Minoxidil: Inhibition of Proliferation of Keratinocytes In Vitro

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Previous studies have suggested that minoxidil stimulates growth of keratinocytes, possibly in a manner similar to the action of epidermal growth factor. Using both a short-term assay, thymidine incorporation, and a longer term assay, cell counting, to assess proliferative growth, we tested the activity of minoxidil in human keratinocyte cultures grown in 0.1 mM Ca^{++} . Minoxidil failed to stimulate growth in these assays. At concentrations of 5–10 μg per ml, minoxidil showed half-maximal inhibition of both EGF- and placental

extract-stimulated thymidine incorporation. Minoxidil also inhibited proliferative growth in the presence or absence of placental extract. Direct measurement of the ability of minoxidil to compete for binding to the EGF receptor indicated that minoxidil probably does not bind to the EGF receptor. Minoxidil was not toxic, as keratinocytes continued to survive and grow, although at a slower rate, in the presence of minoxidil. *J Invest Dermatol* 97:534–536, 1991

Minoxidil after systemic administration is known to stimulate growth of hair at intermittent peak concentrations in plasma of about 155 ng per ml [1] or less. The mechanism by which minoxidil stimulates growth of hair and the target cell or cells of its action are unknown. Minoxidil has been reported to enhance incorporation of thymidine in cultured mouse keratinocytes and to delay differentiation or senescence of keratinocyte cultures at concentrations of 5 μg per ml or higher, far in excess of those apparently effective in stimulating growth of hair in humans *in vivo*. We tested the action of minoxidil in an assay for thymidine incorporation that has been shown to demonstrate the action of some growth stimulators, and we also counted cells after culture in medium containing minoxidil. The results indicate that minoxidil is inhibitory to keratinocyte growth. The concentrations at which minoxidil acts on keratinocytes *in vitro* are high compared to those that appear to be effective *in vivo*. These findings indicate that the interfollicular keratinocyte is probably not a suitable cell for the study of the action of minoxidil.

MATERIALS AND METHODS

Keratinocytes from foreskins of neonates were obtained, cultured, and assayed for thymidine incorporation and for cell growth as previously described [2]. Briefly, subconfluent cells subcultivated twice in MCDB 153 medium were suspended with trypsin and plated in 24-well trays at 10,000 cells per well in 2 ml of MCDB 153 medium supplemented with amino acids, ethanolamine, phosphoethanolamine, hydrocortisone, and insulin as described ([3], basal medium) for 6 h. Placental extract [3], epidermal growth factor (EGF), or minoxidil was then added as indicated in figure legends. After 16 h ^3H -thymidine was added (1 μCi /well) for 6 h, and the cells were washed and assayed for trichloroacetic acid-precipita-

ble radioactivity [2]. Keratinocyte growth was assessed by similarly plating 50,000 cells per 35-mm petri dish and suspending and counting the cells after 7 d. Bovine pituitary extract was not present in these assays.

Binding assays, purification and iodination of EGF, and preparation of placental microsomal membranes were as previously described [4]. Minoxidil was obtained from the Upjohn Company and was dissolved in basal medium at 2 mg per ml. Two different samples of minoxidil gave similar results.

RESULTS

When keratinocytes were plated in basal MCDB 153 medium, *i.e.*, without EGF or bovine pituitary extract (BPE), in the presence of minoxidil and assayed for incorporation of thymidine, the cells showed no significant alteration in the small amounts of radioactivity incorporated in the basal medium, but control cultures containing EGF or placental extract showed substantial stimulation of growth (data not shown). In order to determine whether minoxidil might require some degree of stimulation with a growth factor to produce its effect, we plated cells in the presence of submaximally stimulating concentrations of EGF, but minoxidil inhibited thymidine incorporation in this type of experiment. We therefore tested highly stimulatory concentrations of growth factors in the presence of various concentrations of minoxidil. When keratinocytes were plated in the presence of EGF at 1 ng per ml, a concentration-dependent reduction in thymidine incorporation could be produced by addition of minoxidil (Fig 1); half-maximal reduction was produced by 5–10 μg minoxidil per ml, far below the ranges thought to stimulate growth in previous studies. In order to determine whether the effect of minoxidil was specific for EGF, we examined the ability of minoxidil to affect the stimulation of thymidine incorporation by a different growth factor known not to act through the EGF receptor. When keratinocytes were stimulated with placental extract, thymidine incorporation was reduced by minoxidil with the same effectiveness (Fig 2).

Although the assay for thymidine incorporation used here has been shown to correlate with growth, it was possible that minoxidil was altering thymidine metabolism, possibly by reducing the use of the salvage pathway and shifting growth to dependency on *de novo* synthesis [5] of thymidine and not affecting growth. We therefore measured the effect of minoxidil on keratinocyte growth directly by

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Abbreviations:

BPE: bovine pituitary extract

EGF: epidermal growth factor

Figure 1. Inhibition by minoxidil of EGF-stimulated thymidine incorporation. Cultured keratinocytes were incubated in basal medium with EGF (1 ng/ml) with or without minoxidil at indicated concentrations for 16 h, labeled with ³H-thymidine for 6 h, and counted as in *Materials and Methods*. Results are mean ± range of duplicate wells.

counting cells after 7 d culture in the presence of minoxidil. Cell number was reduced either in the presence or in the absence of a growth factor, placental extract, although the reduction was more severe in the absence of the extract, suggesting that the growth stimulator partially inhibited the action of minoxidil (Fig 3). The concentration of minoxidil required for half maximal effect was similar, 5–10 μg/ml, with or without placental extract, identical to the half maximally inhibitory concentration in the thymidine assay.

Cells cultured in the presence of minoxidil were not killed, as they continued to grow, although at a rate slower in proportion to the concentration of minoxidil. Also, if minoxidil was removed, the cells recovered within 24 h and were able to incorporate thymidine as effectively as control cells. Furthermore, cells treated with minoxidil were not released from the dish and continued to exclude trypan blue as effectively as control cultures (data not shown). Minoxidil appeared therefore to impair the response to growth factors without altering viability, indicating a lack of toxicity.

As the half-maximal effect of minoxidil is the same for two different growth factors, only one of which acts on the EGF receptor, it is unlikely that minoxidil exerts any effect on the EGF receptor. In order to test directly whether minoxidil affects binding of EGF, however, we incubated ¹²⁵I-labeled EGF with placental membranes, known to contain a high concentration of EGF receptor [4], in the

Figure 2. Inhibition by minoxidil of placental extract-stimulated thymidine incorporation. Keratinocytes were assayed as in Fig 1 in basal medium in the presence of placental extract (200 μg/ml). Results are mean ± range of duplicate wells.

Figure 3. Inhibition of growth of keratinocytes by minoxidil. Cells were plated in basal medium with placental extract at 200 μg/ml with or without various concentrations of minoxidil and suspended with trypsin and counted at 7 d. O, placental extract; ●, control.

presence or absence of minoxidil. Although nanomolar concentrations of EGF competed for binding of labeled EGF to the membrane preparation, minoxidil in excess of concentrations inhibitory to growth of keratinocytes was without effect (Fig 4).

DISCUSSION

Two previous studies have described stimulation of interfollicular epidermal keratinocytes by minoxidil. Baden and Kubilus [6] found that cultured human keratinocytes could be subcultivated for longer periods when minoxidil at a concentration of 70 μg per ml (0.3 mM) was present in the culture medium. They suggested that minoxidil had an EGF-like effect and might be delaying in vitro senescence by increasing the number of population doublings of the cells. This study did not actually demonstrate that culturing the cells in the presence of minoxidil increased population doublings, but the inference was made because the cultures could be subcultivated for longer times after confluence. An alternative possibility, in light of the finding that minoxidil can inhibit keratinocyte growth, is that the cells cultured in the presence of minoxidil are retarded with respect to both growth and differentiation; because fewer cells were terminally differentiated in minoxidil-treated cultures, plating efficiency may have been higher. This would be possible if minoxidil were able to retard growth without concomitantly increasing differentiation. The concentrations of minoxidil used by Baden and Kubilus were in excess of those required to substantially retard growth in our cultures.

Cohen et al [7] described an increase in thymidine incorporation associated with a second round of DNA synthesis, beginning 7 d after mouse keratinocytes were initiated into culture. An increase in cell number or quantity of DNA in the cultures was not demonstrated, and the percentage of labeled nuclei was not determined, so the possibility cannot be excluded that minoxidil altered thymidine metabolism, possibly by shifting DNA synthesis in favor of the salvage pathway or by reducing the catabolism of thymidine [8], increasing the amount of incorporated label without stimulating proliferative growth.

Our study was conducted in medium containing 0.1 mM Ca⁺⁺, whereas the earlier studies used stratified cultures containing physiologic concentrations of Ca⁺⁺. This raises the possibility that minoxidil may behave differently under different culture conditions. In addition, the measurements used to determine the effects of minoxidil were all different in our study and the two previous ones. Nevertheless, agents that have been found to stimulate proliferative growth either have been effective under conditions of high and low Ca⁺⁺ (e.g., EGF, hydrocortisone) or have been ineffective, but not inhibitory, under other conditions (e.g., cholera toxin, which is

Figure 4. Competition for binding to the EGF receptor by EGF or minoxidil. Placenta membranes were incubated in triplicate with or without EGF or minoxidil at indicated concentrations for 30 min at 24°C, and ¹²⁵I-labeled EGF was added and incubated for 40 min. The membranes were rapidly diluted, filtered, washed, and counted as described [4]. ○, EGF; ●, minoxidil.

without effect at reduced Ca^{++} concentrations), suggesting that an agent showing inhibition of growth under our conditions is unlikely to be stimulatory under other conditions. Furthermore, the experiments reported here show directly, by cell counts and by assay of thymidine incorporation under conditions in which the effects of growth factors are readily demonstrable [2], that minoxidil at low concentrations compared to those used in other *in vitro* studies is inhibitory to growth. Cell counts were not used in previous studies. Our findings with minoxidil are in concert with findings in other types of cells *in vitro*. Minoxidil has also been shown to be inhibitory to the growth of endothelial cells [9] and fibroblasts [10] and to suppress phytohemagglutinin-stimulated DNA synthesis in lymphocytes [11]. It inhibits the production of prostacyclin in several types of cells [12,13] and inhibits the activity of lysyl hydroxylase activity in fibroblasts [10].

Because minoxidil has an effect on the hair follicle rather than the epidermis in general, it is not surprising that minoxidil fails to stimulate growth of interfollicular keratinocytes. The more likely cellular targets are the keratinocytes of the follicular matrix and the mesenchymal cells of the dermal papilla, and these may be better approached experimentally in an organ culture system using hair follicles. Furthermore, it is well documented that systemic administration of EGF to animals produces aberrant growth and retardation or arrest in growth of hair [14], so it is possible that EGF, even at low concentrations, may not stimulate hair growth. Alternatively, EGF may stimulate follicle growth in physiologic concentrations but inhibit at unphysiologically high concentrations. Recent studies indicate that minoxidil has effects on hair follicles *in vitro* [15], and this model may be more useful for the investigation of the effects of minoxidil on target tissues than isolated cells. Studies of isolated cells from hair follicles may be more difficult to interpret than those of organ cultures using hair follicles; for example, Katsuoka et al [16] found that although EGF stimulated growth of isolated hair bulb papilla cells and root sheath fibroblasts *in vitro*, minoxidil had no effect.

Systemically administered minoxidil stimulates growth of hair at peak plasma concentrations of 100–200 ng per ml, whereas 5–70 μg per ml, or 25–350 times higher concentrations, are required *in vitro* in the various studies cited above. This suggests that minoxidil may be potent in affecting hair growth at much lower concentrations than the growth-inhibitory ones described here if the appropriate *in vitro* system is found. Although recent studies of hair follicles *in vitro* show effects of minoxidil and are promising, the discrepancy between the effective concentrations *in vivo* and *in vitro* remains substantial.

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