However, statistically significant difference ($p<0.05$) was observed only in high concentration of minoxidil (10^{-4}M) than control group. In minoxidil sulfate group there were statistically significant increase in the range of 10^{-8}M, 10^{-7}M and 10^{-6}M ($p<0.05$) than control group, however slight decrease in high concentrations (10^{-5}M, 10^{-4}M), although statistically not significant. (Table 2, Fig. 2)

DISCUSSION

Human and animal models have been mainly used for the evaluation of hair growth promoting drugs. Human models have some limitations for experiment as well as animal models because the hair cycle of most animals is synchronous while human hair cycle is mosaic pattern[18].

Therefore, hair cell culture technique is a new approach for hair research which is already being tried by some authors very recently[2-10, 20]. This is not only useful for selection of new drugs of hair growth promoting effect but also verifying pathogenesis of hair diseases. And it has been developed along with the improvement of keratinocyte culture technique.

The separation and growing hair cells is not as easy as normal skin keratinocyte, and outer root hair sheath cell culture technique is frequently used today because it is relatively simple to culture. Plucked hair cell culture has been firstly tried by Westering et al[1] and it has been improved thereafter by other authors[2-8]. We firstly isolated individual hair follicle using scalpel blade as Philpott[8] tried and treated with collagenase type

I for about 100 minutes and then treated trypsin EDTA for 10 minutes, which we can obtain many dispersed outer root sheath cells. We think this method is superior to plucked hair culture method because plucked hair technique damages hair outer root sheath cells.

Alopecia areata is one of the main skin diseases producing hair loss and various treatments such as minoxidil, dinitrochlorobenzene or similar strong sensitizers, topical steroid have been used for its treatment, however not so satisfactory[21]. Minoxidil which has been known as an antihypertensive drug has hair growth promoting effect and it is being used topically in clinical practice[9]. The mechanism of action of minoxidil is still unknown, however potassium channel opener theory is suggested rather than vasodilator effect[11, 22, 23]. Minoxidil sulfate is known as more active metabolic substance than minoxidil and there are some evidences about it. This is the reason why we have chosen minoxidil and its metabolite in our experiment. Buhl et al[11] suggested modulations of matrix cell proliferation by minoxidil using mouse vibrissae follicular cell, however Dooley[25] mentioned that lower portion of outer root sheath layer is the active site of sulfation by minoxidil sulfotransferase, which suggests that there may be some difference between outer root sheath hair cells and skin nonfollicular basal keratinocytes. In our experiment there were some difference of minoxidil and minoxidil sulfate response between outer root sheath cells and normal human keratinocytes. This may support the idea that both two type of cells are different from each other in certain aspects and there is a recent report that keratin antibody pattern of outer root sheath cells is a little different from basal keratinocytes in the epidermis[12].

We have found that minoxidil and minoxidil sulfate are toxic in higher concentration than 10^{-4}M in our experiment similar to other authors[23, 24]. And in outer root sheath hair cell culture slight toxic effect has been observed even in the concentration of 10^{-5}M of minoxidil sulfate. In our experiment minoxidil sulfate enhanced outer root sheath hair cell growth in lower concentrations (10^{-8}M, 10^{-7}M, 10^{-6}M). This may be related to Buhl's findings[21] that minoxidil sulfate is 14 times more potent than minoxidil in measuring incorporation of radiolabelled cysteine in hair shafts treated with cultured rat vibrissae follicles.

We think that our experiment may be useful in checking growth promoting effects of drugs as an in vitro model of hair growth and other methods such as hair matrix cell culture and co-culture system with hair papilla cells should be developed in the future.

REFERENCES

1. Weterings PJJM, Vermorken AJM, Bloemendal H: *A method for culturing human hair follicle cells. Br J Dermatol 104:1-4, 1981.*
2. Wells J: *A simple technique for establishing cultures of epithelial cells. Br J Dermatol 107:481-482, 1982.*
3. Wells J, Sieber VK: *Morphological characteristic of cells derived from plucked human hair in vitro. Br J Dermatol 113:669-675, 1985.*
4. Arase S, Kuwana R, Sadamoto Y, Nakanishi H, Shigemi F, Takeda K: *Culture of dispersed hair follicle cells from plucked out hairs without a feeder layer. Tokushima J Exp Med 36:87-95, 1989.*
5. Imcke E, Mayer-da-Silva A, Detmar M, Tiel H, Stadler R, Orfanos CE: *Growth of human hair follicle keratinocytes in vitro. J Am Acad Dermatol 17:776-86, 1987.*
6. Messenger AG: *The culture of dermal papilla cells from human hair follicles. Br J Dermatol 110:685-689, 1984.*
7. Katsuoka K, Schell H, Hornstein OP, Deinlein E, Wessel B: *Cmparative morphological and growth kinetics studies of human hair bulb papilla cells and root sheath fibroblasts in vitro. Arch Dermatol Res 279:20-25, 1986.*
8. Philpott MP, Green MR, Kealey T: *Human hair growth in vitro. J Cell Science 97:463-471, 1990.*
9. Lee MG, Yoon MS, Lee JB et al: *A clinical study of Minoxidil therapy in alopecia. Kor J Dermatol 24:181-189, 1986. (English abstract)*
10. Buhl AE: *Minoxidil's action in hair follicles, J Invest Dermatol 96(supplement):73s-74s, 1991.*
11. Buhl AE, Waldon DJ, Baker CA, Johnson GA: *Minoxidil sulfate is the active metabolite that stimulates hair follicles. J Invest Dermatol 95:553-557, 1990.*
12. Kurata S, Itami S, Sonoda T, Takayasu S: *Culture and differentiation of human matrix cells in vitro. 18th World Congress of Dermatology, Book of Abstracts 1992. pp160.*
13. Mosmann T: *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55-63, 1983.*
14. Boyce ST, Ham RG: *Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol 83(supplement):33s-40s, 1983.*
15. Eun HC, Choi SW, Lee AI, Lee MC: *In vitro effect of several irritants using human keratinocyte culture model. Kor J Dermatol 28(4):408-418, 1990. (English abstract)*

16. Yang JM, Eun HC, Lee YS: *A study of irritant potency using patch test: skin organ culture and normal human keratinocyte culture methods. J Kor Med Assoc 34:545-556, 1991. (English abstract)*
17. Liu SC, Parsons CS: *Serial cultivation of epidermal keratinocytes from psoriatic plaques. J Invest Dermatol 81:54-61, 1983.*
18. Rook A, Dawber R: *Disease of hair and scalp. 1st ed., Blackwell Sci Co., Oxford 1982. pp95-98.*
19. Limat A, Humziker T, Boillat C, Bayrenther K, Nosen F: *Post-mitotic human dermal fibroblasts efficiently support the growth of human follicular keratinocytes. J Invest Dermatol 92:758-762, 1989.*
20. Twentyman PR, Luscombe M: *A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56:279-285, 1987.*
21. Chun DK, Eun HC, Lee YS: *Treatment of alopecia areata with diphencyprone. Kor J Dermatol 29(3):407-413, 1991. (English abstract)*
22. Buhl AE, Waldon DJ, Kawabe TT, Holland JM: *Minoxidil stimulates mouse vibrissae follicles in organ culture. J Invest Dermatol 92:315-320, 1989.*
23. Meisheri KD, Cipkus LA, Taylor CJ: *Mechanism of action of minoxidil sulfate-induced vasodilation: a role for increased K^+ permeability. J Pharm Exp Ther 245:751-759, 1988.*
24. Cohen RL, Alves MEAF, Weiss VC, West DP, Chambers DA: *Direct effect of minoxidil on epidermal cells in culture. J Invest Dermatol 82:90-92, 1984.*
25. Dooley TP, Walker CJ, Hirshey SJ, Falany CN, Diani AR: *Localization of minoxidil sulfotransferase in rat liver and the outer root sheath of anagen pelage and vibrissa follicles. J Invest Dermatol 96:65-70, 1991.*