

## ENHANCED IN VITRO HAIR GROWTH AT THE AIR-LIQUID INTERFACE: MINOXIDIL PRESERVES THE ROOT SHEATH IN CULTURED WHISKER FOLLICLES

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### SUMMARY

Inasmuch as hair follicles are difficult to maintain in culture, the study of hair biology using cultured hair follicles has met with only limited success. In our attempts to solve the problem of follicle degeneration, we cultured follicles at the air-surface interface on a modified collagen matrix (Gelfoam). In follicles cultured at the air-surface or submerged, we examined follicular morphology, hair shaft growth, sulfotransferase levels, cysteine incorporation, an expression of a tissue inhibitor of metalloproteinase (TIMP), and ultra-high sulfur keratin (UHSK). 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 fibroblasts, Full Thickness Skin®, or 5- $\mu$ m polycarbonate filter also supported hair growth, whereas Gelfilm, GF-A glass filter, filter paper, or 1- $\mu$ m polycarbonate filter did not. The UHSK expression was significantly higher in the air-liquid interface cultures compared to the submerged culture. Several potassium channel openers, including minoxidil, a minoxidil analog, and the pinacidil analog (P-1075), all stimulated significant cysteine incorporation in follicles. Minoxidil and its analog specifically preserved the follicular root sheath, in contrast to P-1075 which did not, indicating a difference in the two drug types. The preservation of the root sheath was measured by increased TIMP expression and sulfotransferase activity and indicates that the root sheath is a target tissue for minoxidil. Our results show that follicles cultured at the air-liquid interface maintain a better morphology and produced greater hair growth than follicles cultured on tissue culture plastic.

*Key words:* hair growth; minoxidil; rogaïne; pinacidil; hair culture; P-1075; potassium channel opener.

### INTRODUCTION

Although follicles are intact organs containing both the dermal and epidermal tissue held in the proper tertiary structure, culture conditions that support hair growth have been difficult to ascertain. Many different techniques have been tried. In 1949, Hardy (9) reported follicle development and growth from embryonic skin cultured in hanging drop or watch glass culture. Examples of other techniques that have been tried include culturing follicles between two cover slides clamped on each side of a rubber ring (Rose chamber) (24), suspending follicles on polycarbonate supports (22) mixed into a collagen matrix (13), or cultured inside rolled glass tubes (18). Recently a free-floating method that maintained human hair shaft growth for 10 days (19) was reported. The various culture methods have been shown to support in vitro hair growth but the hair growth is limited to short culture periods, and in each case follicles eventually degenerated and failed to maintain hair growth in culture.

Our efforts to maintain hair growth in vitro have previously demonstrated hair formation from mouse whisker explants grown under very simple culture conditions (22). These cultured follicles required minoxidil or another hair growth stimulator to maintain an organized follicular structure that retained some level of proliferation and differentiation of the matrix epithelium. Even with the arti-

ficial growth stimulators, these follicles could only retain hair growth for 4 days.

We attempted to mimic the natural skin environment by implanting follicles in a collagen sponge positioned at the air-liquid interface. Gelfoam, was an ideal material because it is sterile, natural collagen and pliable enough to allow easy imbedding of follicles. We hypothesized that by culturing follicles in more aerobic conditions would help support follicular metabolism because studies indicated that follicles use aerobic glycolysis (1,20). Imbedded follicles proved otherwise, and these follicles grew no better than follicles cultured in the original submerged method. Fortunately, a few follicles that were laid on the surface of the Gelfoam as a control, grew and demonstrated an enhanced hair growth.

### MATERIALS AND METHODS

*Culture methods.* Procedures for whisker dissection have been previously reported (4). Briefly, whisker pads were surgically removed from mouse pups and the follicles dissected from the tissue. The follicles were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum (GIBCO). The air-liquid interface method requires a growth substrate such as Gelfoam (Upjohn, Kalamazoo, MI) positioned at the medium surface. The substrate was supported by a wire mesh to position it at the air-liquid interface. This culture method was referred to as the raised follicle method. Gelfoam was first

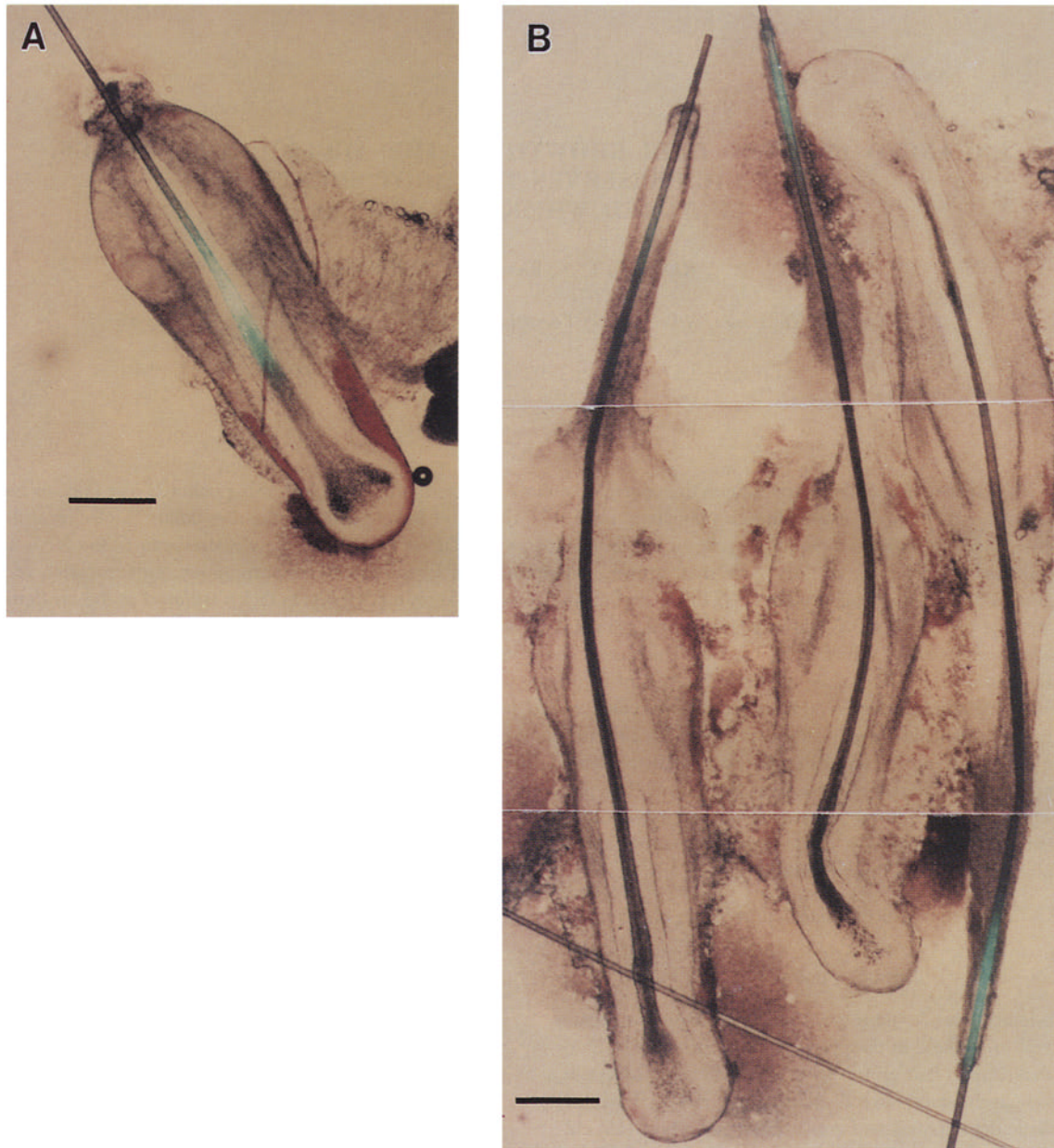


FIG. 1. Before and after photographs of follicles cultured in the raised culture method without any drug treatment. *A*, freshly dissected follicle at the start of culture; hair shaft has a fluorescent band that marked the point of active keratinization at the time of injection of the label. *B*, follicle cultured at the air-interface after 5 days; increase in the hair shaft is demonstrated by the movement of the fluorescent band. Bar = 0.10 mm.

rehydrated in medium and then placed on the mesh. Medium was added to the dish to bring the level up to the bottom of the substrate. The substrate remained saturated with medium and the follicles are placed on top of the substrate. The follicles were exposed to the air but nourished by the culture medium from below. The other substrates tested were Gelfilm (Upjohn, Kalamazoo, MI), a nylon mesh seeded with fibroblasts (Marrow-Tech, La Jolla, CA), Full Thickness Skin (Organogenesis, Cambridge, MA), GF-A glass filter (Whatman, Maidstone, England), filter paper (Schleicher & Schuell, Keene, NH), 5- $\mu$ m polycarbonate membrane (Nuclepore, Pleasanton, CA), and 1  $\mu$ m polycarbonate filter (Nuclepore, Pleasanton, CA).

The original submerged follicle method, where the follicles are cultured on plastic at the bottom of the culture dish, has been previously described

(4). In this procedure, follicles are dissected and transferred to a culture dish. The follicles are cultured on the bottom of a 24-well tissue culture plate (Corning, Corning, NY) in 2 ml of medium. For simplicity, this culture method was referred to as the submerged follicle method.

**Chloramphenicol acetyltransferase (CAT) assay.** The CAT assay is a quick and sensitive method to determine hair specific differentiation of the matrix epithelium in our transgenic mice (21,25); the procedure has been previously described in detail (26,27). These transgenic mice express the CAT gene under the control of an ultrahigh sulfur keratin-related protein (UHSK) gene promoter, from an insertion from a pKER-CAT construct (17). Briefly, follicles were transferred to 0.5-ml microcentrifuge tubes and disrupted by ultrasonication for 4 s at 80% rate in 200  $\mu$ l tris buffer 250

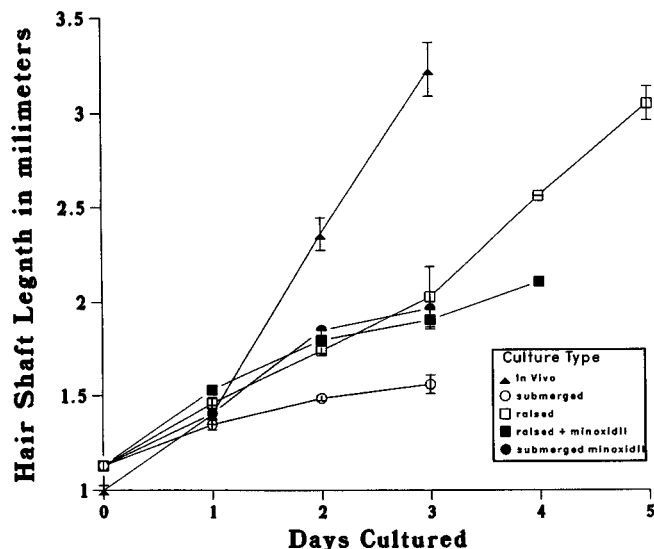


FIG. 2. Comparison of the in vivo hair growth to the in vitro growth rates for the raised and submerged culture methods. In vivo growth rate was 0.76 mm/day. In vitro hair growth was 0.38 mm/day at the air-interface. Growth was only 0.14 mm/day in the submerged cultures. Minoxidil-treated follicles had growth rates of 0.23 mm/day for the submerged culture method and 0.29 mm/day at the air-interface.

mM, pH 7.8. To the tube, 20  $\mu$ l of 4 mM acetyl coenzyme A, and 0.5  $\mu$ l of  $1 \times 10^{-3}$  M BoDipi-chloramphenicol (Molecular Probes Inc., Eugene, OR) was added. The sample was incubated and the reaction was stopped and extracted by ethyl acetate. The dried sample was placed in the autosampler and resuspended in 300  $\mu$ l high pressure liquid chromatography (HPLC) buffer just before the injection. Individual sample runs were stored and processed using the Spectra Physics 4270-310 Computing integrator and down loaded into a WIN 386 PC computer (26).

**Photographic documentation.** To permit quantitation of the new hair formation, 0.1 ml of a 1% fluorescein solution in saline was injected i.p. 1 h before the start of the whisker dissections into the pup's peritoneal cavity. The fluorescein branded the follicle hair shaft with a fluorescent band at the location of the hair keratinization zone (16). This mark on the hair shaft, visible by fluorescent emission, permitted any new hair formation to be directly appraised as length from repeated photographs. Each day a different set of follicles were removed from culture and placed on glass slides in a drop of water and only sealed with a cover slip. The follicles were then photographed through an Olympus BH-2 with the Olympus camera system, which employed a double exposure of visible and then fluorescent light. From this photograph the length of the hair shaft was measured by a dial micrometer (Starret Co., Athol, MA). The hair shaft length was gauged as the length from the bulb end of the follicle to the farthest end of the fluorescent band. The growth rates for all the groups were averaged to show length increase in millimeters per day for the duration of each culture by using the slope of the line for mean daily growth for each day.

**Cysteine incorporation assay.** To assess follicle growth,  $^{35}$ S-cysteine was added to the culture at the start of Day 2. The label was added to give a final concentration of 5  $\mu$ Ci/ml. At the end of the 3-day culture the follicles were removed and the incorporated cysteine was counted by liquid scintillation. The follicles were processed to remove the outer connective tissue so that only the hair shaft and the root sheath remained. The drugs tested were minoxidil at  $5 \times 10^{-3}$  M, minoxidil analog U-86414 at  $5 \times 10^{-4}$  M, and pinacidil analog P-1075 at  $5 \times 10^{-5}$  M. Minoxidil and P-1075 are potassium channel openers and each have been shown to stimulate hair growth in other models (3,8,10).

**Sulfotransferase assay.** Sulfotransferase activity was determined in the outer root sheath of cultured mouse whisker follicles to assess effects on this follicular tissue. The follicles were cultured under the two different methods with or without 1 mM minoxidil. To achieve detectable enzyme levels, the

follicles were dissected and 30 follicles were pooled for each group. Freshly dissected follicles were used as a positive control. The assay total, thermal stable minoxidil sulfotransferase activity (thermal labile by difference) utilizing  $^{35}$ S-PAPS, has been described (11).

**Tissue inhibitor of metalloproteinase.** Tissue inhibitor of metalloproteinase (TIMP) expression was used to assess culture and drug treatment because anagen follicles express TIMP to the inner root sheath (12). TIMP transgenic animals were TIMP-lacZ transgenic mice generated from a CD-1 parental strain (7). Briefly, follicles were fixed for 15 min in 0.5% glutaraldehyde in phosphate buffered saline (PBS) at room temperature. After the fixation period, the tissue was washed 3 times in PBS and permeabilized in PBS, 2 mM  $MgCl_2$ , 0.01% sodium deoxycholate, and 0.02% NP40 for 10 min. The tissue was stained in X-gal (5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside) solution (5) overnight at 37°C. After staining, the tissue was rinsed in PBS, dehydrated in a graded alcohol series, cleared in xylene, and embedded in paraffin. Sections were cut on a rotary microtome at 6  $\mu$ m and counterstained with eosin. As controls, non-transgenic mouse tissues were examined and found to be routinely negative for  $\beta$ -gal activity.

All experiments were analyzed using analysis of variance with a one-sided *t* test. A significance level of  $P < 0.05$  was used for these experiments. All experiments were replicated and support results of the presented data.

## RESULTS

The effectiveness of culturing follicles at the air interface could be seen in the growth of the hair shaft in culture by the movement of a fluorescent band in photographs of follicles (Fig. 1). The freshly dissected follicle at the start of culture has a fluorescent band positioned near the follicular apex. After 5 days, the follicles cultured at the air interface grew new shaft and the band had moved away from the follicular bulb as the hair grew. This method clearly demonstrated that new hair growth was formed in culture and that follicular morphology remained intact with a clear distinction of the proliferative epithelium and dermal papilla. These follicles retained an anagen-like morphology and still did not show follicular degeneration or tissue necrosis in culture after 10 days without any drug treatment.

The comparison of hair growth rates for both raised and submerged culture methods revealed changes in the hair growth dependent on culture method and minoxidil treatment (Fig. 2). In vivo growth represented the normal hair shaft growth rate of 0.76 mm/day. The fastest in vitro hair growth rate was 0.38 mm/day of non-minoxidil-treated follicles using the raised culture method. The slowest shaft growth was that of the nondrug-treated follicles in the submerged culture at only 0.14 mm/day. Minoxidil-treated follicles had growth rates of 0.23 mm/day for the submerged culture method and 0.29 mm/day for the raised follicle method. Hair growth rates of the nontreated submerged follicles were significantly less than the growth in all other groups by Day 3. Minoxidil treatment caused a two-fold increase in the hair growth over the submerged follicle culture independent of culture method.

By Day 3, all the minoxidil-treated follicles showed a decline in the hair growth, whereas the non-minoxidil-treated follicles in the raised culture continue to maintain their original growth rate. Minoxidil was not replaced in these cultures and may be limited after 3 days. Interestingly, follicles cultured with minoxidil showed a reduction in the size of the follicular bulb, which correlated with the termination of hair growth around Day 6. If cultured without minoxidil, the follicles continue to retain an anagenlike morphology. The longest culture times investigated were 10 days. The number of follicles that continued to grow well declined with the age of the culture.

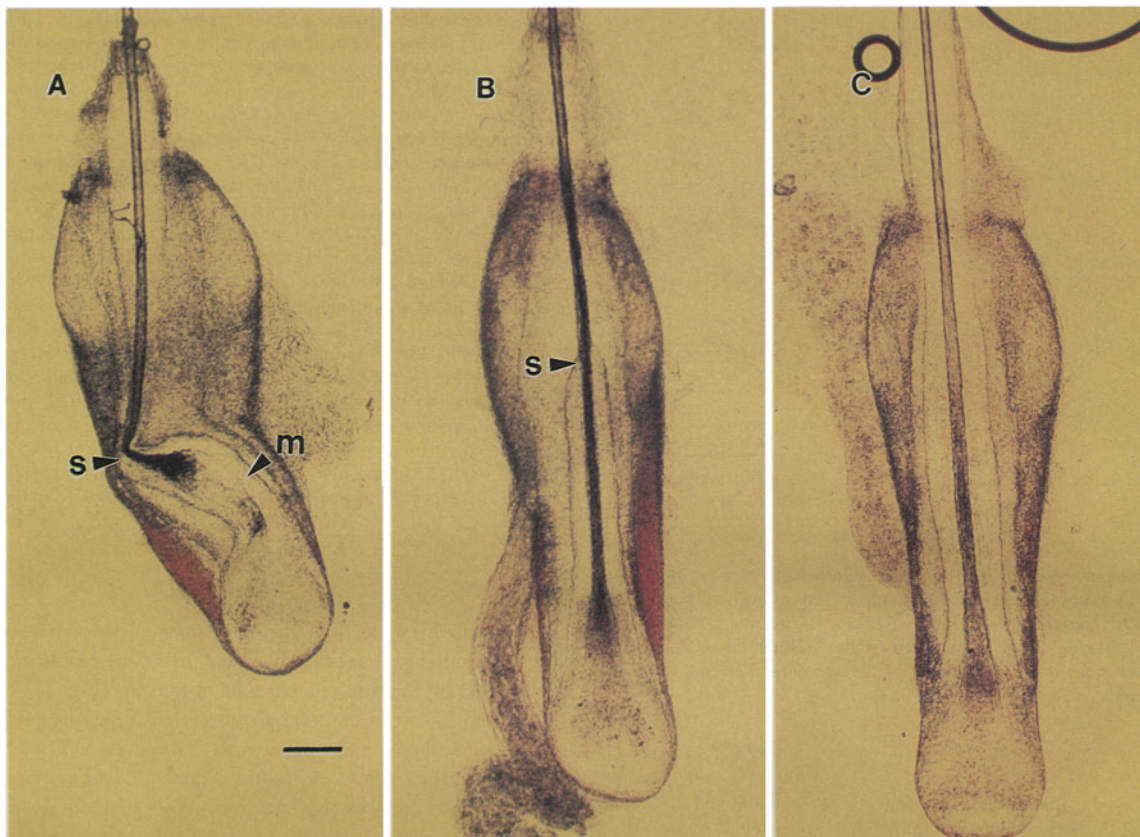


FIG. 3. Cultured follicles after 3 days at the air-interface or submerged showed morphologic differences. A, follicles grown in a submerged culture retain little follicular structure; there is little preservation of epithelial matrix (*m*) or root sheath (*s*) and the hair shaft was usually bent and malformed. B, raised, air-interface culture method preserved an excellent hair shaft and follicle structure with an anagen-like morphology. The root sheath (*s*) disappears at the site of the keratogenous zone when the culture was initiated. Follicles regenerate the root sheath in culture, from the cells of the follicular bulb. C, Minoxidil treatment produced identical follicular morphology for submerged or air-interface culture methods. Minoxidil preserved the follicular root sheath. Minoxidil specifically protect these cells independent of the culture method. Bar = 0.10 mm for all photographs.

Follicle morphology in the raised and submerged non-treated follicles was found to be drastically different (Fig. 3). Follicles grown for three days in a submerged culture showed major abnormalities. There was no maintenance or preservation of epithelial matrix or root sheath and the hair shaft was usually bent and malformed. In contrast the follicles cultured at the air-interface showed excellent hair shaft structure and follicle structural preservation. These follicles retained an anagen like morphology without additional drug treatment.

Minoxidil-treated follicles had a morphology much like that found in the raised follicle cultures except that minoxidil preserved the follicular root sheath along the entire hair shaft. The raised culture method only preserved the root sheath at the bulb end of the follicle. Minoxidil preserved those cells of the root sheath that were near the keratogenous zone at the initiation of the culture. Whatever the cause of the morphologic change, minoxidil and the minoxidil analog were specifically able to protect these cells independent of the culture method. The P-1075-treated follicles retained a good follicular morphology in the raised follicle culture method but the P-1075 did not preserve the root sheath in the follicles (data not shown).

Follicles treated with minoxidil, a potent minoxidil analog, or the

P-1075 increased the cysteine incorporation into cultured follicles (Fig. 4). Cysteine incorporation in follicles at the air interface was greater than cysteine incorporation in follicles grown submerged. The raised culture condition elevated cysteine incorporation, but this effect was independent of drug treatment because both treated and nontreated follicles had the same relative increase. Drug treatment significantly increased cysteine uptake over the nontreated control for both the submerged follicle and raised follicle method. This significant increase in cysteine was an effect due to the drug treatment and culture independent. The P-1075 produced the greatest cysteine incorporation at a dose 100-fold less than minoxidil.

Follicles cultured on Gelfoam with minoxidil show distinct TIMP staining along the hair shaft in the inner root sheath (Fig. 5). A similar effect was found when grown on the bottom of the culture dish in the presence of minoxidil; without minoxidil much less TIMP staining was seen in the follicle. The follicle maintains a straight morphology when grown on Gelfoam but less TIMP stain is present. Follicles grown on the bottom of the culture plate without minoxidil show a distorted morphology in addition to the light TIMP stain. Furthermore, the TIMP stain seems to end abruptly at the bend in the shaft.

We found that culture method and minoxidil treatment affected

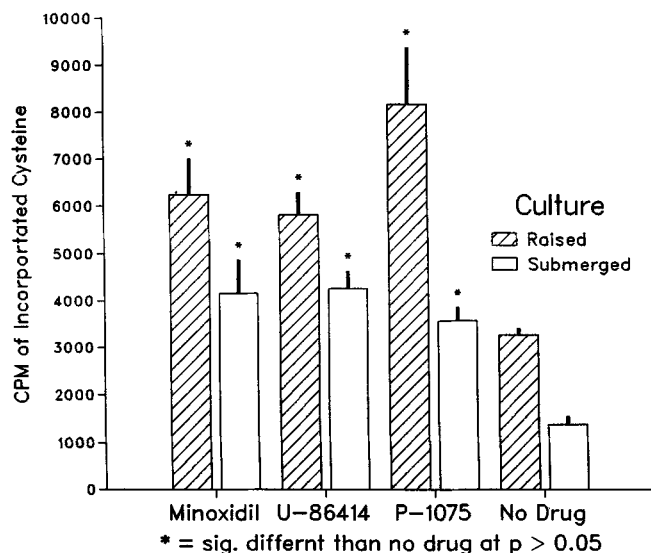


FIG. 4. Incorporation of cysteine into follicles treated with a potassium channel agonist. Air-interface cultures resulted in follicles with greater cysteine incorporation than follicles grown submerged. All the tested compounds were shown to significantly increase cysteine incorporation in both culture methods.

the sulfotransferase activity. Assay results (Table 1) demonstrated that the fresh follicles had 8 to 20 times more total enzyme activity than cultured follicles. Follicles in the raised cultures with minoxidil

had the most total activity, whereas submerged cultures with minoxidil had the least. Thermal stable enzyme activity was greatest in the raised cultures with minoxidil, even greater than the fresh follicles. Submerged cultures with minoxidil had no detectable thermal stable enzyme activity. In addition, it is interesting that the thermal stable activity of the follicles in the raised culture with minoxidil was greater than in the fresh follicles. This may represent selective preservation of the thermal stable form of the enzyme.

Comparison of follicular morphology and UHSK of follicles cultured at the air interface on different substrates (summarized in Table 2) revealed that material such as filter paper and glass fiber did not support follicle growth. Other inert materials such as the polycarbonate filter also did not work with the small pore size (1  $\mu\text{m}$ ) but growth did occur with the larger pore of 5  $\mu\text{m}$ . Gelfilm, made of the same material as Gelfoam but without the open lattice structure, did not support the follicle growth as well as Gelfoam. The substrate that showed better results than Gelfoam was the dermal fibroblast-impregnated nylon mesh. This substrate supported the best follicle growth and produced the best UHSK expression.

#### DISCUSSION

The results of all these experiments show clearly that follicles cultured at the air interface maintain a functional hair biology better than the previously used submerged culture method. We found that the raised follicle culture supported an anagenlike morphology and continued hair growth for over 10 days, but this hair growth was still less than that measured in vivo. In our comparisons of the culture methods we found differences in growth potential of the follicles, the

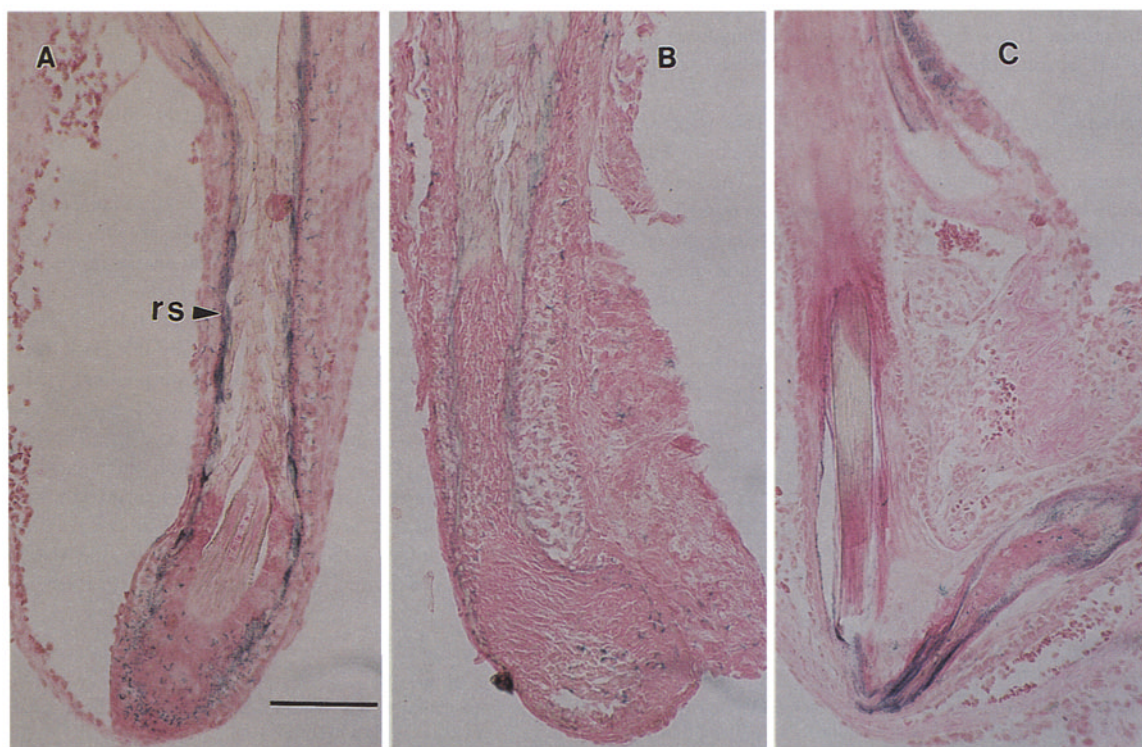


FIG. 5. Tissue inhibitor of metalloproteinase staining in cultured follicles. A, follicles cultured with minoxidil show TIMP staining in the inner root sheath (rs) in both the raised and submerged cultures. B, follicle maintains a straight morphology when grown on Gelfoam but less TIMP stain is present. C, follicles grown submerged without minoxidil show a distorted morphology in addition to the light TIMP stain. Bar = 0.10 mm for all photographs.

amount of cysteine incorporation, and changes in the keratin gene expression. The culture method dramatically affects the follicle hair growth in the non-minoxidil-treated follicles. The raised follicle culture doubled the hair growth rate and more than doubled the time follicles could be maintained *in vitro*.

A substantial difference has been consistently observed in the morphology of follicles cultured with or without minoxidil in the submerged cultures (4). However, in the raised follicle method the follicular bulb and proliferative epithelium are preserved independent of the treatment of minoxidil. The non-drug-treated follicles cultured at the air interface survived longer and produced more hair than drug-treated follicles. However, minoxidil and the minoxidil analog clearly have a protective effect on the root sheath.

The preservation of the root sheath resulted in higher levels of sulfotransferase activity and TIMP expressed in the minoxidil-treated follicles and even to a greater extent in the raised follicle culture when treated with minoxidil. Preservation of the root sheath was of interest because the sulfotransferase enzyme has been localized to the root sheath in follicles (6) and it is the sulfotransferase that converts minoxidil to the active metabolite minoxidil sulfate (2). It is unclear if this preservation of the root sheath is related to minoxidil's mechanism of action for hair growth because the upper root sheath is not directly involved in hair formation (14,23). One might suggest that if the preservative action of minoxidil on the root sheath epithelium was nonspecific enough to also protect other hair epithelium, then this may explain the preservation of hair growth.

The pinacidil analog P-1075 was different from minoxidil or the minoxidil analog in that P-1075 does not preserve the follicular root sheath. Inasmuch as all these compounds can effect hair growth, this preservation effect on the follicular root sheath by minoxidil and its analog U-86414 demonstrates a difference between these two types of compounds. This may indicate that the compounds, both described as K<sup>+</sup> channel openers, do not have identical effects on follicular tissues.

The increased cysteine incorporation and UHSK expression found in follicles cultured at the air interface indicated a higher protein anabolism than in the submerged cultures. The increased cysteine found with minoxidil treatment could be due to the preservation of the follicular root sheath, because actual shaft growth is not significantly different between minoxidil or non-minoxidil-treated follicles in the raised culture method. Of the substrates

TABLE 1

## SULFOTRANSFERASE ACTIVITY IN CULTURED FOLLICLES

Culture Method	Production of Minoxidil Sulfate pmol/mg Protein			
	Total	Stable	Labile	Stable, %
Fresh	16.9	0.4	16.4	4%
Raised	2.1	0.2	1.9	12%
Submerged	2.4	0.2	2.2	10%
Raised + minoxidil	3.1	1.4	1.7	45%
Submerged + minoxidil	0.8	0	1.8	0%

<sup>a</sup> Fresh follicles had 8 to 20 times as much total activity as the other follicles. Of the cultured follicles, the raised culture with minoxidil had the most total activity and the submerged culture with minoxidil had the least total activity. For the stable enzyme, the raised culture, with minoxidil, had the most activity, even more than the fresh follicles and submerged culture, with minoxidil, had no activity.

TABLE 2

SUBSTRATE EFFECT ON MORPHOLOGY AND KERATIN<sup>a</sup>

Substrates	Morphology	UHSK
Plastic (submerged)	—	—
Gelfoam	+++	+++
Gelfilm	+	—
Organogenesis skin	+++	++
Dermis mesh	+++	+++
5 $\mu$ m polycarbonate	+	+++
1 $\mu$ m polycarbonate	—	—
GF-A glass filter	—	—
Filter paper	—	—

<sup>a</sup> Growth substrate on follicle morphology and UHSK gene expression in the raised follicle culture method. Morphology was rated for hair shaft growth and preservation of internal follicular structure.

tested, those that support quick attachment supported better follicle morphology and keratin gene expression. The nylon mesh with fibroblasts was the best substrate, but the material is not readily available. Gelfoam gave very similar results and has the advantages of being sterile and commercially available.

In contrast to our results, culturing human hair follicles on Gelfoam at the air-surface interface does not enhance hair growth. This is evident by comparing a study using Gelfoam at the air-surface interface with another study done using submerged follicles on plastic (6,15). Submerged follicles on plastic yielded greater shaft growth rates and longer durations of hair growth. Unfortunately, direct comparisons using human follicles in the submerged vs. air-surface conditions with and without Gelfoam have not been done; thus the differences between these studies may be due to factors other than culture conditions or the growth substrate.

Quick attachment of the follicles to the growth substrate may facilitate follicle growth by allowing the follicle to minimize the trauma induced by the dissection. In contrast to the Philpott method of follicle dissection in which follicles are sheared off to facilitate dissection (18), we found that any direct trauma, even a small cut to the follicle, resulted in diminished hair growth. The cause of this cessation of growth due to injury remains unknown.

The maintenance of hair growth and other associated growth and differentiation end-points demonstrated that follicles cultured at the air-surface interface are well suited for the study of hair growth biology. Maintenance of the anagen morphology for longer durations renders the air-liquid culture model especially suitable for studies of growth regulation and hair cycling. The follicular root sheath, being a target tissue for minoxidil, may be useful for studies to better understand the drugs effect on hair growth. Future studies to understand the mechanisms involved in preservation of follicle growth, by culture at the air-surface interface, may shed light on this perplexing problem of maintaining hair growth in organ culture.

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