Acta Derm Venereol (Stockh) 1996; 76: 348–352

Immunohistochemical Studies of Proinflammatory Cytokines and Their Receptors in Hair Follicles of Normal Human Skin

AHMED ABDELAZIZ AHMED[1], KLAS NORDLIND[2], MARIANNE SCHULTZBERG[3], JUST BRAKENHOFF[4], JESPER BRISTULF[5], DANIELA NOVICK[6], STEFAN B. SVENSON[7], MOHAMMED AZIZI[8] and STURE LIDÉN[1]

[1]Department of Dermatology, Karolinska Hospital, Stockholm, [2]Department of Dermatology, Academic Hospital, Uppsala, [3]Department of Clinical Neuroscience and Family Medicine, Geriatric Medicine, Karolinska Institute, Novum, KFC, Huddinge, [4]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands, [5]Department of Neurochemistry and Neurotoxicology, Stockholm University, Stockholm, [6]Department of Molecular Genetics and Virology, Weizmann Institute, Rehovot, Israel, [7]The Swedish Institute for Infectious Disease Control, Stockholm and [8]Department of Neurosurgery, Academic Hospital, Uppsala, Sweden

Immunoreactivity to interleukin-1 alpha, interleukin-1 beta, interleukin-6 and tumour necrosis factor-alpha and their receptors, as well as the endogenous interleukin-1 receptor antagonist, was investigated in hair follicles in paraffin-embedded normal human skin.

Interleukin-1 beta- and tumour necrosis factor-alpha-like immunoreactivities were found in the inner root sheath layer of hair follicles, at the suprapapillary level. Interleukin-1 receptor-like immunoreactivity was also found in this layer, while there was a variable immunoreactivity to the interleukin-1 receptor antagonist. In the outer root sheath there was a weak to moderate staining for the four cytokines, in addition to intense staining for their receptors and a weak staining for the antagonist. The fibrous root sheath had a moderate immunoreactivity for interleukin-1 alpha and interleukin-6.

The distribution patterns suggest that these cytokines, particularly interleukin-1 beta and tumour necrosis factor-alpha, may have a protective role in hair formation, while all the investigated proinflammatory cytokines may have a role in the differentiation process. ***Key words: interleukin; tumour necrosis factor.***

(Accepted February 21, 1996.)

Acta Derm Venereol (Stockh) 1996; 76: 348–352.

Dr. A. A Ahmed, Department of Dermatology, Karolinska Hospital, S-171 76 Stockholm, Sweden.

Hair follicles have a cyclic growth, where periods of complex differentiation and high mitotic activity of the matrix are interspersed with apparent mitotic quiescence. An interplay of complex processes mediated by soluble factors and cell-cell as well as cell-matrix interactions regulate the proliferation and differentiation. There is some evidence that cytokines are involved in these processes.

In rat anagen hair follicles the expression of mRNA for transforming growth factor (TGF) beta, insulin-like growth factor (IGF) I, IGF II, nerve growth factor (NGF) beta and interleukin (IL)-1 alpha, respectively, was demonstrated by polymerase chain reaction (PCR) analysis (1). By using in situ hybridization technique mRNA for IL-1 alpha, IL-1 beta and tumour necrosis factor (TNF)-alpha were localized to hair follicular epithelium (2). Immunohistochemical analysis showed cytoplasmic staining for TGF beta in cells of the dermal papilla, the outer and inner root sheath and the bulb matrix (1).

Furthermore, IL-1 beta has been shown to offer protection from cytarabine-induced alopecia in rats, and a potential usefulness for cytokines in the prevention of chemotherapy-induced alopecia was suggested (3, 4). In contrast, IL-1 alpha was reported to inhibit growth of human hair follicles and the production of hair fibres in a whole-organ culture system (5).

IL-1, IL-6 and TNF-alpha are multifunctional proinflammatory cytokines, which have been shown to be released from keratinocytes (6–10). IL-1 has its own natural antagonist, interleukin-1 receptor antagonist (IL-1RA), which is produced in the same cells as IL-1 alpha (see 11). In skin, the balance between IL-1RA and IL-1 alpha may control terminal differentiation as well as inflammation.

In the present investigation the occurrence of IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha as well as their receptors, and the IL-1RA was studied in hair follicles in paraformaldehyde-fixed paraffin-embedded normal human skin.

MATERIALS AND METHODS

Skin biopsy specimens

Biopsy specimens were obtained from normal scalp (1 male and 2 females, age 34–56 years) and axillary skin (6 males and 4 females, age 24–63 years). The specimens were fixed in isotonic 4% buffered paraformaldehyde for 12 h, embedded in paraffin wax and stored until used.

Reagents

Polyclonal antisera to IL-1 alpha, IL-1 beta and TNF-alpha were raised in rabbits and diluted in phosphate-buffered saline (PBS), containing 0.25% bovine serum albumin (BSA) and 0.01% NaN_3, pH 7.4. These antisera were used at dilutions of 1:400 (IL-1 alpha), 1:400 and 1:800 (IL-1 beta), 1:200 and 1:400 (TNF-alpha) (for details see 12, 13). IL-1RA was identified using the IgG fraction of an antiserum diluted 1:50 and 1:100, raised against recombinant human (rh) IL-1RA (a generous gift from Synergen, Boulder, CO, USA), or mouse monoclonal antibodies (I4 and I5, at a concentration of 10 μg/ml), a kind gift by Dr. A. E Berger (Pharmacia&Upjohn, Kalamazoo, MI, USA). A rabbit antiserum against human type I IL-1 (receptor)R was a generous gift from Dr. J. E. Sims (Immunex, Seattle, USA), and used at a dilution of 1:100. IL-6R was visualized using rabbit polyclonal antiserum (diluted 1:100) or a mouse monoclonal antibody (34–4) at a concentration of 130 μg/ml (14). A mouse monoclonal IL-6 antibody (CLB. IL-6/7 (15)), directed against human IL-6, was used at a concentration of 20 μg/ml. Mouse monoclonal antibodies htr-9 (15 μg/ml) directed against the p55 TNF-R and utr-1 (20 μg/ml) directed against the p75 TNF-R were kindly provided by Dr. M. Brockhaus (Hoffman-La Roche, Basel, Switzerland) (16). Peroxidase-conjugated rabbit anti-mouse IgG and swine anti-rabbit Ig were purchased from Dako (Copenhagen, Denmark) and used at a dilution of 1:15. 3-Amino-9-ethylcarbazole was obtained from Sigma (St. Louis, MO, USA).

Recombinant murine (rm) IL-1 alpha with a specific activity of 1.3×10^8 U/mg (115–270, Ro 24–4666) was a generous gift from Dr. P.L. Lomedico (Hoffmann-La Roche, Nutley, NJ, USA). rh IL-6 with a specific activity of $>2.0 \times 10^8$ U/ml was obtained from

 ISSN 0001-5555

Boehringer Mannheim, Germany. rhTNF-alpha with 98% purity, as determined by SDS-PAGE and committed N-terminal sequencing analysis, was obtained from Biosource International (Camarillo, CA, USA), or a kind gift from Reanal (Budapest, Hungary) (MI 148). rh IL-1 R was a generous gift from Dr. J. E. Sims.

Immunohistochemistry

Skin biopsy specimens were cut (4.5-μm thick sections) and the sections were mounted on gelatin-coated slides and incubated for 1 h at 60°C. Deparaffinization was performed by Bio-Clear (Bio-Optica, Milano, Italy) for 10–15 min and was followed by rehydration in graded concentrations of ethanol (100, 95 and 70%, 2 min for each). Endogenous peroxidase activity was blocked by immersing the sections in a freshly prepared mixture of concentrated methanol, containing 0.5% hydrogen peroxide, for 10 min. After preincubation with 10% normal rabbit or swine serum for 15 min, the sections were incubated with primary antibodies over night at +4 °C in a humid atmosphere, followed by incubation with the peroxidase-conjugated secondary antibodies for 30 min and aminoethylcarbazole as substrate. Finally, the sections were counterstained with haematoxylin for 5 min. Micrographs were taken using Kodak Ectachrome 64.

Specificity control

To confirm the specificity of the monoclonal and polyclonal antibodies for their respective antigen, the polyclonal antibodies were preincubated over night at +4°C either alone or in the presence of the antigens. rmIL-1 alpha was used at a concentration of 6.4 μg/ml, rh IL-1 beta at 100 μg/ml, rh IL-6 at 0.02 μg/ml, after being boiled for 40 min at 100°C, rh TNF-alpha at 5 μg/ml, and soluble human type I IL-1R at a concentration of 1.2 mg/ml. After preabsorption, the solutions were centrifuged at 10,000 g for 5 min before use. Other controls consisted of sections incubated with other idiotypic antibodies instead of the primary antibodies.

Microscopy and analysis of staining

The immunolabelling was assessed under a light microscope (Leitz, Germany), independently by two observers (A.A. and K.N.). The intensity of staining was graded according to the following system: −, negative; +−, variable; +, weak, ++, moderate; +++, intense.

RESULTS

Partly overlapping and partly differential staining was observed for the four cytokines, IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha, in hair follicles in normal human skin. Also, the localization of the staining for each cytokine and its respective receptor protein, as well as the antagonist to IL-1R, partly coincided. The polyclonal and monoclonal antibodies to IL-6R gave a similar signal, although the most intense staining was seen with the latter antibody. The two monoclonal antibodies against TNF-R gave a similar staining, but a somewhat stronger signal was seen with the antibody to the 55 kD fragment. The monoclonal antibody I4 to IL-1RA gave a positive staining signal, while the antibody I5 gave a negative staining.

A moderate immunoreactivity for IL-1 alpha was observed in the cuticle (Fig. 1 a, Table I), whereas IL-1 beta, IL-6 and TNF-alpha could not be detected in this area.

Intense cellular staining for IL-1 beta and TNF-alpha, but not IL-1 alpha or IL-6, was seen in the inner root sheath at the border close to the outer sheath, corresponding to the Henle layer, and beginning at the suprapapillary level (Fig. 1 d,f). An intense staining was seen for the type I IL-1R (Fig. 2b) and a variable staining for the IL-1RA (Fig. 1g).

A weak to moderate staining of the outer root sheath could be seen for all four cytokines (Fig. 1a,b), and an intense staining for their receptors (Fig. 2a-c) and a weak staining for the IL-1RA (Fig. 1g).

In the fibrous root sheath, a moderate immunoreactivity for IL-1 alpha and IL-6 was observed (Fig. 1a,b).

The isthmus exhibited a moderate staining for IL-1 alpha (Fig. 1 c), IL-1 beta and TNF-alpha, while a weak staining was seen for IL-6. Immunoreactivity for all three receptors as well as the IL-1 RA was also found here.

In the upper part of the hair follicle, the infundibulum, there was a weak immunoreactivity for all four cytokines. The intensity of the signal was similar to that seen in the epidermis, except in the case of IL-6, which exhibits a very weak/variable signal in the epidermis. An intense signal for IL-1R and IL-1RA was seen in the infundibulum, while a moderate staining was found for IL-6R and a weak staining for TNF-R.

After preabsorption of the antibodies with the respective antigen, the immunoreactive signals were abolished (Fig. 1e). No signal was observed when the primary antisera were omitted, or when other idiotypic antibodies were used.

DISCUSSION

Analysis of normal human skin by immunohistochemistry revealed a partly similar localization, but specific patterns of IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha in the hair follicles. Furthermore, there was a partial mismatch between the occurrence of receptors and their respective ligands.

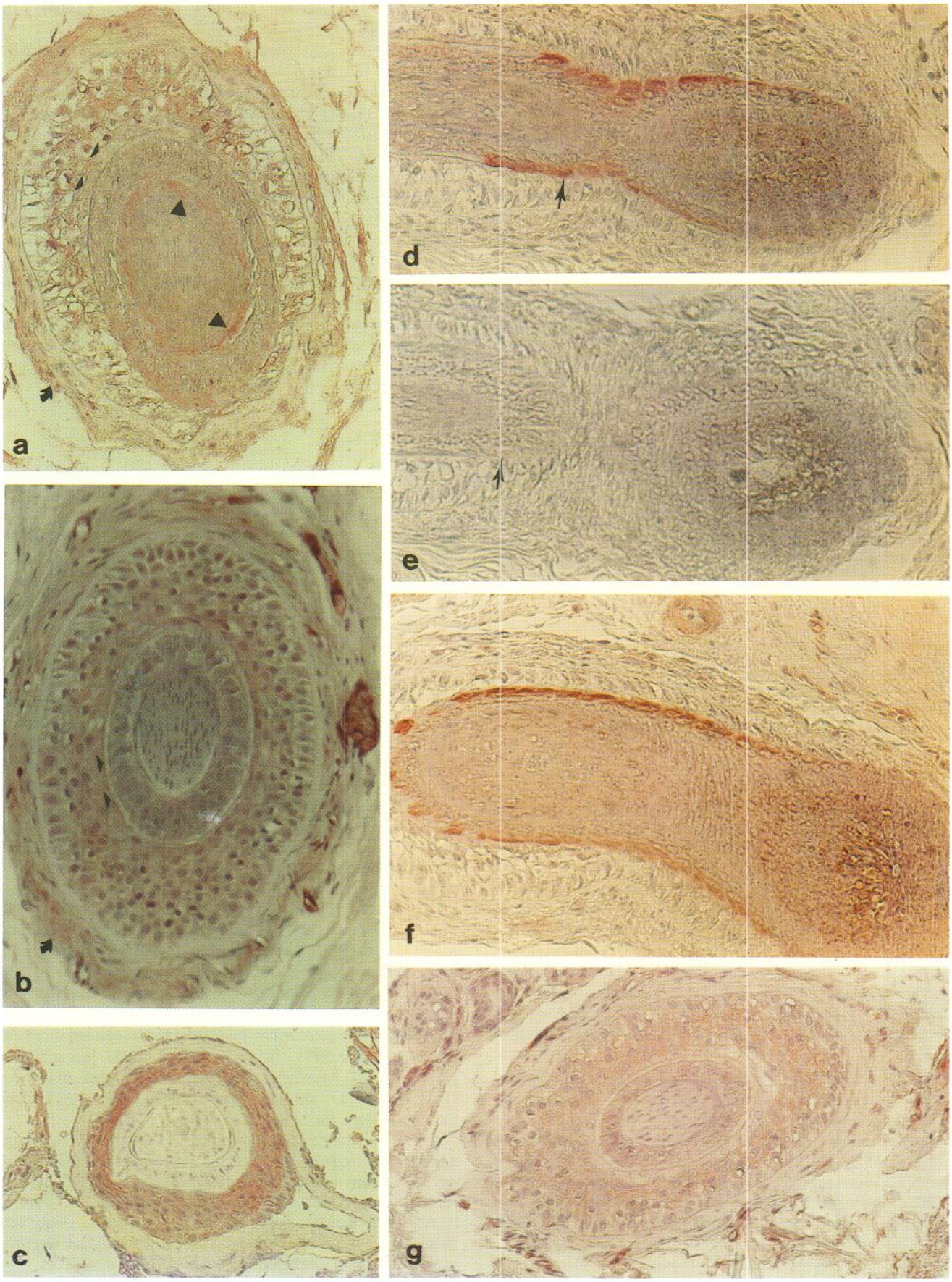
IL-1 beta and TNF-alpha exhibited an intense staining of the inner root sheath, while there was a variable and intense staining for IL-1RA and IL-1R, respectively. This labelling, most likely representing localization in the Henle layer, may indicate a role for these cytokines in hair formation. The inner root sheath, with the Henle layer, hardens before the hair within it, and it is thought to control the definitive shape of the hair shaft and establish a firm coat around the soft central part of the hair follicle. Whether or not the similarity in distribution of IL-1 beta and TNF-alpha is reflective of the fact that TNF-alpha is considered the closest biological relative of IL-1 (see 11) is presently unknown.

IL-1 is a proinflammatory cytokine, which may also induce protective and beneficial changes, possibly through the induction of acute-phase hepatic proteins, antioxidants or hematopoietic growth factors (see 11). A protective effect of IL-1 beta in hair follicles may be considered.

The occurrence of staining for both ligand (IL-1 beta) and receptor (IL-1R) in the inner root sheath would support a functional role, possibly of an autocrine or paracrine nature, of IL-1 beta in these cells. The IL-1 receptor exists in two forms, type I and type II. mRNA for the type I receptor but not the type II receptor has been detected in epithelial cells of the hair follicle (17). In general, IL-1 alpha has a higher affinity for IL-1RI, whereas IL-1 beta has a higher affinity for IL-1RII (see 11), although it is assumed that IL-1RI is the most important for biological activity.

All four cytokines were observed in the outer root sheath, and the staining for the receptor proteins was moderate to intense. In this location the cytokines may be of importance for growth and differentiation of the epithelial cells. There is increasing evidence that IL-1 alpha rather than IL-1 beta acts as an intracellular autocrine growth factor regulating terminal differentiation of epithelial cells (11).

The localization of TNF-alpha in the inner root sheath and


a
b
c
d
e
f
g

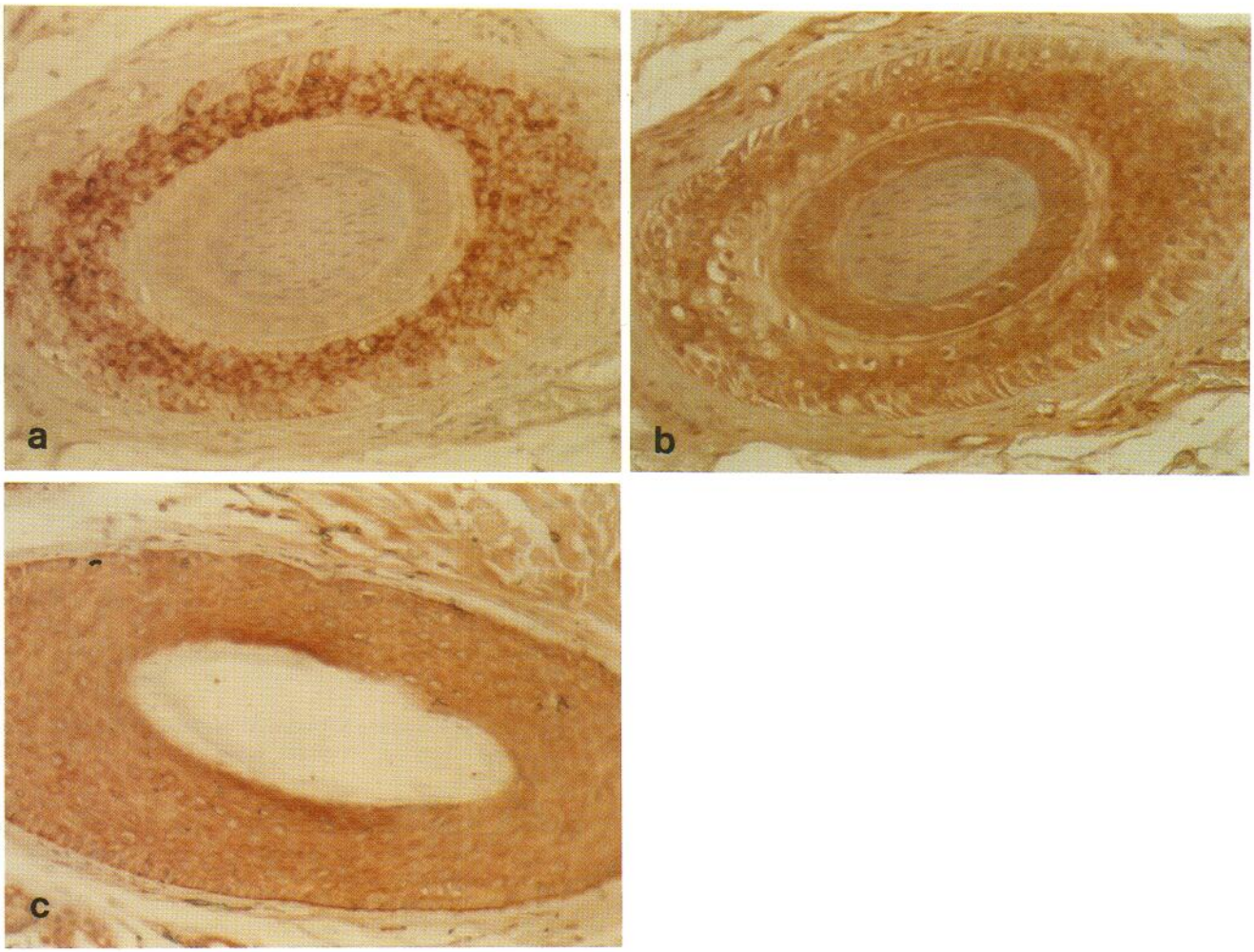


Fig. 2 (a-c). Immunoperoxidase labelling in sections of human skin after incubation with antibodies to IL-6R (*a*), IL-1R (*b*) and TNF-R (*c*). (*a*) Hair follicle with intense staining for IL-6R of the outer root sheath. (*b*) Intense staining for IL-1R in the outer and inner root sheath. (*c*) Intense staining for TNF-R in the outer root sheath. Magnifications are 400 ×.

Fig. 1 (a-g). Immunoperoxidase micrographs of paraffin-embedded sections of hair follicles from normal human skin after incubation with antibodies to IL-1 alpha (*a, c*), IL-1 beta (*d, e*), IL-6 (*b*), TNF-alpha (*f*) and IL-1RA (*g*).
(*a*) Section of hair follicle with a moderate staining for IL-1 alpha of the cuticle (*big arrow-head*) and weak staining of the outer root sheath (*small arrow-heads*) and the fibrous sheath (*curved arrow*). (*b*) Diffuse weak staining for IL-6 in the outer root sheath and fibrous sheath (*curved arrow*). (*c*) Moderate labelling for IL-1 alpha in the isthmus part. (*d*) Intense labelling for IL-1 beta in the outer layer of the inner root sheath (*arrow*). (*e*) Lack of staining after incubation with IL-1 beta antiserum, which was preabsorbed with rhIL-1 beta (*arrow*). (*f*) Intense TNF-alpha staining in the outer layer of the inner root sheath. (*g*) Variable labelling for the inner root sheath and weak labelling for the outer root sheath for IL-1RA. Magnifications are 400 × (*a-c, g*) and 1000 × (*d-f*).

of IL-1 alpha and IL-6 in the fibrous root sheath did not correlate with the occurrence of their respective receptor proteins in these tissues. Mismatch between ligands and their endogenous receptors has been reported earlier for neuropeptides (18).

The finding of IL-6-like immunoreactivity in the fibrous root sheath is consistent with in vitro studies showing an increase in IL-6 production by postmitotic human dermal fibroblasts in direct cell to cell contact with keratinocytes or outer root sheath cells (19).

A medium staining intensity for IL-1 alpha, IL-1 beta and TNF-alpha could be seen in the isthmus zone of the hair follicle. In the case of IL-1 alpha and IL-1 beta, this staining was more intense than in the outer root sheath. This is interesting in view of a recent hypothesis suggesting that follicular stem cells reside in the bulge zone of the hair follicle, being a subpopulation of keratinocytes with a superior proliferative potential (see 20).

In conclusion from the present results, the localization of IL-1 beta and TNF-alpha may indicate protective effects on the hair follicles, while all the investigated proinflammatory cytokines may have a role in differentiation during hair formation.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Welander/Finsen Foundation. We are grateful to Dr. P.L. Lomedico, Hoffman-La

Table I. *Evaluation of cytokine ligand, receptor and antagonist staining intensity in hair follicles of human skin*

	IL-1 α	IL-1 β	IL-6	TNF-α	IL-1R	IL-6R	TNF-R	IL-1RA
Epidermis	+	+	+−	+	+++	++	++	++
Infundibulum	+	+	+	+	+++	++	+	+++
Isthmus	++	++	+	++	++	++	++	+++
Fibrous root sheath	++	−	++	−	−	−	−	−
Outer root sheath	+	+	+	++	+++	+++	+++	+
Inner root sheath	−	+++	−	+++	+++	−	−	+−
Cuticle	++	−	−	−	−	−	−	−
Hair shaft	−	−	−	−	−	−	−	−
Matrix	−	−	−	−	−	−	−	−

Labelling: −, negative; +−, variable; +, weak; ++, moderate; +++, intense

Roche, Dr. J.E. Sims, Immunex, and to Reanal, for generously providing rmIL-1 alpha, rhIL-1R and rhTNF-alpha, respectively, to Dr. M. Brockhaus, Hoffman-La Roche, for providing antibodies against TNF-R, and to Dr. A.E. Berger, Pharmacia&Upjohn, and Dr. J.E. Sims, for providing IL-1RA and IL-1R(type 1) antibodies, respectively.

REFERENCES

1. Little JC, Westgate GE, Evans A, Granger SP. Cytokine gene expression in intact anagen rat hair follicles. J Invest Dermatol 1994; 103: 715–720.
2. Boehm KD, Yun JK, Strohl KP, Elmets CA. Messenger RNAs for the multifunctional cytokines interleukin-1 alpha, interleukin-1 beta and tumor necrosis factor-alpha are present in adnexal tissues and in dermis of normal human skin. Exp Dermatol 1995; 4: 335–341.
3. Jimenez JJ, Wong GHW, Yunis AA. Interleukin 1 protects from cytosine arabinoside-induced alopecia in the rat model. FASEB J 1991; 5: 2456–2458.
4. Jimenez JJ, Sawaya ME, Yunis AA. Interleukin 1 protects hair follicles from cytarabine (ARA-C)-induced toxicity in vivo and in vitro. FASEB J 1992; 6: 911–913.
5. Harmon CS, Nevins TD. IL-1 alpha inhibits human hair follicle growth and hair fiber production in whole-organ cultures. Lymphokine Cytokine Res 1993; 12: 197–203.
6. Luger TA. Epidermal cytokines. Acta Derm Venereol (Stockh) 1989; 69 (Suppl 151): 61–76.
7. Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL-6 and related molecules (IL-1 and TNF). FASEB J 1990; 4: 2860–2867.
8. Barker JNWN, Mitra RS, Griffiths CEM, Dixit VM, Nickoloff BJ. Keratinocytes as initiators of inflammation. Lancet 1991; 337: 211–214.
9. Oxholm A. Epidermal expression of interleukin-6 and tumor necrosis factor-alpha in normal and immunoinflammatory skin states in humans. APMIS 1992; 100 (Suppl 4): 5–32.
10. Nickoloff BJ, Turka LA. Keratinocytes. Key immunocytes of the integument. Am J Pathol 1993; 143: 325–331.
11. Dinarello CA. The interleukin-1 family: 10 years of discovery. FASEB J 8: 1314–1325, 1994.
12. Schultzberg M, Andersson C, Undén A, Troye-Blomberg M. Interleukin-1 in adrenal chromaffin cells. Neurosci 1989; 30: 805–810.
13. Schultzberg M, Svenson S, Undén A, Bartfai T. Interleukin-1-like immunoreactivity in peripheral tissue. J Neurosci 1987; 18: 184–187.
14. Novick D, Engelmann H, Wallach D, Revel M, Leitner O, Rubinstein M. Monoclonal antibodies to the soluble human IL-6 receptor: affinity purification, ELISA, and inhibition of ligand binding. Hybridoma 1991; 10: 137–145.
15. Brakenhoff JPJ, de Hon FD, Fontaine V, ten Boekel E, Schooltink H, Rose-John S, et al. Development of a human interleukin-6 receptor antagonist. J Biol Chem 1994; 269: 86–93.
16. Espevik T, Brockhaus M, Loetscher H, Nonstad U, Shalaby R. Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. J Exp Med 1990; 171: 415–426.
17. Deyerle KL, Sims JE, Dower SK, Bothwell MA. Pattern of IL-1 receptor gene expression suggests role in noninflammatory processes. J Immunol 1992; 149: 1657–1665.
18. Kuhar MJ. The mismatch problem in receptor mapping studies. Trends NeuroSci 1985; 8: 190–191.
19. Limat A, Hunziker T, Waelti ER, Inaebnit SP, Wiesmann U, Braathen LR. Soluble factors from human hair papilla cells and dermal fibroblasts dramatically increase the clonal growth of outer root sheath cells. Arch Dermatol Res 1993; 285: 205–210.
20. Yang J-S, Lavker RM, Sun T-T. Upper human hair follicle contains a subpopulation of keratinocytes with superior in vitro proliferative potential. J Invest Dermatol 1993; 101: 652–659.