Interleukin-1β–Induced Inhibition of Hair Growth *In Vitro* Is Mediated by Cyclic AMP

Rolf Hoffmann, Wolfgang Eicheler, Elke Wenzel, and Rudolf Happle
Department of Dermatology, Philipp University, Marburg, Germany

Interleukin (IL)-1 has been shown to be a potent inhibitor of hair growth *in vitro*. We hypothesized that this cytokine might be a decisive factor causing hair loss during the lymphocytic attack in alopecia areata. Neither the intracellular pathways involved in hair growth inhibition mediated by IL-1β nor the signal transduction processes within hair follicles in general are known. We therefore investigated the intracellular signals involved in human hair growth *in vitro*. Hair follicles were isolated from scalp biopsies by microdissection, and hair growth was measured daily by image analysis. We assessed intracellular signal transducing elements using specific inhibitors or activators either alone or in combination with IL-1β. The calcium ionophore A 23187 induced a rapid and complete arrest of hair growth, and phorbol-12-myristate-13-acetate (PMA), genistein, or IL-1β decreased hair growth by approximately 60%–80%. IL-1β–elicited hair growth arrest was not antagonized by calphostin C, a specific inhibitor of protein kinase C. In contrast, coincubation of IL-1β with pertussis toxin or H 1004 neutralized the effect of IL-1β, and dibutyryl-cAMP and cholera toxin, an activator of adenylate cyclase, inhibited hair growth. These data suggest that cAMP acts as a second messenger for IL-1β–induced inhibition of hair growth. Moreover, our data indicate that *in vitro* hair growth is dependent on intracellular Ca^{2+} levels and activation of tyrosine kinase as well as protein kinase C. We were unable to detect a signal transducing element responsible for enhanced hair growth *in vitro*. *Key words: human/alopecia areata/cytokines/adenylate cyclase.* *J Invest Dermatol 108:40–42, 1997*

Factors that influence the growth of the anagen follicle or initiate the switch to a catagen growth pattern have not been discovered, but there is increasing evidence that cytokines and growth factors are involved in these processes (Philpott *et al*, 1990). In line with this concept, interleukin (IL)-1 has been shown to be a potent inhibitor of hair follicle growth *in vitro* (Harmon and Nevis, 1993). Moreover, transgenic mice that overexpress interleukin (IL)-1α in basal keratinocytes characteristically show patchy hair loss.[1] Because IL-1β was found to be aberrantly expressed in active alopecia areata (Hoffmann *et al*, 1994), we have hypothesized that IL-1β might lead to hair loss during the lymphocytic attack in alopecia areata (Hoffmann and Happle, 1995). If this hypothesis holds true, an effective treatment of alopecia areata should antagonize lesional IL-1β or antagonize IL-1β–elicited intrafollicular pathways leading to hair loss. The intrafollicular signal transduction pathways involved in IL-1β–mediated arrest of hair growth are unknown, but it is possible to pharmacologically manipulate the signal transduction processes to identify the pathways that are: (i) responsible for IL-1β–mediated inhibition of hair growth, (ii) required for augmented or prolonged hair growth, and (iii) involved in changes in morphology of hair follicles *in vitro*. With this approach we wanted to elucidate signal transduction pathways in single human hair follicles after incubation with IL-1β and transduction elements that are generally important for hair growth *in vitro*.

Manuscript received December 29, 1995; revised September 3, 1996; accepted for publication September 30, 1996.

Reprint requests to: Dr. med. Rolf Hoffmann, Zentrum für Hautkrankheiten, Philipps-Universität, Deutschhausstrasse 9, 35033 Marburg, Germany.

Abbreviations: dbcAMP, dibutyryl-cAMP.

[1] Groves RW, Williams IR, Sarkar S, Nakamura K, Kupper TS: Analysis of epidermal IL-1 family members *in vivo* using transgenic mouse models. *J Invest Dermatol* 102:556, 1994 (abstr.).

MATERIALS AND METHODS

Chemicals L-Glutamine, insulin, transferrin, sodium selenite, Fungizone, penicillin, streptomycin, and phorbol ester (PMA) were from Sigma (Deisenhofen, Germany). Recombinant human IL-1β was bought from Laboserv (Giessen, Germany). Williams E medium was purchased from GIBCO (Heidelberg, Germany). Calphostin C, melittin, U 73122, propanolol, pertussis toxin, cholera toxin, dibutyryl-cAMP (dbcAMP), methylene blue, dibutyryl-cGMP, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), calcium ionophore A 23187, KT-5823, C2-ceramide, sphingiosine-1-phosphate, and genistein were obtained from BIOMOL (Plymouth Meeting, PA).

Isolation, Cultivation, and Maintenance of Single Human Hair Follicles After informed consent had been obtained, excisional scalp biopsies were taken under local anesthesia from 20 healthy volunteers. Intact, viable anagen hair follicles were isolated by microdissection as previously described (Philpott *et al*, 1989, 1992). In brief, scalp specimens were placed in supplemented Williams E medium in petri dishes. Under a stereo-dissecting microscope, a scalpel blade was used to remove the epidermis and upper parts of the corium. The intact hair follicles were isolated from the subcutaneous fat with a watchmaker's forceps by gently gripping the outer root sheath of the hair follicle with subsequent gentle traction. The isolated single hair follicles did not show visible damage and were maintained in 500 μl of Williams E medium containing 2 mM L-glutamine, insulin (10 μg per ml), transferrin (10 μg per ml), sodium selenite (10 ng per ml), Fungizone (2.5 μg per ml), and penicillin/streptomycin (100 U per ml:100 μg per ml). Follicles were incubated in a humidified atmosphere of 5% CO_2/95% air and were kept up to 6 d in

0022-202X/97/$10.50

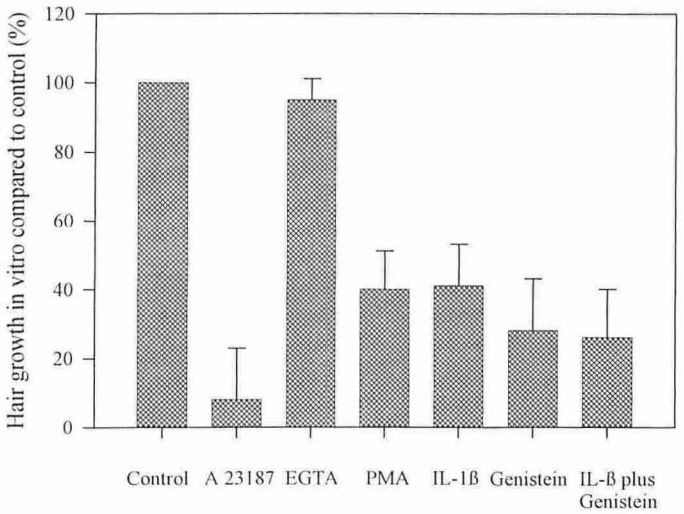

Figure 1. Inhibition of human hair growth *in vitro*. Six single hair follicles were incubated with a given substance for 6 d. The length of the hair follicles was measured by digital image analysis, and the growth rate was compared to control hair follicles (100%). Immediate inhibition of hair growth was elicited by the calcium ionophore A 23187 (2 μM). Phorbol ester (PMA, 1 μM), genistein (50 μM), or IL-1β (100 ng per ml) inhibited hair growth almost to the same degree, by approximately 60%, after 6 d in culture. IL-1β–elicited arrest of hair was augmented by coincubation with genistein. EGTA (1 μM) had no effect on hair growth. Experiments were performed at least in triplicate. Values are means ± SD (*bars*).

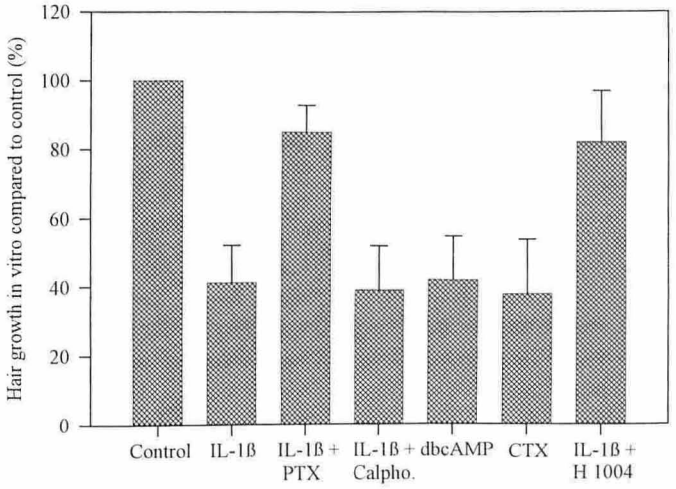

Figure 2. IL-1β–elicited hair growth inhibition is mediated by cAMP. Incubation of hair follicles with IL-1β (100 ng per ml) led to consistent inhibition of hair growth, by approximately 60%, compared to unstimulated hair follicles. This growth inhibition was nearly completely antagonized by addition of pertussis toxin (100 ng per ml, PTX) or H 1004 (2 μM). When used alone, the cAMP analog dbcAMP (500 μM) or the adenylate cyclase activator cholera toxin (1 μg per ml, CTX) inhibited hair growth *in vitro* to the same degree as IL-1β. Coincubation of IL-1β with calphostin C (0.1 μM, Calpho.) did not influence inhibition of hair growth. Experiments were performed at least in triplicate. Values are means ± SD (*bars*).

culture. At the beginning of the experiments all hair follicles were nearly equal in length. The culture medium was changed every 3 d, and viability of hair follicles was assessed by light microscopy and daily measurement of hair growth.

Analysis of Signal Transduction Pathways in Single Human Hair Follicles Hair follicles were kept in 24-multiwell plates at one follicle per well in 500 μl of supplemented Williams E medium. Every experiment for a given substance included six single hair follicles from one donor and was performed at least in triplicate. Daily measurements of hair growth were performed by the use of an inverted microscope connected to a video camera and a digital imaging processing unit (Lucia M, Nikon, Düsseldorf, Germany). Our experimental objective was (i) to antagonize or (ii) to simulate the IL-1β effect by the use of either inhibitors or activators of signal-transducing elements within hair follicles. Inhibitors were used together with IL-1β; activators were used alone. One hour after isolation of the hair follicles, the substances were added to the culture dishes. The experiments were stopped after 6 d.

RESULTS

Inhibition of Human Hair Growth *in Vitro* Hair growth was immediately inhibited by calcium ionophore A 23187 (2 μM) whereas EGTA (1 μM) had no effect on hair growth. PMA (1 μM) and IL-1β (10 ng per ml) inhibited hair growth not immediately, but slowly and nearly equally, by approximately 60%, after 6 d in culture. Genistein (50 μM) inhibited hair growth by approximately 80% and augmented the IL-1β–elicited hair growth inhibition. **(Fig 1)**. Treatment of hair follicles with propanolol (1 μM), KT 5823 (0.5 μM), dibutyryl-cGMP (100 μM), C2-ceramide (0.5 μM), sphingiosine-1-phosphate (1 μM), methylene blue (1 μM), U 73122 (1 μM), or melittin (1 μg per ml) alone or in combination with IL-1β had no effect on hair growth *in vitro* (data not shown). The morphology of the hair follicles was not altered by any treatment (data not shown).

IL-1β–Elicited Hair Growth Inhibition Is Mediated by cAMP The IL-1β–elicited hair growth arrest was nearly completely antagonized by addition of pertussis toxin (100 ng per ml) or H 1004 (2 μM) into the culture dishes but was not influenced by addition of calphostin C (0.1 μM). When used alone, the cAMP analog, dbcAMP (500 μM) or the adenylate cyclase activator cholera toxin (1 μg per ml) inhibited hair growth as did IL-1β **(Fig 2)**.

DISCUSSION

The exact mechanism of IL-1 signaling in hair follicles is unknown. Because of its pleiotropic effects, IL-1 utilizes different signaling pathways (Foxwell *et al*, 1992) and has been shown to activate, e.g., phospholipase A2 (Chang *et al*, 1986), protein kinase C (PKC)-, cAMP-activated protein kinase-, and cGMP-activated protein kinase-dependent signal transduction pathways in various cells (Rosoff *et al*, 1988; Beasley *et al*, 1991), (Leszcynski *et al*, 1994). Furthermore, ceramide and sphingomyelin, which are linked to phospholipid hydrolysis, have been identified as mediators of some of the effects of IL-1β (Ballou *et al*, 1992; Mathias *et al*, 1993).

As reported for other cells (Shirikawa *et al*, 1988; Onozaki *et al*, 1992), our data suggest that IL-1β–elicited hair growth inhibition is mediated by cAMP. This conclusion is based on the following pieces of evidence: (i) IL-1–elicited hair growth inhibition is similar to hair growth inhibition evoked by the cAMP analog dbcAMP, (ii) nontoxic concentrations of the adenylate cyclase inhibitor, pertussis toxin, inhibited the IL-1β–induced arrest of hair growth, (iii) nontoxic concentrations of the cAMP-dependent protein kinase A inhibitor H 1004 antagonized the IL-1β–induced arrest of hair growth, (iv) a cAMP-elevating agent (cholera toxin) could substitute for IL-1 in inhibiting hair growth. Due to the limited numbers of hair follicles present in small scalp biopsies obtained from healthy volunteers, we were unable to measure cAMP levels or adenylate cyclase activity in isolated hair follicles.

It has been hypothesized that IL-1 might be a crucial mediator in the pathophysiology of various diseases accompanied by hair loss and a regulator of the cyclical hair growth pattern (Harmon and Nevis, 1993). IL-1α and IL-1β share common receptors, and the biologic functions are indistinguishable. The mechanisms within the hair follicle that are evoked by IL-1 and the IL-1-responsive

hair follicle cells are not known, but an inhibition of matrix cell differentiation and proliferation has been assumed (Harmon and Nevis, 1994). We performed experiments with hair follicles that were isolated by a technique similar to that described by previous authors (Westgate *et al*, 1993), which reported a continuous matrix cell division and formation of keratinized hair shafts. It has been shown that IL-1 is able to inhibit hair loss induced by cytosine arabinoside (Jimenez *et al*, 1991). This cytostatic drug interferes with normal DNA synthesis and is therefore selectively toxic to highly proliferating cells. In this situation IL-1 may inhibit matrix cell differentiation and this, in turn, protects the hair follicle from the toxic effects mediated by the chemotherapeutic agent.

We were unable to confirm previous studies showing that increased PKC activity augments the IL-1β–induced arrest of hair growth (Harmon and Nevis, 1993). In our hands, IL-1β–elicited inhibition of hair growth was independent of PKC activity, since addition of a selective inhibitor of PKC, calphostin C, to IL-1β–stimulated hair follicles was without effect on hair growth. Nevertheless, our data suggest that PKC activity is important to maintain hair growth *in vitro*, because PMA stimulation led to a significant growth inhibition. PMA is known to activate PKC transiently in several cells, but prolonged exposure of cells to PMA downregulates the activity of PKC. Hence, our data suggest that decreased PKC activity is responsible for inhibition of hair growth, rather then PMA-elicited PKC activation as previously suggested (Harmon and Nevis, 1993). Moreover, our data indicate that hair growth *in vitro* is dependent on intracellular Ca^{2+} levels and tyrosine kinase because A 23187 treatment resulted in an immediate and complete arrest of hair growth, whereas genistein inhibited hair growth to a lesser degree. In our system, the inhibition of other possible signal-transducing elements, such as phospholipase A2, guanylate cyclase, tyrosine kinase, or phospholipase C/D, did not antagonize the IL-1β–mediated arrest of hair growth.

Our results provide evidence that constitutive activation of several intracellular signal-transducing elements maintains the growth of single human hair follicles *in vitro* and that inhibition of hair growth by IL-1β is mediated by cAMP. Modulation of these factors *in vivo* may be a future tool for the manipulation of hair growth.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ho 1598/1-2), Bonn, Germany, and from the Kempkes Stiftung, Marburg, Germany.

REFERENCES

Ballou LR, Chao CP, Holness MA, Barker SC, Raghow R. Interleukin-1-mediated PGE2 production and sphingomyelin metabolism. *J Biol Chem* 267:20044–20049, 1992

Beasley D, Schwartz JH, Brenner BM. Interleukin-1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J Clin Invest* 87:602–608, 1991

Chang J, Gilman SC, Lewis AJ. Interleukin-1 activates phospholipase A2 in rabbit chondrocytes: A possible signal for IL-1 action. *J Immunol* 136:1283–1287, 1986

Foxwell BMJ, Barrett K, Feldmann M: Cytokine receptors: structure and signal transduction. *Clin Exp Immunol* 90:161–169, 1992

Harmon CS, Nevis TD: IL-1α inhibits human hair follicle growth and hair fibre production in whole-organ cultures. *Lymphokine Cytokine Res* 12:197–203, 1993

Harmon CS, Nevis TD: Hair fibre production by human hair follicles in whole organ culture *Br J Dermatol* 130:415–423, 1994

Hoffmann R, Wenzel E, Huth A, Henninger HP, Steen P, Schäufele M, Happle R: Cytokine mRNA levels in *alopecia areata* before and after treatment with the contact allergen diphenylcyclopropenone. *J Invest Dermatol* 103:530–533, 1994

Hoffmann R, Happle R: Does interleukin-1 induce hair loss *in vivo*? *Dermatology* 191:273–275, 1995

Jimenez JJ, Wong GH, Yunis AA: Interleukin-1 protects from cytosine arabinoside-induced alopecia in the rat model. *FASEB J* 5:2456–2458, 1991

Leszczynski D, Josephs MD, Foegh ML: IL-1β-stimulated leucocyte-endothelial adhesion is regulated in part, by the cyclic-GMP-dependent signal transduction pathway. *Scand J Immunol* 39:551–556, 1994

Mathias S, Younes A, Kan CC, Joseph C, Orlow I, Kolesnick RN: Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1β. *Science* 259:519–522, 1993

Onozaki K, Kato K, Inui S, Takuwa Y, Akiyama Y: Cyclic AMP mimics IL-1 action in augmenting the differentiation of a mouse myeloid leukemic cell line. *J Pharmacobiodyn* 15:491–500, 1992

Philpott MP, Green MR, Kealey T: Human hair growth *in vitro*. *J Cell Sci* 97:463–471, 1990

Philpott MP, Green MR, Kealey T: Rat hair growth *in vitro*. *Br J Dermatol* 127:600–607, 1992

Philpott MP, Green MR, Kealey T: Studies on the biochemistry and morphology of freshly isolated and maintained rat hair follicles. *J Cell Sci* 93:409–418, 1989

Rosoff PM, Savage N, Dinarello CA. Interleukin-1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. *Cell* 54:73–81, 1988

Shirakawa F, Yamashita U, Chedid M, Mizel SB: Cyclic AMP-an intracellular second messenger for interleukin-1. *Proc Natl Acad Sci USA* 85:8201–8205, 1988

Westgate GE, Gibson WT, Kealey T, Philpott MP: Prolonged maintenance of human hair follicles *in vitro* in a serum-free medium. *Br J Dermatol* 129:372–379, 1993