# Effect of Minoxidil on Cultured Keratinocytes

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Minoxidil has been shown to stimulate hair growth and these studies were undertaken to determine whether the drug had a direct effect on keratinocytes. Cultures of human epidermal cells were treated with minoxidil and it was found that they survived longer than control cultures. In addition, minoxidil prolonged the time that cells could be passed after reaching confluence. The results suggest that minoxidil slows the senescence of keratinocytes, which is similar to what has been found with epidermal growth factor.

Minoxidil, a drug used for the treatment of hypertension [1,2], has the unique side effect of increasing the growth of vellus hair [3,4]. This has been observed following both oral and topical use [5–7]. The mechanism for the stimulation of hair growth is unknown, but it has been postulated that vasodilatation of skin blood vessels could be responsible for the effect. Since vasodilatation of the skin is not consistently associated with enhanced hair growth, we considered that minoxidil might stimulate hair matrix cells directly and chose to study epidermal cells, from which hair is derived. Epidermal cells can be grown and studied in tissue culture [8] but hair matrix cells cannot. We now report that minoxidil slows the senescence of cultured human epidermal cells, which is similar to the action of epidermal growth factor [9].

## MATERIALS AND METHODS

Epidermal cells were isolated from newborn human infant foreskins and cultured on mitomycin C-treated murine 3T3 cells using Dulbecco's modified Eagle's medium containing 20% fetal calf serum with 0.4 μg/ml of hydrocortisone and 50 μg/ml of gentamycin as described previously [10]. Epidermal growth factor (EGF) (Collaborative Research, Waltham, Massachusetts), cholera toxin (CT) (Sigma Chemical Co., St. Louis, Missouri), and minoxidil (kindly provided by The Upjohn Company) were dissolved directly in culture medium and these solutions, after sterilization by filtration, were added to the cells as needed for a particular experiment. These drugs were always added 3 days after plating to be certain that the number of cells that sat down were the same in control and treated culture. The concentration of minoxidil used between 3 and 7 days never exceeded 20 μg/ml, since at higher levels the morphology of the feeder cells was altered. When minoxidil was used at higher concentrations than 20 μg/ml, it was added after 7 days of growth. In order to produce a permanent record of the cultures, the plates were fixed with 10% formalin and stained with Giemsa's stain.

The isolation of proteins from cultured cells was accomplished by rinsing the plates with phosphate-buffered saline (PBS), scraping off

the cells, and homogenizing them in 0.05 M Tris, pH 7.5, with a ground glass homogenizer at 4°C. After centrifugation at 30,000 g for 30 min at 4°C the insoluble pellet was extracted sequentially with 0.1 M citrate buffer, pH 2.65, at 4°C and 0.05 M Tris buffer, pH 9.0, containing 8 M urea and 0.1 M β-mercaptoethanol (TUM) at room temperature [11].

The cell envelope precursor proteins were identified by homogenizing the cells in 0.05 M Tris, pH 7.5, with 0.001 M EDTA and 0.5 mg/ml dansyl cadaverine [12]. After centrifugation the supernatants were made 0.01 M in calcium and 0.002 M in dithiothreitol and incubated at 37°C for 3 h. The reaction mixture was then prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein was determined by the Bio-Rad method and SDS-PAGE was done by the Laemmli technique [13]. Fluorescent bands were detected by examining the gels with a Wood's lamp in the dark and the amount of protein in Coomasie-stained bands was quantitated using a scanning densitometer.

The percent of cells having a cornified envelope was determined by counting them in a hemocytometer before and after treatment with 10 mM phosphate buffer, pH 7.2, containing 2% SDS and 25 mM dithiothreitol for 15 min at 90°C [14]. This was done on cells shed into the culture medium and those trypsinized from the culture plates.

## RESULTS

Human epidermal cells cultured on a 3T3 feeder layer attached within 48 h, formed colonies which expanded by peripheral growth, and reached confluence in about 7–10 days. The doubling time was about 24 h when only hydrocortisone was present in the medium. The optimum time to pass cells was just before they reached confluence, since at later times the total number of cell generations that could be reached decreased. Cells that were maintained at confluence gradually lost the capacity to be passed and eventually peeled off the dish. The time after confluence when cells could be passed varied from 2–8 weeks in different cell lines. This time was always shorter in late-passage cells compared to early-passage ones using the same cell line.

The toxicity of minoxidil for cultured keratinocytes was tested by addition of the drug at different concentrations to confluent cultures. Cultures treated for 1 week with minoxidil at concentrations of 70 μg/ml and below showed no difference in morphology compared to normal controls. Above 100 μg/ml the cells showed a progressive change in their appearance and above 200 μg/ml the cultures separated from the plates after 18 h of treatment. Keratinocytes treated for 48 h with 70 μg/ml of minoxidil and then passed had a plating efficiency identical to control cells and reached confluence at the same time—further evidence for lack of toxicity.

The effect of minoxidil on the survival of cultures after reaching confluence was studied in different cell lines at a concentration of 70 μg/ml. In all cases the minoxidil-treated plates remained intact longer than control cultures, as shown in Fig 1.

Minoxidil treatment (70 μg/ml) was also found to prolong the time after confluence that cultured keratinocytes could be passed. The control and treated cells were plated into fresh medium without minoxidil at different times after confluence using 200,000 cells/35-mm Petri dish. With some cell lines no difference was observed between control and treated cells for up to several weeks. The earliest observable minoxidil effect was a difference in the size of the colonies in the passed cells, as shown in Fig 2. When stained cultures were examined by light microscopy, it was clearly evident that the increase in size of colonies was associated with increased number of cells per
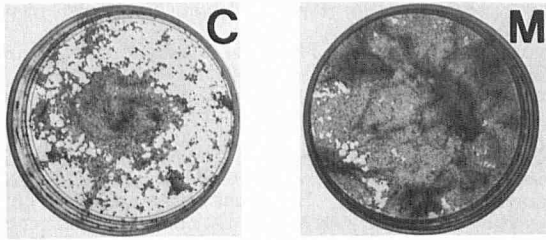
FIG 1. Appearance of control (C) and minoxidil (M) cultures 7 weeks after confluence. Many of the cells have peeled off the control plate leaving open space, while the minoxidil-treated plate is almost totally intact.
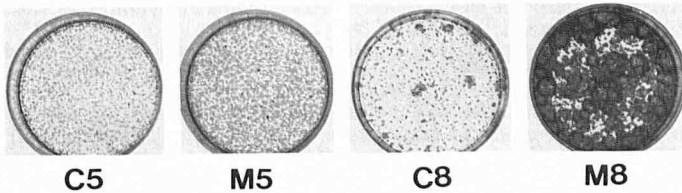


FIG 2. Appearance of cultures that were passed after 5 and 8 weeks at confluence and then allowed to grow for 1 and 2 weeks, respectively. The minoxidil (M5 and M8)-treated cultures have larger colonies than the control cultures (C5 and C8). In addition, some of the cultures passed after 5 and 8 weeks were allowed to continue growing. The 5-week minoxidil and control cultures grew to confluence but the latter took longer. The 8-week minoxidil culture grew to confluence but the control culture did not.



FIG 3. a, Appearance of cultures that were passed after 2 weeks at confluence and then allowed to grow for only 1 week. C is control, E is EGF, M is minoxidil, and E + M has both EGF and minoxidil. In addition, some of the passed cultures were allowed to continue growing. All the cultures grew to confluence with E + M the fastest, E and M next, and the control the slowest. b, Appearance of cultures that were passed after 4 weeks at confluence and then allowed to grow for only 1 week. In addition, some of the passed cultures were allowed to continue growing. C did not grow further, E + M became almost confluent, and E and M reached about 50% confluence.

TABLE I. Protein in extracts of control and minoxidil treated cultures.

| | Percent of Total Protein ± SE | | | |
| | Neutral Buffer | Citrate Buffer | TUM Buffer | Total Extractable Protein per 35mm Dish (mg ± SE) |
|---|---|---|---|---|
| Control[a] | 32 ± 3 | 28 ± 3 | 38 ± 4 | 0.76 ± .07 |
| Minoxidil[a] | 39 ± 5 | 22 ± 2 | 37 ± 3 | 0.72 ± .06 |
| Control[b] | 36 ± 4 | 24 ± 1 | 40 ± 6 | 1.42 ± .11 |
| Minoxidil[b] | 31 ± 5 | 20 ± 2 | 48 ± 5 | 1.35 ± .09 |

[a] Ten days after plating.
[b] Eighteen days after plating.

TABLE II. The percent of cells in the culture that are cornified at different times after plating. The cells were confluent at 7 days.

| Time (days) | Percent of Cells ± SE | |
| | Control | Minoxidil |
|---|---|---|
| 18 | 8.8 ± .7 | 6.0 ± .7 |
| 26 | 8.2 ± .8 | 6.6 ± .5 |
| 76 | 2.4 ± .3 | 2.0 ± .1 |
| 100 | 2.7 ± .2 | 2.4 ± .3 |

colony. This growth difference became more pronounced in passages started at later times after confluence. Eventually, the control cultures failed to grow at all even though some keratinocytes appeared to attach.

The minimal concentration of minoxidil that prolonged the survival of cultures after reaching confluence was 4–20 µg/ml. The lowest concentration tested that prolonged the time that cultured keratinocytes could be passed after reaching confluence was 20 µg/ml.

Since EGF was shown to increase the culture lifetime of epidermal cells, its ability to prolong the time after confluency that keratinocytes could be passed was investigated [9]. EGF (10 µg/ml) gave results identical to that observed with minoxidil, as shown in Fig 3. Furthermore, the EGF and minoxidil effects were additive (Fig 3). CT can also increase the culture lifetime of keratinocytes [15], but cells treated with this cAMP-elevating agent behaved similar to control cultures.

The total amounts of protein and its distribution in the neutral, citrate, and TUM buffers were similar for control and minoxidil-treated cultures, as shown in Table I. SDS-PAGE patterns of the same extract of control and treated cells showed no consistent differences. SDS-PAGE patterns of dansylated neutral soluble proteins showed fluorescent bands at molecular weights of 125,000 and 12,000. The intensity of the fluorescence relative to the amount of stained protein band at that position was similar for minoxidil-treated and control cultures. Scanning of the stained patterns with a densitometer indicated that the bands were present in similar amounts in extracts of control and treated cells.

The percent of cells that were cornified in control and minoxidil (70 µg/ml)-treated cultures was very similar, as shown in Table II and the values appeared to decrease with aging of the cultures. The value for sloughed cells varied between 7–25% and was lowest for the oldest cultures.

## DISCUSSION

These studies indicated that minoxidil has a rather characteristic effect on cultured epidermal cells. The optimal time of passing keratinocytes is just prior to confluency but cells can be passed up to se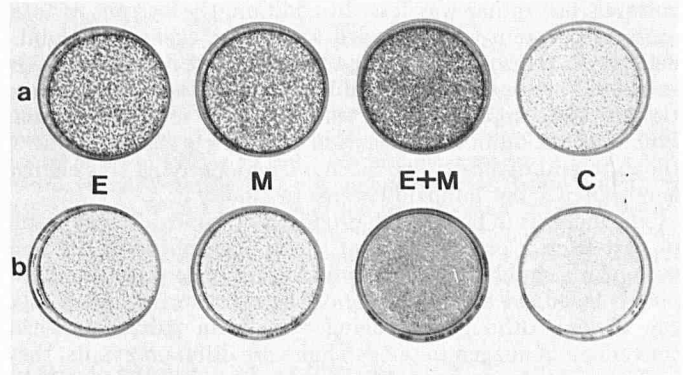veral weeks later. Minoxidil appears to lengthen the time period during which cells can be successfully passed. Examination of Fig 2 might suggest that the greater rate of colony expansion in minoxidil-treated cultures was due to more rapid cell division. However, there were some experiments done shortly after confluency in which no difference was observed between control and treated cultures. Furthermore, as shown in Fig 2, growth was not sustained in some control cultures that showed early evidence of colony formation. A better explanation for our findings is that minoxidil has an EGF-like action, as described by Rheinwald and Green [9], in that it delays senescence of cells. Indeed, in experiments in which minoxidil and EGF were compared, the cell behaviors were identical. However, the mechanism of action is likely to be different since the two drugs had an additive effect and we were working at the optimal concentration of EGF.

It has been suggested that failure of cells to divide results from terminal differentiation, which in vivo and in vitro occurs as cells traverse the various epidermal layers and is associated with formation of the cornified envelope, disulfide cross-linking of fibrous proteins and death [16,17]. These were not reflected in the biochemical changes that have been observed. The percent of cells that were cornified was not greater in our oldest

cultures, but rather was less. In addition, the percent of total extracted protein which needed a reducing agent for solubilization was the same. Thus, the failure of further cell division in old cultures is not explainable simply as terminal differentiation. Cells appear to lose their capacity for most cellular functions, including the formation of products characteristic of the differentiated cell. The mechanism underlying this change is not known, but minoxidil seems to modify it.

The increase in hair length produced by minoxidil may result in part from a prolongation of the anagen phase of the hair cycle. This could be viewed as a slowing of senescence of the matrix cells and a lengthening of the time during which cells can divide. Although the aging of cells in culture and the conversion of anagen to telogen hairs are different events, they both very likely are brought about by some type of metabolic signal. A possible mechanism for minoxidil action would be preventing release of such a signal or blocking the response to it. Further studies are necessary to determine which or whether either of these possibilities is correct.

## REFERENCES

1. Dargie HJ, Dollery CT, Daniel, J: Minoxidil in resistant hypertension. Lancet 2:515–518, 1977
2. Devine BL, Fife R, Trust PM: Minoxidil for severe hypertension after failure of other hypotensive drugs. Br Med J 2:667–669, 1977
3. Burton JL, Marshall A: Hypertrichosis due to minoxidil. Br J Dermatol 101:593–595, 1979
4. Earhart RN, Ball J, Nuss DD, Aeling JL: Minoxidil-induced hypertrichosis—treatment with calcium thioglycolate depilatory. South Med J 70:442–443, 1977
5. Zappacosta AR: Reversal of baldness in patient receiving minoxidil for hypertension. N Engl J Med 303:1480–1481, 1980
6. Seidman M, Westfried M, Maxey R, Rao TKS, Friedman EA: Reversal of male pattern baldness by minoxidil. A case report. Cutis 28:551–553, 1981
7. Weiss VC, West DP, Mueller CE: Topical minoxidil in alopecia areata (Letter to Editor). J Am Acad Dermatol 5:224–226, 1981
8. Rheinwald JG, Green H: Serial cultivation of stains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331–344, 1975
9. Rheinwald JG, Green H: Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature 265:421–424, 1977
10. Kubilus J, Macdonald MJ, Baden HP: Epidermal proteins of cultured human and bovine keratinocytes. Biochim Biophys Acta 578:484–492, 1979
11. Baden HP, Lee LD: Fibrous protein of human epidermis. J Invest Dermatol 71:148–151, 1978
12. Kubilus J, Baden HP: Isolation of two immunologically related transglutaminase substrates from cultured human keratinocytes. In Vitro 18:447–455, 1982
13. Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680–685, 1970
14. Rice RH, Green H: The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. Cell 11:417–422, 1977
15. Green H: Cyclic AMP in relation to proliferation of the epidermal cell: a new view. Cell 15:801–811, 1978
16. Green H: Terminal differentiation of cultured human epidermal cells. Cell 11:405–416, 1977
17. Steinert PM, Idler WW: Postsynthetic modifications of mammalian epidermal α-keratin. Biochemistry 18:5664–5669, 1979

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# Effects of Amino Acid Treatments on 12-*O*-Tetradecanoylphorbol-13-acetate-Induced Ornithine Decarboxylase Activity in Mouse Epidermis In Vivo and In Vitro*

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**We have compared the effects of several amino acid treatments on the induction of ornithine decarboxylase activity and the accumulation of putrescine, spermidine, and spermine by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse epidermis in vivo and in vitro. Incubation of isolated epidermal cells with mM concentrations of glycine, asparagine, glutamic acid, canavanine, arginine, and/or lysine inhibited dramatically the induction of ornithine decarboxylase activity by the tumor promoter. These remarkable inhibitory effects were concentration-dependent and additive. Arginine and its analog, canavanine, inhibited to the same degree TPA-induced ornithine decarboxylase activity, and potentiated to the same extent the inhibitory effects of glutamic acid, asparagine, and glycine on this enzyme. However, the inhibitory effects of arginine and canavanine were not additive. Similar alterations of tumor promoter-induced epidermal ornithine decarboxylase activity were observed in vivo when 62.5 μmol of the amino acids were injected i.p. 2 h before the topical application of 8.5 nmol of TPA to mouse skin. The results suggest the possibility that treatments with glycine, asparagine, glutamic acid, and arginine, the amino acids that were the most effective in inhibiting the tumor promoter-induced accumulation of polyamines in vivo, may reduce the tumor-promoting ability of TPA.**

Abbreviations:
cyclic AMP: adenosine cyclic 3′:5′-monophosphate
DMBA: 7,12-dimethylbenz[a]anthracene
Hepes: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
ODC: L-ornithine carboxy-lyase, EC 4.1.1.17
TPA: 12-*O*-tetradecanoylphorbol-13-acetate

The inhibitory effect of amino acid imbalance (as opposed to