Growth Factor and Growth Factor Receptor Localization in the Hair Follicle Bulge and Associated Tissue in Human Fetus

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The bulge region of the hair follicle has been thought to contain follicular stem cells. The bulge in the human follicle is a collection of undifferentiated cells that is prominent only in the fetal period. Antibodies that recognize epidermal growth factor (EGF), transforming growth factor–α (TGF-α), EGF receptor, platelet-derived growth factor (PDGF) A and B chains, PDGF α and β receptors, and the low-affinity nerve growth factor receptor (p75) were used to study the bulge and associated mesenchymal cells in this fetal period. Weak EGF and TGF-α immunoreactivities were seen in the bulge. Confocal laser scanning microscopic images revealed intracytoplasmic and intranuclear punctate patterns of immunoreactivities in the bulge cells labeled by anti-EGF and anti–TGF-α antibodies. All the bulge cells stained strongly for EGF receptor. Cells within the bulge were labeled both with PDGF A chain and with PDGF B chain, although the immunoreactivities were weak in the outermost layer of cells. The follicular sheath was strongly immunoreactive with antibodies against both PDGF α and β receptors. p75 was expressed in mesenchymal cells around the hair follicle and in the lower portion of the bulge. These differential labeling patterns suggested that EGF, TGF-α, and nerve growth factor may be involved in regulation of the growth and differentiation of bulge cells and that PDGFs may have related functions in the interaction arising between the bulge and associated tissue during follicle morphogenesis. ***Key words: epidermal growth factor/transforming growth factor–α/platelet-derived growth factor/nerve growth factor receptor. J Invest Dermatol 106: 391–396, 1996***

During the mid-bulbous hair peg stage of human fetal hair follicle development, at 16–18 wk of estimated gestational age (EGA), the bulge is a prominent hemispherical protrusion of cells that are contiguous with the outer root sheath (ORS) located just distal to the opening of the sebaceous duct (Madsen, 1964). It becomes a less conspicuous swelling of the ORS after birth. Although the bulge was identified over a hundred years ago, its function and significance have only recently been revealed. Using tritiated thymidine labeling, Cotsarelis *et al* (1990) showed that the bulge area of mouse hair follicle contains a population of slow-cycling cells and suggested that the bulge is the site of follicular stem cells. The bulge has commanded the attention of many scientists as a stem cell site associated with regulation of the hair cycle (Cotsarelis *et al*, 1990; Sun *et al*, 1991) and skin tumor formation (Cotsarelis *et al*, 1990; Leshin and White, 1990; Lavker *et al*, 1991), and also as a target site of graft-*versus*-host disease (Murphy *et al*, 1990; Sale and Beauchamp, 1993; Sale *et al*, 1994). The "bulge-activation hypothesis," proposing that hair cycles reflected activation and inactivation of stem cells in the bulge, has been developed on the basis of the identification of putative hair follicle stem cells in the bulge region (Cotsarelis *et al*, 1990; Sun *et al*, 1991). Certain kinds of skin neoplasms are known to be follicular in origin (Ghadially, 1961), and the bulge as a stem cell site is thought to be involved in skin tumor formation (Cotsarelis *et al*, 1990; Leshin and White, 1990; Lavker *et al*, 1991). Damage to the bulge cells appears to be very important in graft-*versus*-host disease, as are other stem cell sites such as the rete ridge of the epidermis (Sale *et al*, 1985) and the murine forestomach (Sale *et al*, 1991), which are preferred targets in this disease.

The human bulge consists of densely packed cells of relatively uniformly small size; their cytoplasm is less dense than that of other "regular" ORS cells (Akiyama *et al*, 1995). Ultrastructurally, the human bulge cells, especially the interior cells, have features characteristic of undifferentiated cells including abundant free ribosomes, glycogen particles, and sparse cytoplasmic organelles (Akiyama *et al*, 1995).

To date, little is known about the growth regulation of the bulge cells and the role of the bulge in hair follicle development. In the present study, we paid special attention to the presence of certain soluble growth factors and growth factor receptors—epidermal growth factor (EGF), transforming growth factor–α (TGF-α), EGF receptor (EGFR), platelet-derived growth factors (PDGFs), PDGF receptors (PDGFRs), and the low-affinity nerve growth factor receptor (NGFR) (p75)—in the bulge at specific stages of hair follicle development. The known activities of these growth factors

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Abbreviations: EGA, estimated gestational age; EGFR, epidermal growth factor receptor; NGFR, nerve growth factor receptor; PDGFR, platelet-derived growth factor receptor; PGP9.5, protein gene product 9.5.

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and their receptors in other systems can provide insight into the possible mechanism for their action in follicle morphogenesis. EGF, TGF-α, and PDGFs are known to mediate the proliferation and differentiation of various types of cells. EGF and TGF-α are the most extensively studied members of the EGF family of growth factors. They are structurally related and bind to the same receptor, EGFR. EGF and TGF-α are known as potent positive regulators of skin fibroblasts and epidermal cell growth (Cohen, 1965; Carpenter and Cohen, 1976; Barrandon and Green, 1987). In addition to its mitogenic activities, PDGF is a chemoattractant for fibroblasts (Seppa *et al*, 1982) and has been shown to stimulate increased levels of extracellular matrix production by fibroblasts in culture (Narayanan and Page, 1983). p75 is expressed in a number of tissues that are not known to respond to nerve growth factor (NGF) or other neurotrophins, and in skin mesenchyme during development (Heuer *et al*, 1990; Wyatt *et al*, 1990). The possibility remains that p75 plays a more general role in embryonic development (Bothwell, 1990).

Using immunohistochemical, immunofluorescence, and confocal laser scanning microscopic techniques, we clearly demonstrated the localization of EGF, TGF-α, EGFR, PDGFs, PDGFRs, and a low-affinity NGFR (p75) in human developing hair follicle bulge and its associated tissues. These results suggest that EGF, TGF-α, and NGF are involved in regulation of the growth and differentiation of bulge cells and that PDGFs have related functions in the interaction between the bulge and the associated tissue.

MATERIALS AND METHODS

Tissue Human fetal tissue was acquired through the Central Laboratory of Human Embryology at the University of Washington, Seattle, with the approval of the Human Subjects Review Board and in accordance with the United States DHEW policies. Human fetal skin was obtained from abortuses of 100–160 d EGA, a stage when the bulge is most prominent ontogenically. Fetal age was estimated by crown-rump length, foot length, and menstrual age. Skin specimens were taken from limbs, trunk, and scalp, and processed for observation.

Antibodies The rabbit anti-human EGF polyclonal antibody (Ab-3) was purchased from Oncogene Science, Inc. (Uniondale, NY). The mouse anti-human TGF-α monoclonal antibody (Ab-2) (Sorvillo *et al*, 1990), which does not inhibit TGF-α binding to the receptor, was also purchased from Oncogene Science. The anti-human EGFR monoclonal antibody, which recognizes the extracellular domain of EGFR and competes with EGF for the EGF binding site on human cells, was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). The rabbit anti-human PDGF A chain and anti-human PDGF B chain polyclonal antibodies used in this study were generated by immunizing rabbits with purified recombinant PDGF-AA or PDGF-BB, respectively, as previously described (Hart *et al*, 1990). Both the mouse anti-human PDGFRα and anti-human PDGFRβ monoclonal antibodies are described elsewhere (Hart *et al*, 1987; Ansel *et al*, 1993). All the anti-PDGF and anti-PDGFR antibodies were kind gifts from Dr. Charles E. Hart (ZymoGenetics Inc., Seattle, WA). The generation and characterization of the mouse anti-human p75 (a low-affinity NGFR) monoclonal antibody used in the present study (a generous gift from Dr. Mark Bothwell, University of Washington, Seattle) have been previously described (Marano *et al*, 1987). Rabbit polyclonal anti-neurofilament antibody (Advanced Immuno Chemical Inc., Long Beach, CA), mouse monoclonal anti-protein gene product 9.5 (PGP9.5) antibody (Accurate Chemical & Scientific Corp., Westbury, NY), and rabbit polyclonal anti-human calcitonin gene-related peptide antibody (Peninsula Laboratories Inc., Belmont, CA) were used as neuronal markers to detect a development nerve network in the dermis.

Immunohistochemistry Fresh fetal skin specimens mounted in OCT compound (Tissue-Tek) were quick-frozen using an ethanol bath cooled with dry ice, and sections were cut in the cryostat. Sections were fixed for 8 min in 100% acetone (in 0.1% glutaraldehyde solution for the labeling with anti-PDGFRs α and β). For the labeling with anti-human neurofilament protein, anti-PGP9.5 and anti-calcitonin gene-related peptide antibodies, skin specimens were fixed in Zamboni's fixative (Stefanini *et al*, 1967). All 6-μm sections were stained by the avidin-biotin-peroxidase complex method (Hsu *et al*, 1981). The dilutions for anti-EGF, TGF-α, and PDGFRα antibodies were 1/20, and the dilutions for anti-EGFR and anti-PDGF A chain antibodies were 1/100. The dilutions for anti-PDGF B chain antibody, anti-PDGFRβ antibody, anti-calcitonin gene-related peptide antibody, and normal rabbit serum were 1/50, 1/1000, 1/40, and 1/100, respectively. The dilutions for anti-p75 antibody, anti-neurofilament antibody, anti-PGP9.5 antibody, and the control ascites fluid were 1/400. Sections were incubated in normal horse (goat [changes for polyclonal antibodies are given in parentheses]) serum, and endogenous peroxidase activity was blocked in 0.01% hydrogen peroxidase solution, then incubated in monoclonal antibody (polyclonal antibody) for 2 h, biotin-conjugated horse anti-mouse IgG and IgM (goat anti-rabbit IgG) for 30 min, and then in avidin mixed with biotin-conjugated peroxidase for 30 min (Vector Laboratories, Inc., Burlingame, CA). Sections were rinsed extensively with phosphate-buffered saline between incubations. Color was developed by incubation in freshly prepared substrate solution containing 50 mM Tris-HCl, pH 7.6, 3,3′-diaminobenzidine-HCl (0.05 mg/ml), and 0.01% hydrogen peroxide at room temperature for 5 min. Counterstaining with hematoxylin was performed when it was necessary to identify the bulge area.

Immunofluorescence Labeling Six-micrometer-thick sections of fresh fetal skin cut by cryostat were used. The sections were fixed for 8 min in 100% cold acetone (in 0.1% glutaraldehyde solution for the labeling with anti-PDGFRs α and β), incubated in normal horse and goat sera for 30 min, and then incubated in primary antibody solution (the same concentration or a two to four times higher concentration than that used in immunohistochemistry) for 1 h in 37°C, followed by fluorescein isothiocyanate-conjugated to horse anti-mouse IgG and IgM (Vector Laboratories) for 30 min at room temperature. Sections were extensively washed with phosphate-buffered saline between incubations. Counterstaining was done by incubating the sections in 10 μg/ml propidium iodide (to demonstrate nuclei) (Sigma Chemical Co., St. Louis, MO) for 30 sec or in 2 μg/ml 4′,6-diamidino-2-phenylindole (Sigma) for 5 min. Stained sections were mounted with a cover slip in Vectashield (Vector Laboratories) mounting medium. For double-labeling, binding of monoclonal antibodies was detected by incubating sections in biotin-conjugated horse anti-mouse IgG and IgM (Vector Laboratories) solution diluted to 1/200 for 40 min and in rhodamine$_{600}$-avidin D (Vector Laboratories) solution diluted 1/1000 for 40 min. Binding sites of polyclonal antibodies were demonstrated using the same method as that of the single-labeling described above.

Confocal Laser Scanning Microscopy Laser scanning confocal microscopy was performed with a Nikon epifluorescence microscope equipped with a Bio-Rad MRC-600 Laser Scanning Confocal Imaging System (Bio-Rad Laboratories, Richmond, CA). The images were observed with a ×10 objective 0.45 NA or a ×60 objective 1.4 NA.

RESULTS

The results of the immunostaining with growth factor and growth factor receptor antibodies in human fetal skin in the second trimester are summarized in **Table I**.

Bulge Cells Show EGF, TGF-α, and EGFR Immunoreactivities Weak EGF and TGF-α immunoreactivities were seen in the bulge **(Fig 1*A*,*E*)**, inner root sheath (IRS) **(Fig 1*B*,*F*)** and epidermal basal cells; the intermediate cells of the epidermis, the inner part of the ORS **(Fig 1*B*,*F*)** and the sebaceous gland **(Fig 1*A*,*E*)** exhibited moderate to strong EGF and TGF-α staining. Matrix cells of the bulb showed weak EGF immunoreactivity and strong TGF-α immunoreactivity **(Fig 1*C*,*G*)**. EGF immunoreactivity was present in some periderm cells. Confocal laser scanning microscopy revealed punctate staining for EGF **(Fig 1*D*)** and TGF-α **(Fig 1*H*)** in both the cytoplasmic and nuclear compartments of the bulge cells; the nuclear compartment was differentiated from the cytoplasm by propidium iodide nuclear stain.

The bulge (both the interior cells and the outermost cells), the sebaceous gland **(Fig 1*I*)**, matrix cells **(Fig 1*K*)**, and epidermal basal cells exhibited strong EGFR immunoreactivity. Moderate-to-weak EGFR immunoreactivity was present in ORS, IRS **(Fig 1*J*)** and epidermal intermediate cells. The bulge cells showed diffuse cytoplasmic EGFR staining by confocal microscopy **(Fig 1*L*)**.

Bulge Cells Exhibit PDGF Immunoreactivity and Mesenchymal Cells around the Bulge Show PDGFR Immunoreactivity Moderate PDGF A chain immunoreactivity was detected in the interior cells of the bulge **(Fig 2*A*)**, IRS, matrix cells **(Fig 2*B*)**, the sebaceous gland **(Fig 2*A*)**, and intermediate cells in the epidermis, and weak reaction was seen in the outermost cells of the bulge **(Fig 2*A*)** and epidermal basal cells. The PDGF A chain

Table I. Localization of Immunoreactivity of Growth Factors and Growth Factor Receptors in Fetal Skin in the Second Trimester[a]

	Hair Follicle										Epidermis		
	Bulge						Hair						
	Inferior cells	Outermost Cells	Infundibulum	Outer Root Sheath	Inner Root Sheath	Matrix Cells	Cortex	Medulla	Sebaceous Gland	Perifollicular Dermis	Basal Cells	Intermediate Cells	Periderm
EGF	+	+	++	− (innermost layer ++)	+	+	+	++	++	−	+	++	+/−
TGF-α	+	+	++	− (innermost layer ++)	+	+++	+	++	+++	−	+	++	−
EGFR	+++	+++	++	+	+	+++	−	−	+++	−	+++	++	−
PDGF A	++	+	++	−	++[b]	++	++	++	++	−	+	++	−
PDGF B	++	+	++	−	+++	−	−	−	+	−	+	++	+/−
PDGFR α	−	−	−	−	−	−	−	−	−	++ (dermal papilla −)	−	−	−
PDGFR β	−	−	−	−	−	−	−	−	−	++ (dermal papilla −)	−	−	−
p75	− (distal part ++)	− (distal part ++)	−	−[c]	−	−	−	−	−	−/+[d]	−	−	−

[a] +++, strong; ++, moderate; +, weak; −, absent.
[b] The labeling was lost in the lower bulb.
[c] ++ only between the bulge and the bulb.
[d] ++ in the dermal papilla and between the sebaceous gland and the bulge.

staining in IRS and matrix cells was reduced in the terminal portion of the bulb **(Fig 2*B*)**.

Cells within the bulge were labeled with PDGF B chain although the immunoreactivity was weak in the outermost cells **(Fig 2*F,I*)**. IRS **(Fig 2*G*)** and the intermediate cells in the epidermis showed moderate-to-strong PDGF B chain immunoreactivity. Weak immunoreactivity with anti-PDGF B chain antibody was present in the sebaceous gland, the epidermal basal cells, and some periderm cells.

By confocal microscopy, the bulge cells and IRS showed mainly diffuse, cytoplasmic PDGF A chain **(Fig 2*C*)** and B chain **(Fig 2*H*)** staining together with punctate pattern.

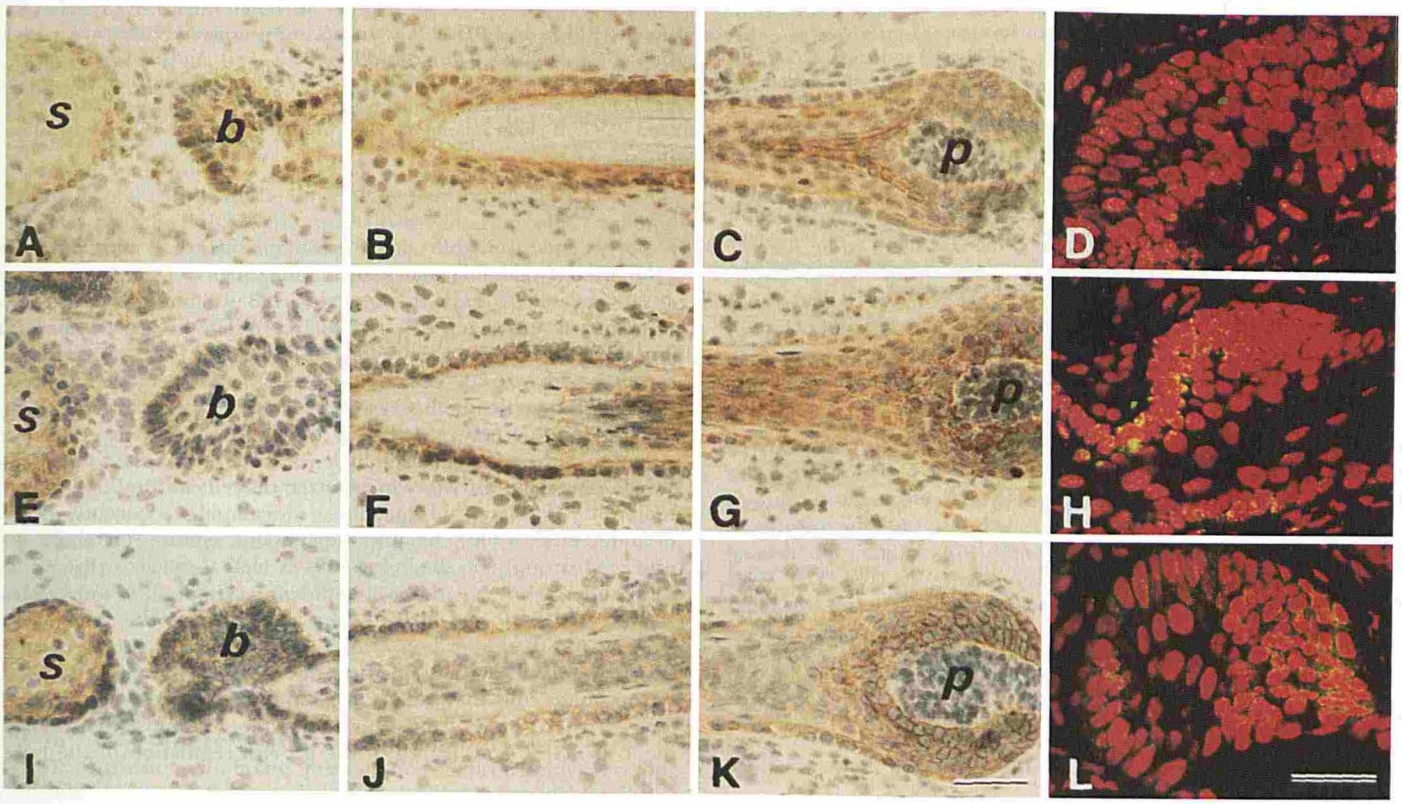

Figure 1. Weak, punctate EGF and TGF-α immunoreactivities and strong EGFR immunoreactivity are seen in the bulge of human fetal hair follicles at 16–18 wk EGA. *A–D*) Anti-EGF. *E–H*) Anti-TGF-α. *I–L*) Anti-EGFR. Bulge (*b*) and sebaceous gland (*s*) (*A,E,I*), ORS (*B,F,J*), bulb and dermal papilla (*p*) (*C,G,K*), confocal microscopic images of the bulge (propidium iodide nuclear stain) (*D,H,L*). EGF (*A*) and TGF-α (*E*) immunoreactivities are present in the bulge (*b*) and EGFR immunoreactivity (*I*) is also seen in the bulge (*b*). Confocal microscopy reveals the punctate staining in the bulge for EGF (*D*) and TGF-α (*H*) and diffuse cytoplasmic staining for EGFR (*L*). *Scale bars*, 50 μm.

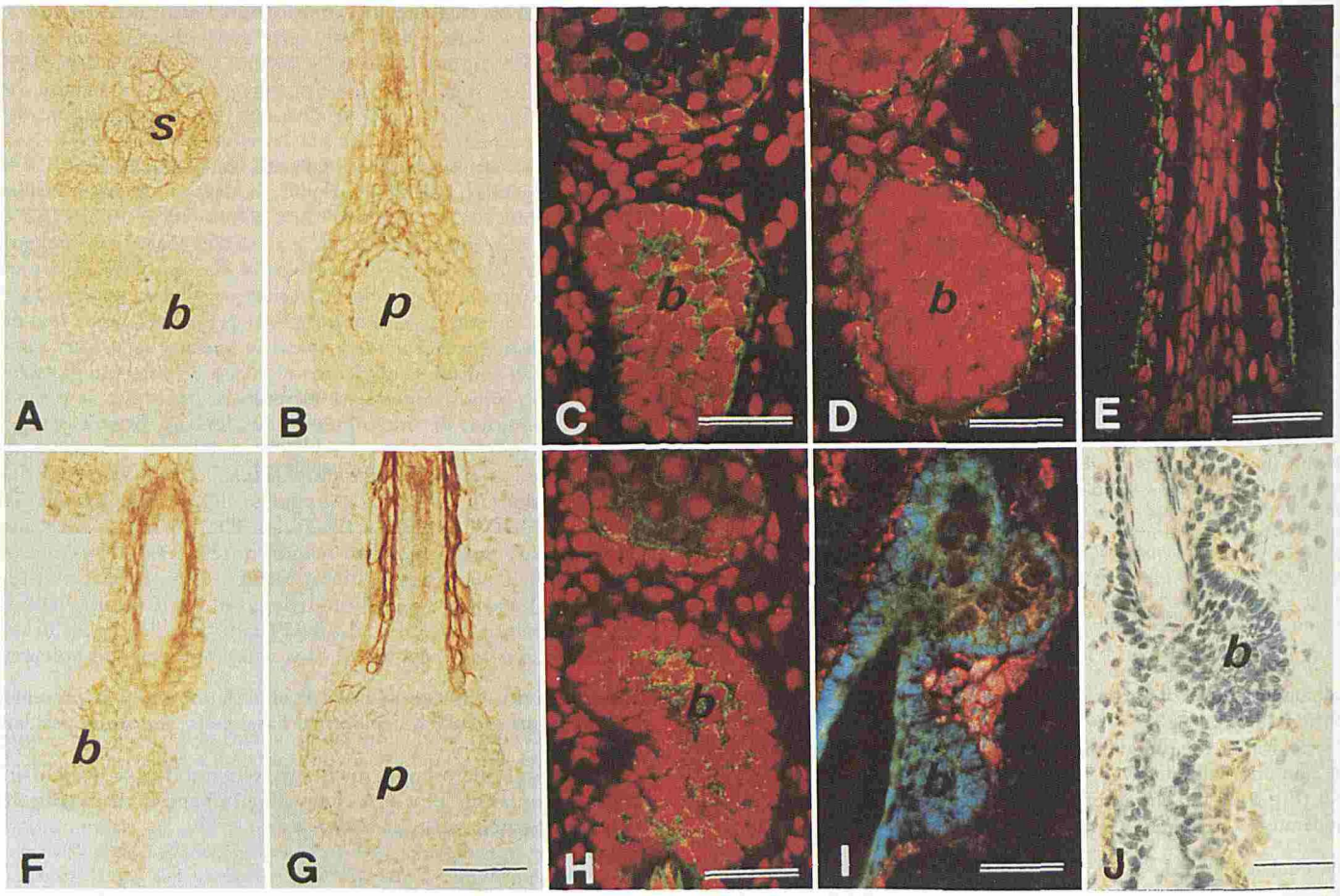

Figure 2. PDGF A chain and B chain immunoreactivities are observed in the bulge and PDGFR α and β immunoreactivities are seen in the mesenchymal cells around hair follicles (16–18 wk EGA). *A–C*) Anti-PDGF A chain. *D,E*) Anti-PDGFR α. *F–I*) Anti-PDGF B chain. *J*) Anti-PDGFR β. Double-labeled with anti-PDGF B chain (fluorescein isothiocyanate) and anti-PDGFR β (rhodamine) (*I*), confocal microscopic images (propidium iodide nuclear stain) (*C,D,E,H*), bulge (*b*) and sebaceous gland (*s*) (*A,C,D,F,H,I,J*), bulb and dermal papilla (*p*) (*B,G*), follicular sheath (*E*). The bulge (*b*) cells, especially the interior cells, exhibit PDGF A chain staining (*A,C*) and PDGF B chain staining (*F,H,I*). Mesenchymal cells around the follicle show PDGFR α (*D,E*) and PDGFR β (*I,J*) immunoreactivities. *Scale bars*, 50 μm.

The expression of PDGFRs α and β did not seem to be specific to the bulge area **(Fig 2*D,I,J*)**. The follicular sheath was immunoreactive with both anti-PDGFR α **(Fig 2*D,E*)** and β **(Fig 2I,*J*)** antibodies although the dermal papilla cells showed no reaction. The follicular sheath in some area around the bulge seemed to exhibit even less immunoreactivity for PDGFRs than the other part of follicular sheath.

A Low-Affinity NGFR (p75) Is Expressed in the Lower Portion of the Bulge and in Mesenchymal Cells around the Bulge p75 was expressed in mesenchymal cells around the hair follicle, especially in cells between the bulge and the sebaceous gland and those surrounding the bulb **(Fig 3*A,C*)**; the dermal papilla cells showed moderate immunoreactivity **(Fig 3*D*)**. This distribution of p75-positive cells between the bulge and the sebaceous gland and around the bulb overlapped with the localization of nerve fibers that were detected by antibodies recognizing neurofilament protein, calcitonin gene-related peptide, and PGP9.5 **(Fig 3*B*)**. In addition, p75 antibody labeled cells of the lower bulge and ORS distal to the bulge **(Fig 3*C*)**. p75 immunoreactivity was absent in all other parts of the epithelium including the hair follicle and interfollicular epidermis.

DISCUSSION

EGF, TGF-α, EGFR, and the Bulge Both EGF and TGF-α are synthesized as transmembrane precursor molecules and cleaved by proteolysis to smaller mature soluble factors (Davis, 1990). EGF is a polypeptide mitogen of 53 amino acids that stimulates the proliferation of epidermal and epithelial cells. TGF-α, a peptide closely related to EGF, is composed of 50 amino acids and also functions as a mitogen (Massagué, 1990). The biological effects of both EGF and TGF-α are mediated by a 170-kDa transmembrane receptor with tyrosine kinase activity. It is known that EGF receptor and ligand complexes cluster and are internalized into the cell after the binding (Carpenter, 1983).

In the human fetal hair follicle in the bulbous hair peg stage, EGFR immunoreactivity was reported on cells of the ORS, matrix region of the hair bulb, and the developing sebaceous glands (Nanney *et al*, 1990). In our study, EGFR immunoreactivity was observed strongly on the bulge cells as well as matrix cells and the sebaceous gland. In addition, punctate, cytoplasmic and nuclear staining for EGF and TGF-α were observed in the bulge cells. Together with the finding that the bulge cells strongly exhibited EGFR immunoreactivity, the punctate staining for EGF and TGF-α in the bulge cells may reflect the binding of exogenous EGF and TGF-α to the bulge cells. Our observations support the involvement of EGFR and ligands in the growth regulation of the bulge cells.

PDGF A Chain, B Chain, PDGFRs α and β, and the Bulge PDGF is a dimeric glycoprotein of approximately 31 kDa and is composed of disulfide-linked monomeric chains A and B (Hart *et al*, 1990; Hammacher *et al*, 1988). PDGF is a potent mitogen for many mesenchymal cells including cells in the dermis (Ross *et al*, 1986).

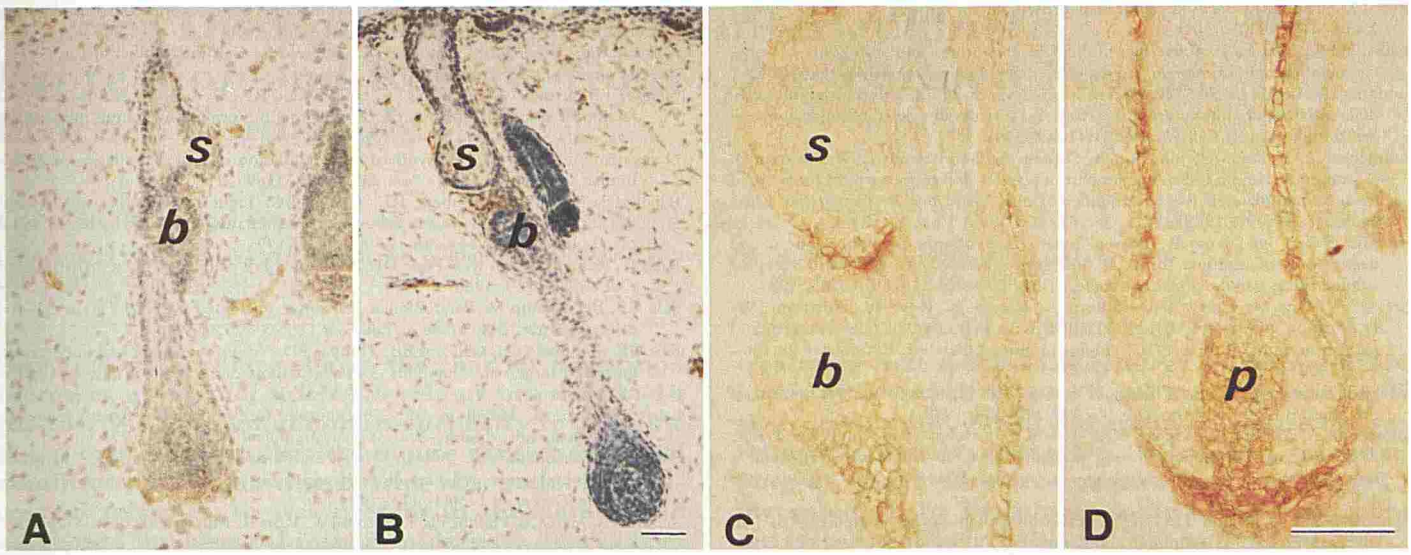

Figure 3. A low-affinity NGFR (p75) is expressed in the lower part of the bulge as well as in the mesenchymal cells around hair follicles (16–18 wk EGA). *A,C,D)* Anti-p75. *B)* Anti-PGP9.5. Cells stained with anti-p75 and anti-PGP9.5 antibodies are seen between the bulge (*b*) and the sebaceous gland (*s*) (*A,B*). p75 immunoreactivity is present in the lower part of the bulge (*b*), in the ORS between the bulge and the bulb, and in the dermal papilla (*p*) (A,C,D). *Scale bars*, 50 μm.

The biological activities of PDGF are mediated via binding to cell-surface receptors termed α and β (Seifert *et al*, 1989). Human keratinocytes are thought to be a major source of cutaneous PDGF (Ansel *et al*, 1993).

The role of PDGF (A chain and B chain) in hair follicle development has not been clarified, although *in vivo* proliferation of dermal papilla cells of rat hair follicles and their secretion of stromelysin were stimulated by exogenous PDGF (Goodman and Ledbetter, 1992). In the early stages of the developing follicle—the hair germ and hair peg stages—the PDGF A chain was found within the outer cells of the follicles, cells which are believed to be derived from the basal layer (Holbrook *et al*, 1993). Immunoreactivity of the PDGFRs was found only on mesenchymal cells around the developing hair follicle.

In the present study of the developing hair follicle in the bulbous hair peg stage, the bulge cells, especially the interior cells, exhibited PDGF immunoreactivity and the follicular sheath showed PDGFR staining. These results suggested that PDGF is secreted by the bulge cells and that interaction occurs between the bulge and parafollicular mesenchymal cells via PDGFs.

In addition, the finding that PDGFRs immunoreactivities were absent in the dermal papilla cells was interesting in that it does not concur with the results of the *in vitro* study of rat dermal papilla cells (Goodman and Ledbetter, 1992); according to the study in rats, the secretion of stromelysin by dermal papilla cells was regulated by the PDGF. In the human, different from the rat, the action of PDGF may be indirect to the dermal papilla cells.

In some area around the bulge, immunoreactivity for PDGFRs in mesenchymal cells seemed to be weak in the present study. This finding may reflect the fact that PDGFR immunoreactivity is absent in the arrector pili muscle attached to the bulge. However, we cannot exclude the possibility that this weak staining of PDGFR in some area of the follicular sheath around the bulge implies its similarity to the dermal papilla cells.

p75 (a Low-Affinity NGFR) and the Bulge p75, a 75- to 80-kDa intrinsic membrane protein and neurotrophic receptor molecule, binds all five members of the neurotrophin family of proteins, including NGF (Bothwell, 1991). The follicular and interfollicular epidermis is known both as a site and as a source of NGF (Davies *et al*, 1987). The close relationship of nerves to the developing follicles has been suggested by the localization of NGFRs around the follicles (Holbrook *et al*, 1993; Ribeiro-da-Silva *et al*, 1991) and observations in rats and mice have suggested that interaction between the hair follicle epithelial cells and the sensory nerves is mediated in part by NGF.

In our study, p75 immunoreactivity was expressed in the lower part of the bulge and ORS as well as in mesenchymal cells around the hair follicles. This p75 localization suggested that the interaction between the bulge and ORS cells and the nerves might be mediated in part by soluble neurotrophic factors.

Further knowledge of growth factor and receptor interactions in regulating the hair follicle bulge cells and associated tissue will help us understand the development, growth, and pathology of skin.

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