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Expression of transforming growth factor ß isoforms and their receptors during hair growth phases in mice

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Summary. Transforming growth factor ß (TGF-ß) is a family of potent growth inhibitor proteins, often produced as a precursor and often secreted in a complex with the latent TGF-ß binding protein (LTBP). We investigated the expression of TGF-ß1, -ß2, -ß3, LTBP, TGF-ß receptor proteins type I and type II (TßR-I and -II) during induced hair growth in C57 BL-6 mice. We here demonstrated that TGFßs and TßR-I are expressed in hair follicle epithelium and have found a positive reactivity for LTBP and TßR-I in sebocytes. Dermal tissue was weakly stained for LTBP and TGF-ß3. In early anagen the inner hair root sheath epithelium expressed TGF-ß1, whereas outer hair root was positive for TßR-I during anagen/catagen switch. TßR-II was found in sebaceous glands without significant variations during the hair cycle. We may conclude that in follicle epithelium TGF-ß1 is not produced in a complex together with LTBP. On the other hand, it is possible that other types of LTBP, like LTBP-2 and LTBP-3, are present, which are not detected by the antibody we used. Furthermore, a very rapid secretion of LTBP from-producing cells may prevent immunohistochemical detection. TGF-ß1 released by inner hair root sheath may regulate outer root sheath growth. A bidirectional interaction of sebocytes and hair follicle epithelium in the TGF-ß/LTBP seems possible. Sebocytes can be considered to be a target for TGFs since they express both TßR-I and -II. The general properties of TGF-ß as a growth inhibitor of epithelial cells may suggest a possible involvement in either the abrogation of extensive growth at the end of anagen or the initiation of catagen for the follicle epithelium as well as growth control for sebaceous glands.

Key words: Hair follicle, Sebaceous gland, Transforming growth factor, Transforming growth factor receptor, Latent transforming growth factor-ß binding protein

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Introduction

The regulation of hair growth phases involves different mechanisms including calcium binding proteins, phosphotyrosine and epidermal growth factor receptor, and neuropeptides (Paus et al., 1994; Wollina et al., 1995).

Transforming growth factor-ß (TGF-ß) is a family of multifunctional proteins. Three different isoforms, TGF-ß1, -ß2, and -ß3, have been identified in mammals. TGF-ßs belong to a larger TGF-ß superfamily, including activins, inhibins, bone morphogenic proteins, Müllerian inhibiting substance and glial cell-line-derived neurotrophic factor. TGF-ß was originally identified as a factor which, together with TGF-α, stimulated the growth of normal rat kidney fibroblasts on soft agar (Assoian et al., 1983). Later, TGF-ß was found to be of a potent growth inhibitor on most cell types, possessing a number of other biological effects on different cell types; i.e. regulation of cellular differentiation, migration, production of extracellular matrix and adhesiveness to the matrices, and modulation of immune functions (Roberts and Sporn, 1990; Miyazono et al., 1991; Sporn and Roberts, 1992). Disruption of the TGF-ß1 gene in mice results in severe inflammatory responses in many organs after birth, indicating that TGF-ß1 plays critical roles in immune suppression in vivo (Shull et al., 1992; Geiser et al., 1993; Kulkarni et al., 1993).

TGF-ßs exert their functions through the interaction with various receptors and binding proteins on the cell surface (Massagué, 1992; Kingsley et al., 1994; Miyazono et al., 1991; ten Dijke et al., 1994). Type I receptor (TßR-I), type II receptor (TßR-II), betaglycan or endoglin, and type III receptor have been characterized. TßR-I and TßR-II are widely expressed on many different cell types and both are necessary for signal transduction (Lin et al., 1992; Wrana et al., 1992). After binding of the ligands, TßR-I and TßR-II form heteromeric receptor complexes, and transduce intracellular signals (Wrana et al., 1992, 1994; Franzén et al., 1993). Betaglycan and endoglin are structurally

similar surface proteins (Gougos and Letarte, 1990; Lopez-Casillas et al., 1991; Wang et al., 1991), that are not directly involved in the signal transduction of TGF-ß.

Recently, several latent TGF-ß binding proteins (LTBP) have been purified, cloned and subsequently characterized (Kanzaki et al., 1990; Olofsson et al., 1991). The LTBP-1 is involved in the secretion of TGF-ß complex as well as compartmentalization of TGF-ß in the extracellular matrix (Miyazono et al., 1991), which may be important for the regulation of the different functions of TGF-ß in vivo.

TGF-ß acts as growth inhibitor on proliferating basal cells in the skin (Choi and Fuchs, 1990), and TGF-ß2 has been shown to be expressed in the stratifying keratinocytes in developing mouse epidermis (Lyons et al., 1989), and by keratinocytes during the terminal differentiation process (Glick et al., 1989, 1990). Growing keratinocytes in culture secrete TGF-ß in a latent form. However, they begin to secrete TGF-ß in an active form as they differentiate at high Ca^{2+} concentration or confluency (Kato et al., 1995). The idea of TGF-ß as an endogenous negative regulator of proliferating basal cells has further been strengthened by a targeted overexpression of TGF-ß to the epidermis (Sellheyer et al., 1993) as well as by mice with targeted deletion of the TGF-ß gene (Glick et al., 1993). Moreover, grafts of v-rasHa-initiated TGF-ß null keratinocytes progressed to squamous cell carcinoma in athymic mice, suggesting that TGF-ß acted as a tumor suppressor in the initiated keratinocytes (Glick et al., 1994).

In the present study, we investigated the relationship of TGF-ß and TßR expression in murine skin during initiated hair cycle.

Materials and methods

Animals

Female syngenic C56 BL-6 mice (6-9 weeks old) were purchased from Charles River (Hannover, Germany). They were housed in community cages with 12-hour light periods at the Department of Dermatology at the Rudolf-Virchow-Krankenhaus (Berlin) facilities and fed ad libitum with water and «rat/mouse chow» (Agway, Syaracuse, N.Y., USA). Telogen mice were anaesthesized with 30 mg sodium pentobarbital/kg b.w. and hair was stripped with a mixture of beewax and resin (Paus et al., 1991) to induce anagen. This technique generates mature anagen hair follicles indistinguishable from spontaneously developing anagen follicles (Slominski et al., 1991). Animals were sacrificed by an ethyl ether overdose.

Nine different hair cycle stages were investigated (Chase et al., 1951): spontaneously telogen (day 0), anagen I-VI (days 1-18), post-epilation catagen (day 19), and post-epilation telogen (day 25). For each cycle stage five different mice were studied.

Antibodies

The rabbit polyclonal antibodies, Ab 96, Ab 94, and Ab 95, directed against synthetic peptides corresponding to specific amino acid sequences of the latency associated peptide (LAP) portions of the TGF-ß1, -ß2, -ß3 precursors (Olofsson et al., 1992) as well as against synthetic peptides corresponding to the intracellular juxtamembrane parts of the receptors, TßR-I, and TßR-II (Franzén et al., 1993; Yamashita et al., 1994) were used. Antisera were affinity purified using CNBr-activated Sepharose CL-4B (Pharmacia-LKB) columns with immobilized peptides as described before (Waltenberger et al., 1993a,b). An antiserum, Ab 39, against latent LTBP was used (Eklöv et al., 1993).

Immunohistochemistry

Back skin samples from mice were formalin-fixed, paraffin-embedded, cut at 4 µm and deparaffinated. Sections were kept at -200 ºC up to immunostaining. ABC peroxidase immunohistochemistry was performed essentially as described before (Waltenberger et al., 1993b). Sections were treated with 0.3% H_2O_2 to exhaust endogenous peroxidase activity, preincubated with PBS containing 10% normal goat serum (NGS), and incubated with primary antibodies in a humidified chamber overnight at 40 ºC. The Ab 94, Ab 95, and Ab 96 antibodies were used at a concentration of 5 µg/ml, and anti-TßR-I and anti-TßR-II antibodies were used at a concentration of 3 µg/ml. The Ab 39 antibody was used at a dilution of 1:40. Then tissues were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, C.A., USA) at a 1:200 dilution in PBS containing 1% bovine serum albumin (BSA) for 45 minutes, followed by incubation with Vectastain ABC Elite complex (Vector Laboratories) for 30 minutes. The immunoreaction was visualized by using 3-amino-9-ethylcarbazole (Merck) as a chromogen in the presence of 0.002% hydrogen peroxide for 10-15 minutes.

Between each step tissues were rinsed in PBS, and finally counterstained with Mayer's hematoxylin and mounted in glycerol-gelatin. To exclude the non-specific reactions of secondary antibodies or ABC complexes, primary antibody solutions were replaced by 1% BSA in PBS. Specificities of the affinity-purified antibodies were confirmed by the fading of the stainings, when antibodies had first been preincubated with an excess molar ratio of the corresponding peptide antigens (Eklöv et al., 1993).

Results

General immunoreactivity

The mouse skin remained completely negative for antibodies Ab 94 and anti-TßR-II. Antibodies Ab 96, Ab 95, Ab 39, and anti-TßR-I produced a variable staining dependent on hair cycle phases. Anti-TßR-II gave

Table 1. Expression of TGF-ß isoforms in murine skin.

	ANAGEN	CATAGEN	TELOGEN
Outer hair root sheath	TGF-ß1 TßR-I (late)	TGF-ß1 (early) TßR-I (early)	
Inner hair rootsheath	-	-	-
Bulge	-	-	-
Bulb	-	-	-
Papilla	-	-	-
Sebaceus glands	LTBP TßR-II	TßR-II	LTBP TßR-II
Connective tissue	TGF-ß3 (early)	TGF-ß3	TGF-ß3 TßR-I

positive signals throughout the hair cycle.

Expression of TGF-ß1 and 3 (Fig. 1)

Dermal connective tissue cells expressed TGF-ß3 with moderate intensity during early anagen (days 0-3), and post-epilation catagen and telogen. TGF-ß1 was expressed in the inner hair root sheath during days 5-7 (anagen) and 19-21 (anagen/catagen switch).

Expression of LTBP (Fig. 2)

Antibody Ab 39 against LTBP was fixed by different skin cells. Sebocytes gave moderate to strong immunostainings during days 5-8 (anagen). During telogen a weak to moderate LTBP-positivity was seen in the connective tissue of the dermis (days 25-34).

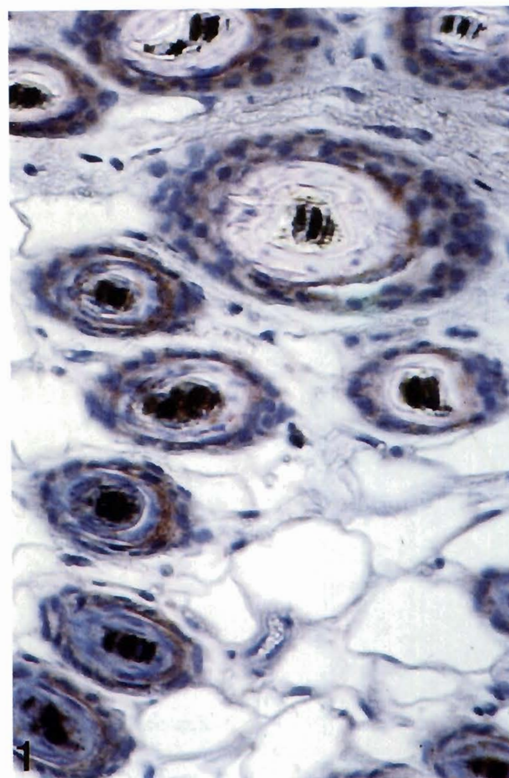
Expression of TßR-s (Fig. 3)

Anti-TßR-II gave positive signals for sebocytes throughout the hair cycle. A moderate connective tissue staining was also seen. Antibody against TßR-I stained sebocytes during early anagen (days 3 to 8). The outer hair root sheath was labeled at the anagen/catagen switch; i.e. days 17 to 19. During telogen, the dermal tissue disclosed a weak staining intensity for TßR-I.

Discussion

The hair follicle is characterized by cyclic growth and regression (Chase et al., 1951). The stem cell reservoir of hair follicles has been identified in the bulge region (Cotsarelis et al., 1990). We have demonstrated recently, that the murine bulge temporarily express the vasoactive intestinal peptide (VIP) receptor protein in late anagen. We assumed, that VIP receptor expression may be related to an abrogation of anagen hair growth (Wollina et al., 1995).

In the present paper expression of another potent growth inhibitor has been investigated. We report the first profile of TGF-ß isoforms and receptor expression and their relationships to neuropeptide VIP in murine



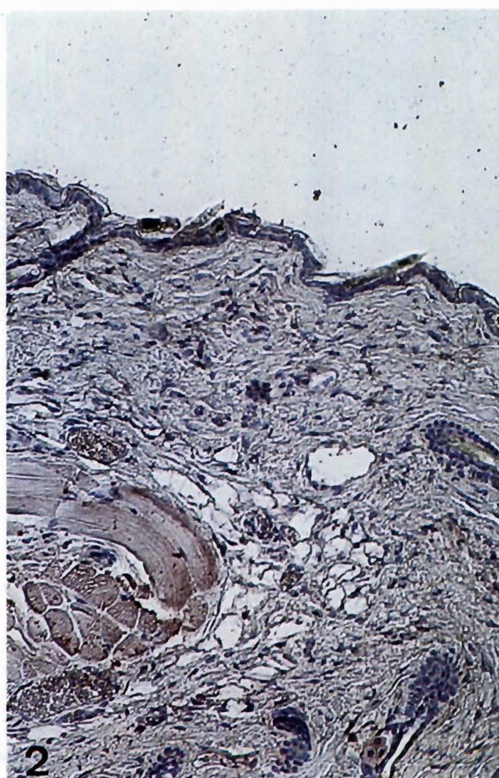
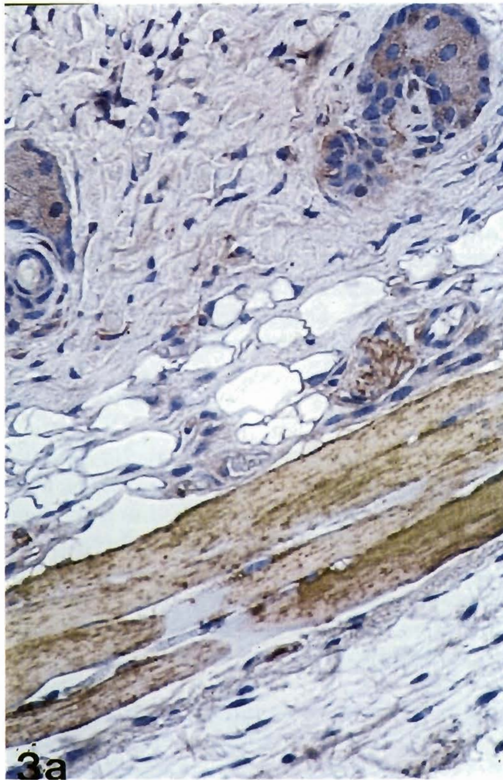


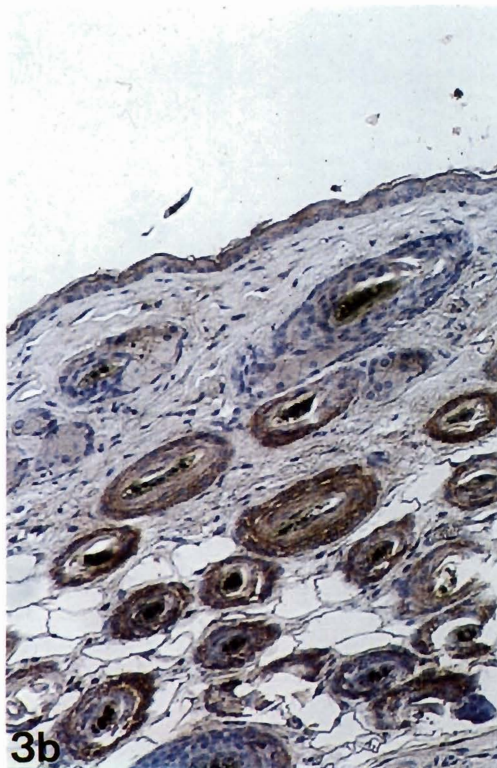
Fig. 1. Expression of TGF-ß1 in inner hair follicle sheath (day 19). x 400

Fig. 2. Expression of LTBP in sebocytes (day 5). x 400



hair follicles. In different types of cells TGF-ßs have been characterized as growth inhibitors (Roberts and Sporn, 1990). In several benign tissues, TGF-ßs are produced in a complex with LTBP (Kanzaki et al., 1990; Eklöv et al., 1993).

TGF-ß1 was expressed in the early anagen and along the anagen/catagen switch in the epithelium of the inner hair root sheath. Sebocytes are another site of TGF-ß expression. Expression of LTBP has been observed in sebocytes. LTBP is involved in secretion and extracellular matrix binding of TGF-ßs (Miyazono et al., 1991; Olofsson et al., 1992). The inner and outer root sheath lacked immunoreactivity for LTBP. It may be possible that TGF-ß1 produced by inner sheath epithelium is bound to sebocytes by LTBP. Probably, TGF-ß-LTBP complex approaches to sebocytes where the latent TGF-ß becomes activated through LTBP and the activated native TGF-ß binds to the receptor. In this case type II receptors are necessary for binding. TGF-ß1 is able to increase fibroblast glycosaminoglycan synthesis in human fibroblasts in a dose-dependent manner. TGF-ß is the only factor known to induce the production of the key elements of extracellular matrix (Roberts and Sporn, 1990; Messadi et al., 1994). Remodelling of the dermal root sheath depends upon matrix synthesis. Dermal tissue, however, gave some staining for LTBP during telogen but lacked antibody binding through anagen and catagen. Recently, LTBP has been cloned from human foreskin fibroblasts



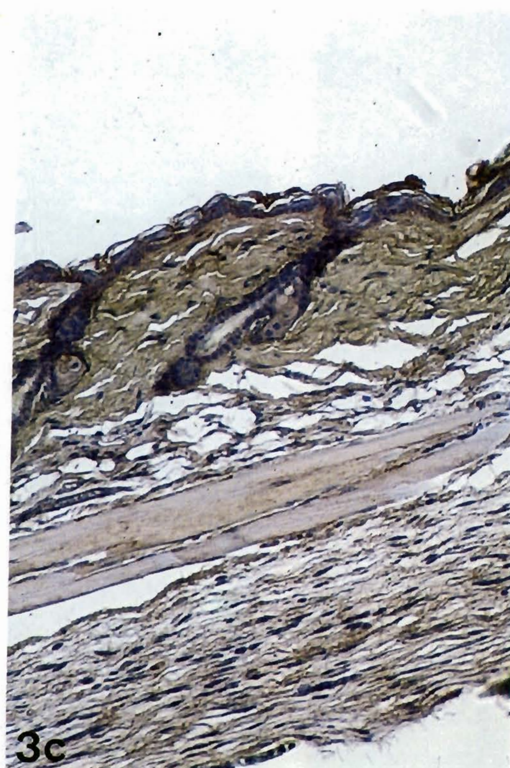


Fig. 3. Expression of TBR-I **a.** Day 3. x 400. **b.** Day 19. x 200. **c.** Day 25. x 200

(Kanzaki et al., 1990). Dermal LTBP immunoreactivity may also result from passive binding after secretion by epithelial cells.

Another mechanism of action of TGF-ß2 is its binding to TßR-I (Lin et al., 1992; Wrana et al., 1992). During early anagen, receptor expression was evident in sebocytes. At the anagen/catagen switch TßR-I was expressed in the outer hair root sheath, but lacked in the inner layer. Antigen expression below the threshold of detectability may explain the lack of binding of antibodies Ab 94. Anti-TßR-II was not bound within the hair follicle epithelium but in sebaceous glands and connective tissue. There was no significant variation of either staining intensity or localization.

The mechanisms of interaction of both the inner and outer hair root sheath have yet not been revealed. Our data may show that the different epithelial layers of the hair follicle are regulated separately. On the other hand, TGF-ß1 expression during early anagen in the inner hair root sheath followed by TßR-I in the outer hair root sheath at the anagen/catagen switch suggests that TGF-ß1 may be a candidate peptide for interactions between epithelial cell layers of the hair follicle. Probably, it is involved in the initiation of catagen. Since TßR-I and -II are expressed in sebaceous glands, they may be another target for the action of TGF-ß.

As the cyclic growth and regression of the hair follicle is a model for developmentally-regulated epithelial-mesenchymal-neuroectodermal interactions, the study of TGF-ß in hair research promises an insight into the interactivity of the epithelial cells layers and sebocytes.

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