Involvement of Transforming Growth Factor-β2 in Catagen Induction During the Human Hair Cycle

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The involvement of transforming growth factor-β isoforms in the induction of the regressing phase (catagen) of human hair follicles were examined *in vivo*. In the growing phase (anagen), transforming growth factor-β1 was detected at the hair cuticle and connective tissue sheath. Transforming growth factor-β2 was restricted to the outermost cell layer of the outer root sheath. Transforming growth factor-β3 was observed in the precortical hair matrix of anagen hair follicles. During the anagen–catagen transition phase, strong transforming growth factor-β2 immunoreactivity appeared in the lower bulb matrix cells adjacent to the dermal papilla. In addition, transforming growth factor-β2 and transforming growth factor-β type II receptor were colocalized in the regressing epithelial strands, where terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling-positive apoptotic cells were also found. Transforming growth factor-β1 and transforming growth factor-β3 were mostly negative in the strand. Using an organ culture system, we investigated whether transforming growth factor-β2 and its antagonists affected the transition process. Elongation of hair was significantly suppressed by transforming growth factor-β2. Next, a neutralizing antibody and fetuin, a potent transforming growth factor-β antagonist was tested. In the presence of the antibody as well as fetuin, hair follicles were markedly elongated in a concentration-dependent manner. These results strongly suggest that transforming growth factor-β2 plays an essential part in the induction of the catagen phase of the human hair cycle. ***Key words: apoptosis/catagen induction/hair cycle/transforming growth factor-β. J Invest Dermatol 118:993–997, 2002***

Transforming growth factor (TGF)-β is a family of multifunctional cytokines. Three isoforms, designated as TGF-β1, TGF-β2, and TGF-β3, are present in mammals (Roberts and Sporn, 1990). The signals of all three TGF-β are mediated through a heteromeric complex of type I and type II TGF-β receptors, which have serine/threonine kinase activities (Massague, 1998). Recently, it was revealed that the signal transduction downstream of the TGF-β receptors involves the Smad family (Hoodless and Wrana, 1998). TGF-β regulate various physiologic reactions, such as cellular growth and differentiation, morphogenesis, angiogenesis, adhesion and chemotaxis, extracellular matrix formation (Massague *et al*, 1992), and apoptotic cell death (Oberhammer *et al*, 1992; Ohta *et al*, 1994). The three TGF-β isoforms are differentially expressed in many tissues, including whisker follicles during mouse embryogenesis (Schmid *et al*, 1991). Three null mutation studies revealed characteristic and only partially overlapping phenotypes (Letterio *et al*, 1994; Dickson *et al*, 1995; Kaartinen *et al*, 1995; Snaford *et al*, 1997), suggesting that these isoforms have distinct functions, depending on their expression sites, in embryogenesis, morphogenesis, and various biologic reactions.

The hair cycle is a highly regulated process. Three phases have been defined for the mammalian hair cycle: anagen (growing phase), catagen (regressing phase), and telogen (resting phase) (Kligman, 1959). It is important to understand the mechanism of hair cycle regulation in order to prevent hair loss. Recent investigations have revealed involvement of the Shh and Wnt signal pathways in hair morphogenesis, as well as in the hair induction process (St-Jacques *et al*, 1998; Chiang *et al*, 1999; Millar *et al*, 1999). A previous study using a single hair follicle organ culture clearly demonstrated that TGF-β could induce morphologic changes and apoptotic cell death indistinguishable from those seen in human catagen hair follicles (Soma *et al*, 1998).

In this study, we report that among TGF-β isoforms, TGF-β2 is the key molecule that modulates catagen entry in the human hair cycle *in vivo and in vitro*. Furthermore, it is shown that TGF-β antagonists suppress catagen-like morphologic changes, resulting in the elongation of hair follicles.

MATERIALS AND METHODS

Materials Williams E medium, Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, streptomycin, and fungizone were supplied by Life Technologies (Rockville, MD). All other tissue culture reagents, including bovine fetuin, were purchased from Sigma (St Louis, MO).

Tissue preparation Human scalp skin specimens were obtained from plastic surgery. Scalp skin pieces and isolated hair follicles washed with ice-cold phosphate-buffered saline (PBS) were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) at 4°C for 4 h, and embedded in paraffin wax. Serial sections of 3–5 μm were cut and mounted on slides precoated with silane (Matsunami, Tokyo, Japan). For double staining of TGF-β receptor type II and apoptotic cells, frozen sections with 10 μm were used.

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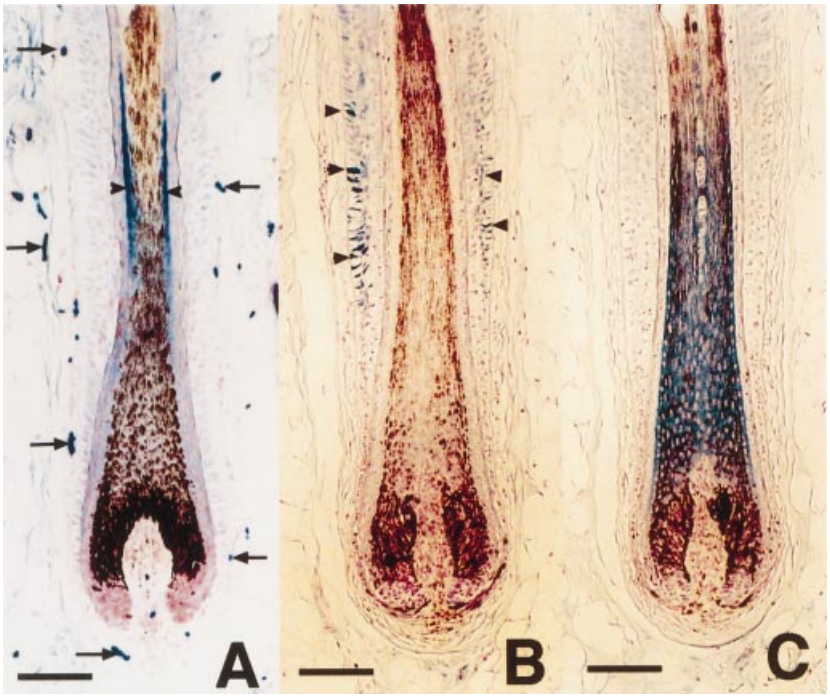

Figure 1. Immunohistochemical detection of TGF-β isoforms in human anagen hair follicles. Human anagen hair sections were prepared and stained with antibodies specific to TGF-β isoforms. TGF-β1 was seen in the connective tissue sheath cells (indicated by *arrows*) and the hair cuticle (indicated by *arrowheads*) (*A*). TGF-β2 immunoreactivity was detected at the outermost cell layer of the outer root sheath in late anagen (indicated by *arrowheads*) (*B*). TGF-β3 immunoreactivity was found in the keratinizing matrix cells (*C*). *Scale bars*: 100 μm.

Keratin adsorption and antigen adsorption of antibodies All antibodies were subjected to keratin treatment in order to reduce nonspecific binding of antibodies to epidermal and follicular keratins. Human cornified cell extract was washed three times with Tris-buffered (pH 7.6) saline, once with acetone, and then air-dried. Antibodies diluted to the working concentration were incubated with 100 mg per ml keratin powder in PBS containing 3% bovine serum albumin with vigorous shaking at 4°C overnight. After centrifugation, supernatants were collected for immunohistochemistry. Rabbit polyclonal antibodies specific to human TGF-β1 (SC-146), TGF-β2 (SC-90), TGF-β3 (SC-82), and TGF-β receptor type II (SC-220) were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-TGF-β1 antibody was used at a dilution of 1:20, and anti-TGF-β2, anti-TGF-β3, and TGF-β type II receptor antibodies were used at 1:100. In order to test the specificity of antibodies, each antibody was incubated with 100 μg per ml of an appropriate immunogen peptide for 1 h at room temperature. After centrifugation, the supernatant was adjusted to the same concentration as the original antibody, and used in immunohistochemistry as a negative control. Peptides used for immunoadsorption were SC-146P, SC-90P, SC-82P, and SC-220P (Santa Cruz) for anti-TGF-β1, anti-TGF-β2, anti-TGF-β3, and anti-TGF-β, type II receptor antibodies, respectively.

Immunohistochemical detection of TGF-β isoforms Paraffin-embedded tissue sections were deparaffinized, rehydrated, and equilibrated in PBS for 10 min at room temperature. After blocking with 10% normal goat serum at room temperature for 20 min, sections were incubated with each antibody treated with keratin powder at 4°C overnight. For TGF-β1 immunostaining, but not others, tissue sections were moderately digested with 10 μg per ml proteinase K (Nakalai Tesque, Tokyo, Japan) in PBS at 37°C for 30 min before the blocking procedure. A biotinylated rabbit anti-mouse IgG (Nichirei, Tokyo, Japan) was used as a secondary antibody followed by reaction with peroxidase-conjugated streptavidin (Nichirei). Tetramethylbenzidine (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was used as a color-developing reagent in Tris-buffer (pH 7.6) containing 0.01% H_2O_2. The sections were counterstained with nuclear fast red (Sigma). In the case of anagen hairs, more than 60 follicles from six patients (minimum 10 follicles from each patient) were used for each antibody staining. For catagen hairs, 20 follicles (two to four catagen follicles per patients) were used for the staining.

Double immunodetection of terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)-positive cells and TGF-β receptor type II For visualization of apoptotic cells and TGF-β receptor type II, cryostat sections were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) at room temperature for 20 min and subjected to immunohistochemical staining using Texas Red® dye-conjugated anti-rabbit IgG (donkey) as a secondary antibody. TUNEL reaction was performed using a fluorescein *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Isolation and culture of human hair follicles Human hair follicles were isolated and cultured as previously described (Soma *et al*, 1998). Isolated anagen follicles were maintained in 1 ml of Williams E medium containing 100 U penicillin per ml, 10 μg streptomycin per ml, and 2.5 μg fungizone per ml (basal medium) at 37°C in 5% CO_2 and 95% air. To evaluate the effects of TGF-β antagonistic molecules, hair follicles were incubated with 10 or 20 μg per ml of anti-TGF-β neutralizing antibody (Genzyme, Cambridge, MA) or 20 μg per ml of normal mouse IgG1 (Chemicon, Temecula CA). In separate experiments, fetal bovine serum fetuin or bovine serum albumin was added to the basal medium. Fetuin is a potent TGF-β antagonist, having a homologous domain to TGF-β type II receptor binding site (Demetrious *et al*, 1996). Culture medium was replaced every 3 d unless otherwise mentioned. Tissue sections of hair follicles were prepared as described above. Ten hair follicles were used for each sample concentration. The same experiment was repeated three times using hair follicles obtained from three different patients.

RESULTS

Each TGF-β isoform is localized in distinct portions of human anagen hair follicles In late anagen hair follicles, TGF-β1 immunoreactivity was detected in the hair cuticle and the connective tissue sheath cells (**Fig 1*A***). The hair cuticle was also strongly positive for anti-TGF-β1 antibody. TGF-β2 was detected at the outermost layer of outer root sheath cells, which showed an elongated and palisade structure (**Fig 1*B***). The lower bulb portion of anagen hair follicles was negative for TGF-β2. Anti-TGF-β3 antibody showed strong staining at the hair cortex and the hair cuticle in the keratogenous zone of the upper hair bulb (**Fig 1*C***). Hair medulla was not stained (data not shown). Treatment of each antibody with the appropriate blocking peptide completely abolished the positive staining for TGF-β1, TGF-β2, or TGF-β3, on hair follicles, confirming the specificity of each antibody

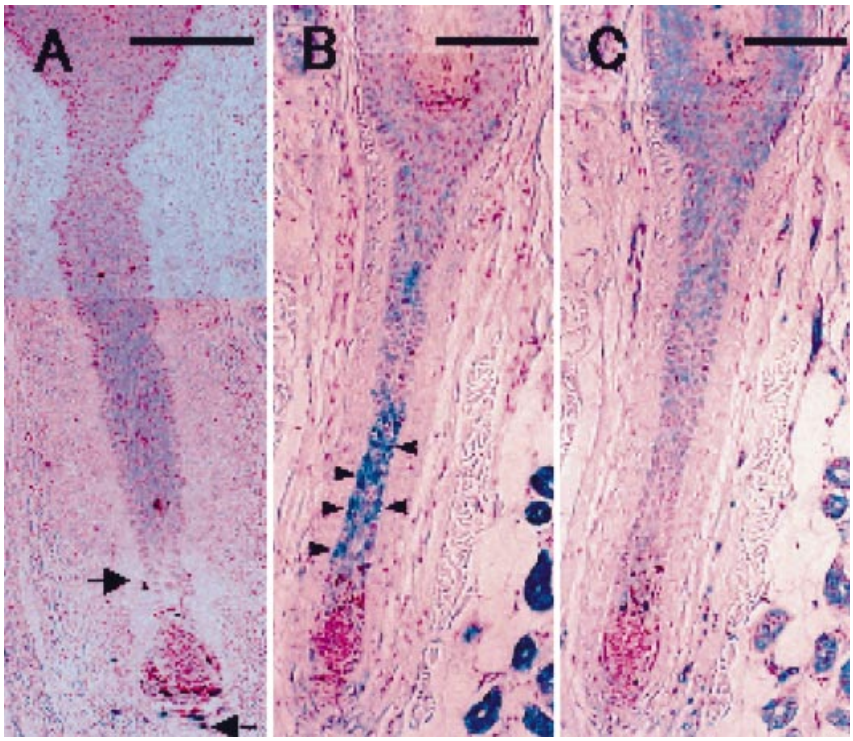

Figure 2. Immunohistochemical detection of TGF-β isoforms in human catagen hair follicles. Catagen hair follicles were identified from serial sections and immunostained with each isoform-specific antibody. Only a few positive cells for TGF-β1 were observed in the connective tissue sheath of catagen hair (indicated by *arrows*) (*A*). TGF-β2 immunoreactivity was detected in the regressing epithelial strand (ES) in late catagen (indicated by *arrowheads*) (*B*). TGF-β3 immunoreactivity was no longer observed in the catagen hair (*C*). *Scale bars*: 100 μm.

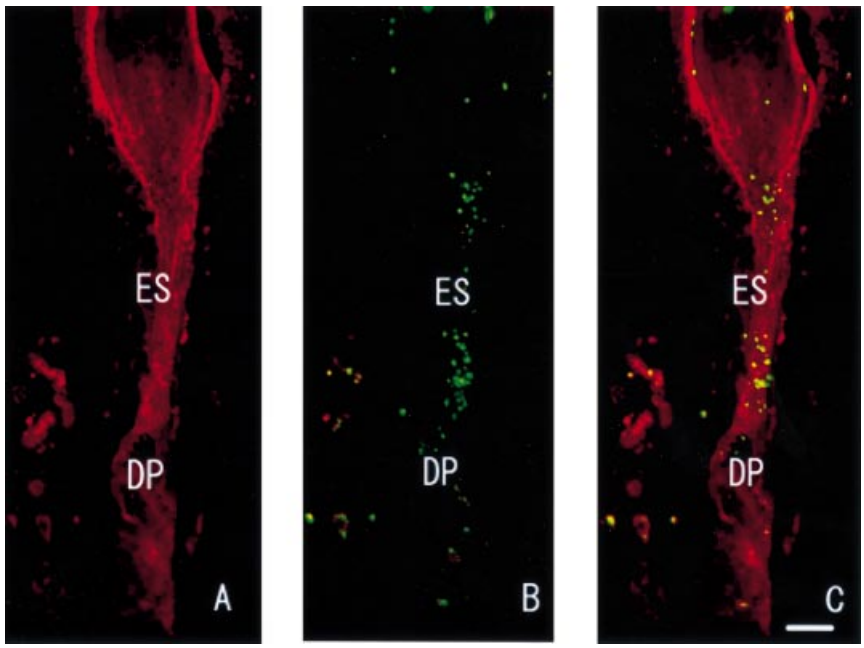

Figure 3. TUNEL-positive cells localized among the TGF-β type II receptor-positive cells. Human catagen hair follicles were isolated and double staining was carried out for the detection of TGF-β type II receptor and apoptotic cells. TGF-β type II receptor immunoreactivity was found in a broad area of regressing epithelial component (*A*) including epithelial strand (ES). TUNEL-positive apoptotic cells were detected in the same area (*B*). Dermal papilla cells (DP) were negative for both stainings. A superposition of images (*A*) and (*B*) is shown in (*C*). *Scale bars*: 100 μm.

used in this study. We also confirmed loss of the positive staining when the primary antibodies were omitted (data not shown).

TGF-β isoforms change their localization at catagen In late catagen hair follicles, only a few cells in the connective tissue sheath were positive for TGF-β1 (**Fig 2*A***). Regressing hair follicles were not stained by anti-TGF-β1 antibody. Strong TGF-β2 immunoreactivity was detected in the regressing epithelial strand (**Fig 2*B***), indicating an important role of TGF-β2 in the catagen progression. Specific staining for TGF-β3 seen in the keratogenous zone in the anagen phase was no longer observed in catagen hair follicles (**Fig 2*C***). Weak staining was still observed in the inner cell layers of the outer root sheath.

Apoptotic cells are present in the type II receptor-positive regressing epithelial strand In order to analyze involvement of the TGF-β signal pathway in apoptotic processes, double staining for TGF-β type II receptor and TUNEL-positive cells was carried out. In late catagen hair follicles, TGF-β type II receptor was found in a relatively wide range of the epithelial component, including the edge of club hair and regressing epithelial strand (**Fig 3*A***). TUNEL-positive apoptotic cells were also detected in this area, although their localization was more restricted (**Fig 3*B***). The superimposed image showed that TUNEL-positive cells were found in the TGF-β type II receptor-positive cells (**Fig 3*C***).

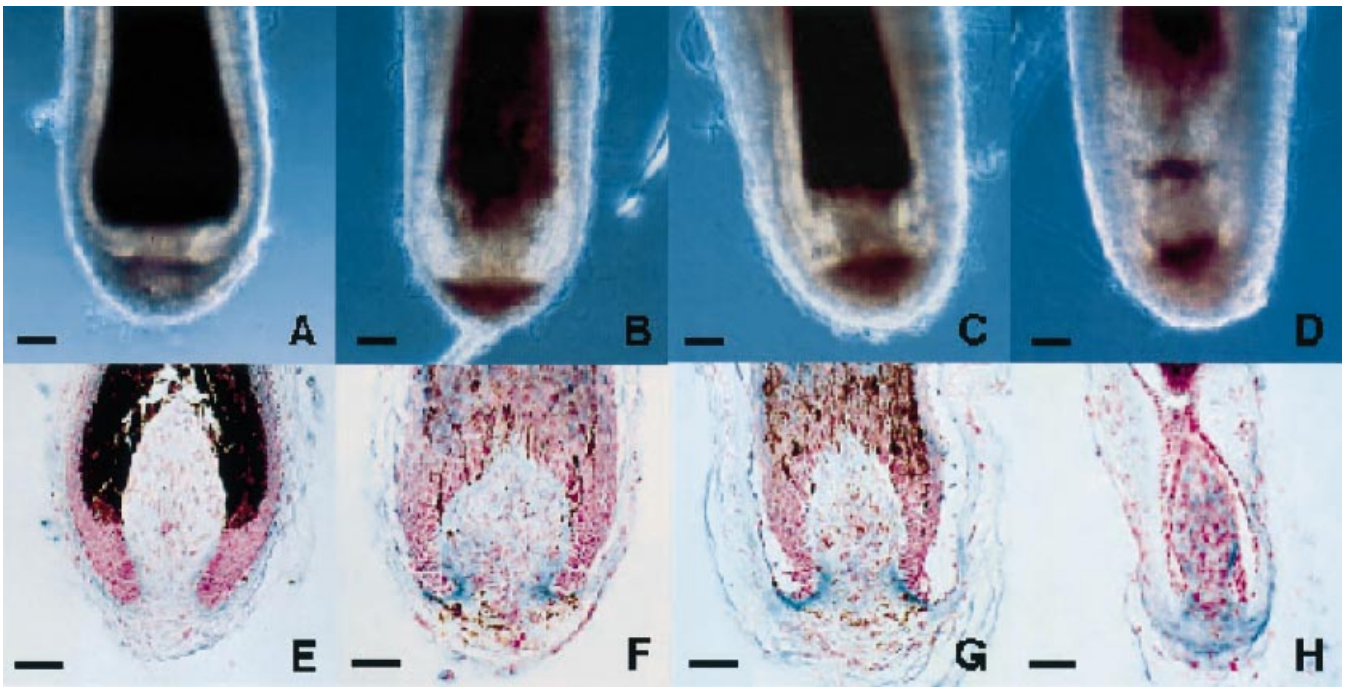

Figure 4. Upregulation of TGF-β2 in the boundary area of germinative hair matrix cells and DP cells in early catagen hair follicles. Hair follicles were isolated and classified according to the degree of catagen morphology. In late anagen hair follicles (*A*), the germinative matrix cells and DP cells were almost negative for TGF-β2 immunoreactivity (*E*). In early catagen hair follicles characterized by the upward movement of the hair shaft from the DP (*B, C*), strong immunoreactivity for TGF-β2 was observed in the DP around the germinative matrix cells (*F, G*). As the catagen phase progressed, the DP and the basal plate became positive for TGF-β2 (*H*).*Scale bars*: 40 μm.

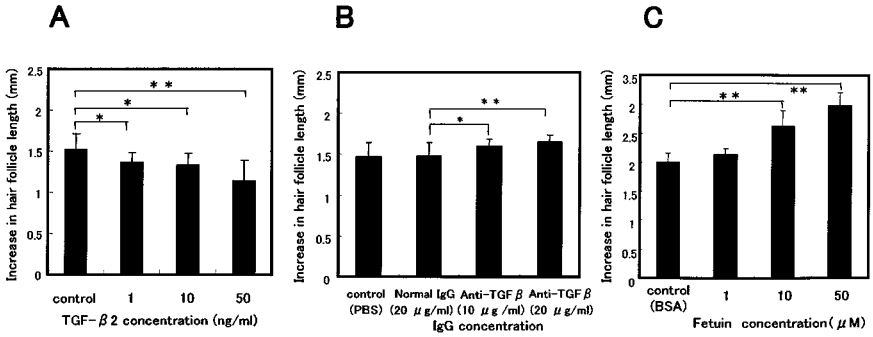

Figure 5. Effects of TGF-β2 and TGF-β antagonists on hair elongation. Anagen hair follicles were isolated and cultured in the presence of TGF-β2 (*A*), neutralizing antibody (*B*), or a potent TGF-β antagonist, fetuin (*C*). Cultures were performed for 5 d for (*A, B*), or 8 d for (*C*). As controls, the same concentrations of normal rabbit IgG was used for (*B*) and bovine serum albumin was added to (*C*). The length of hair follicles was measured under a microscope. Ten follicles were used for each concentration. Results are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ (one-way ANOVA and Dunnett's *post-hoc* procedure).

Strong deposition of TGF-β2 occurs in the hair bulb during the transition phase In order to determine whether TGF-β2 plays a part in the early catagen phase, hair follicles at the transitional stages were isolated from human scalp skin by microdissection based on the early morphologic changes of hair bulbs. They were easily detectable as upward removal of the hair shaft from the dermal papilla cells (DP), decrease of hair color due to the downregulation of melanogenesis and increased thickness of the connective tissue sheath. Hair follicles were immediately fixed and stained for TGF-β2. **Figure 4** shows an example of a series of isolated hair follicles exhibiting anagen–catagen transition (**Fig 4*A–D***). In the late anagen hair follicle, a faint staining for TGF-β2 could be detected around the germinative matrix cells (**Fig 4*E***). In the very early catagen hair follicles, which are characterized by the removal of melanocytes above the DP (**Fig 4*B***, ***C***), strong TGF-β2 deposition was demonstrated in the lower part of the boundary area between the DP and the germinative matrix cells (**Fig 4*F***, ***G***). With the progression of the catagen phase, the intercellular space of DP became positive and the basal plate was mostly positive for TGF-β (**Fig 4*H***). In the organ culture system, we confirmed essentially the same TGF-β2 staining pattern, starting as early as at 3 d of culture (data not shown).

Effects of TGF-β and its neutralizing antibody on hair growth and morphology in organ culture system We then tested the effect of TGF-β2 on the elongation of hair follicles cultured *in vitro*. Hair elongation was suppressed in the presence of

TGF-β2, especially at 50 ng per ml concentration ($p < 0.01$) (**Fig 5*A***). Overall elongation of control hair follicles reached 1.5 mm from the starting point, whereas that of TGF-β2-treated follicles was approximately 1.2 mm during the 5 d culture period. The anti-TGF-β neutralizing antibody (Genzyme), which inhibits TGF-β action, can neutralize all three TGF-β isoforms in cell culture (Dasch *et al*, 1989). To block endogenous TGF-β2 seen around the germinative matrix cells, we used this antibody in our organ culture system. Hair growth was stimulated in the presence of the neutralizing antibody and showed about 7% ($p < 0.1$) and 11% increases ($p < 0.01$) at the concentrations of 10 μg per ml and 20 μg per ml of the antibody, respectively (**Fig 5*B***).

A recent study revealed that fetuin and some synthetic peptides derived from both fetuin and TGF-β type II receptor could work as antagonists for TGF-β activity (Demetrious *et al*, 1996). We also examined whether fetuin could have similar effect, as seen by the neutralizing antibody. In the presence of fetuin, hair growth was markedly and significantly stimulated at day 8 in a concentration-dependent manner, compared with the effect of bovine serum albumin (**Fig 5*C***).

DISCUSSION

We examined the regressing phase of the human hair cycle to identify the key molecule responsible for catagen induction. Our results revealed that TGF-β2 plays an essential part in the induction of catagen. TGF-β is not only a potent growth inhibitor (Glick *et al*, 1991; Alexandrow and Moses, 1995) but also an apoptosis inducer (Oberhammer *et al*, 1992; Ohta *et al*, 1994) of epithelial cells, including interfollicular and follicular epithelium. Recently, we demonstrated that TGF-β2 suppressed the proliferation of hair follicle cells and accelerated the catagen-like morphologic changes associated with increased apoptosis of these cells (Soma *et al*, 1998). The most striking finding of our study was the strong deposition of TGF-β2 at the boundary area between the germinative matrix and the DP during the anagen–catagen transition (**Fig 3**). The germinative matrix cells cease to grow as soon as the hair follicle enters the catagen phase. In this study, it was also demonstrated that TGF-β2 markedly inhibited hair elongation in the organ culture system. Furthermore, as the catagen phase proceeds, TGF-β2- and TUNEL-positive cells were found in similar areas of the regressing epithelial strand (**Figs 2*B*** and **3*B***), where the TGF-β type II receptor was strongly positive (**Fig 3*A***). Taking all these results into account, induction of catagen is suggested to take place as follows. In late anagen, deposition of TGF-β2 occurs around the germinative matrix cells, where proliferating cells reside. High levels of TGF-β2 result in growth inhibition and further lead to the apoptosis of these cells.

An inductive role of TGF-β2 is also supported by the inhibition studies. We tried to suppress TGF-β action by using two antagonistic molecules, a neutralizing antibody and fetuin. In the culture system, we found that the catagen-like morphologic changes well reflected the *in vivo* condition, with the upregulation of TGF-β2 and the same deposition pattern of TGF-β2 in the lower part of the hair bulb. Both antagonistic molecules promoted remarkable hair follicle elongation, suggesting suppression of the catagen entry process by TGF-β2. These lines of evidence strongly indicate that TGF-β2 plays an essential part in catagen induction of human hair follicles. Furthermore, inhibition of TGF-β action can prolong the anagen phase in human hair.

TGF-β may also be involved in the early catagen signaling in the mouse hair cycle. TGF-β2 was expressed immediately before catagen (Seiberg *et al*, 1995). Moreover, TGF-β1 injection in mice resulted in the inhibition of anagen and caused premature catagen induction (Foitzik *et al*, 2000). Maximal immunoreactivity as well as mRNA levels of TGF-β type II receptor was observed in the anagen VI to early catagen transformation in the mouse hair cycle (Paus *et al*, 1997).

In summary, this study has elucidated the spatiotemporal localization of TGF-β isoforms during the human hair cycle. Upregulation and specific localization of TGF-β2 in the anagen–catagen transition phase would be the key to initiate the process of catagen. The effect of antagonistic molecules to TGF-β strongly supports this hypothesis, providing the basis for a fuller understanding of hair cycle regulation.

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