

A Potential Suppressor of TGF- β Delays Catagen Progression in Hair Follicles

Yumiko Tsuji,* Sumiko Denda,* Tsutomu Soma,*† Laurel Raftery,† Takashi Momoi‡ and Toshihiko Hibino*

*Shiseido Life Science Research Center, Yokohama, Japan; †MGH/Harvard Cutaneous Biology Research Center, Charlestown, MA, USA; ‡National Institute of Neuroscience, Tokyo, Japan

TGF- β plays important roles in the induction of catagen during the hair cycle. We examined whether TGF- β 2 could activate a caspase in human hair follicles. Using active caspase-9 and -3 specific antibodies, we found that TGF- β 2 activated these caspases in two regions, the lower part of the hair bulb and the outer layer of the outer root sheath. In addition, we searched for a plant extract that can effectively suppress TGF- β action. We found that an extract of *Hydrangea macrophylla* reduced synthesis of a TGF- β -inducible protein. We con-

firmed that the extract has a potential to promote hair elongation in the organ culture system. Furthermore, it delayed *in vivo* progression of catagen in a mouse model. Our results suggest that the induction of catagen by TGF- β is mediated via activation of caspases and that a suppressor of TGF- β could be effective in preventing male pattern baldness. Keywords: TGF- β /hair cycle/catagen induction/caspase/apoptosis. JID Symposium Proceedings 8:65–68, 2003

Hair loss is the result of premature entry into catagen due to various causes, including androgens (Jahoda, 1998), anticancer drugs (Paus *et al*, 1994), and inflammatory reactions (Galbraith *et al*, 1984). Three distinct 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 catagen induction mechanism in order to find ways to prevent hair loss.

We have previously reported two important findings in this process. First, catagen is characterized by the massive apoptotic cell death of follicular epithelial cells (Soma *et al*, 1998). Second, TGF- β 2 appears in the lower part of the boundary area between the dermal papilla cells and the germinative matrix cells during anagen-catagen transition phase *in vivo*. Using hair follicle organ culture we have clearly demonstrated that exogenous TGF- β could induce morphological changes and apoptotic cell death indistinguishable from that which is seen in human catagen hair follicles. In progressing stages of catagen, TGF- β 2-, TGF- β RII- and TUNEL-positive cells were colocalized at the regressing epithelial strand. Furthermore, we demonstrated that a neutralizing antibody to TGF- β prevented morphological changes and resulted in the elongation of hair shafts (Soma, submitted). These findings strongly suggest that TGF- β plays an essential role in catagen induction via activation of an apoptotic pathway.

Caspases are well-known expeditors of apoptosis and 14 members are known in mammals (Kumar, 1999). Once the caspase cascade is activated, it inevitably leads to apoptotic cell death. It is now widely accepted that sequential activation of caspases is required in apoptosis processes.

In the present study, we investigated whether TGF- β 2 could activate the expedition of apoptosis, the caspase cascade. In addition, we searched for a plant extract that could effectively suppress

TGF- β action in catagen progression. We identified such an extract and confirmed that it can delay catagen progression *in vivo*.

MATERIALS AND METHODS

Culture of human hair follicles Human scalp skin specimens were obtained from plastic surgery. Human hair follicles were isolated and cultured according to the method of Philpot *et al* (1990). In order to analyze the effect of TGF- β on caspase activation, anagen hair follicles were incubated in the presence of TGF- β 2 (20 ng/ml) for 2 days and then 10 μ m frozen sections were prepared. To evaluate TGF- β antagonistic molecules, the length of hair follicles in the presence or absence of test materials was measured by light microscopy. Ten hair follicles were used for each sample to perform statistical studies.

Double immuno-detection of tunel-positive cells and active caspases It was important to identify the site of caspase activation for the better understanding of the catagen induction mechanism. For this purpose, we used cleavage site-directed antibodies to caspase-9 (Fujita *et al*, 2000) and -3 (Kouroku *et al*, 1998), respectively. These antibodies do not recognize proforms, and only active enzymes can be detected. Using these antibodies, we studied the relationship between the appearance of TUNEL-positive cells and caspase activation. Cryostat sections were fixed with acetone at room temperature for 20 min and incubated with the active caspase-3 or -9 specific antibodies at 4°C overnight. Texas Red[®] dye-conjugated anti-rabbit IgG (donkey) was used as a secondary antibody. The TUNEL reaction was performed using a fluorescein *in situ* cell death detection kit (Roche Diagnostics) according to the manufacturer's instructions.

TGF- β suppression activity of plant extracts Dermal papilla (DP) cells were isolated according to the method of Itami *et al* (1990) and used within 4 passages. DP cells were incubated with plant extracts in the presence of TGF- β 2 (1 ng/ml) for 24 h. TGF- β suppression was assessed by monitoring the amount of plasminogen activator inhibitor 1 (PAI-1), a TGF- β -responsive gene product, in culture medium using TintElize PAI-1 (Biopool, CA). Cytotoxicity of every extract was tested using AlamarBlue (Biopool) according to the manufacturer's instructions.

***In vivo* test of catagen suppression** Female C57BL/6 mice (approximately 8 weeks of age) were used in this study, since the hair

Accepted for publication February 1, 2003

Reprint requests to: Toshihiko Hibino, Shiseido Life Science Research Center, 2-12-1 Fukuura, Kanazawa-ku, Yokohama 236-8643, Japan; E-mail: toshihiko.hibino@to.shiseido.co.jp

cycle is well characterized in this mouse (Muller-Rover *et al*, 2001). Hair induction was obvious at day 10 after depilation by wax. An extract was topically applied on the back once a day for 10 days after hair induction. Back skin sections were prepared, stained with HE, and the catagen stage of each hair follicle was scored. Stages of catagen were determined based on the criteria described by Muller-Rover *et al* (2001). One hundred hair follicle sections were graded for each mouse, and 5 mice were used for each group.

RESULTS

TGF- β 2 activates caspases in cultured hair follicles To understand the role of TGF- β 2 in relation to apoptosis, human hair follicles were cultured in the presence of TGF- β 2 for 2 d and then examined for the activation of caspase-9 and caspase-3. We focused on these two caspases, since they represent the initiator caspase and the effector caspase, respectively (Kumar, 1999). Using an active caspase-3 in two regions, including the cells in the lower part of the germinative matrix and the outer layer of the outer root sheath (Fig 1A). Caspase-9 was also activated in similar areas (Fig 1B). Only a few cells were positive for active caspase-3 in hair follicles cultured without TGF- β 2 (Fig 1C). Dual staining for active caspase-3 and TUNEL

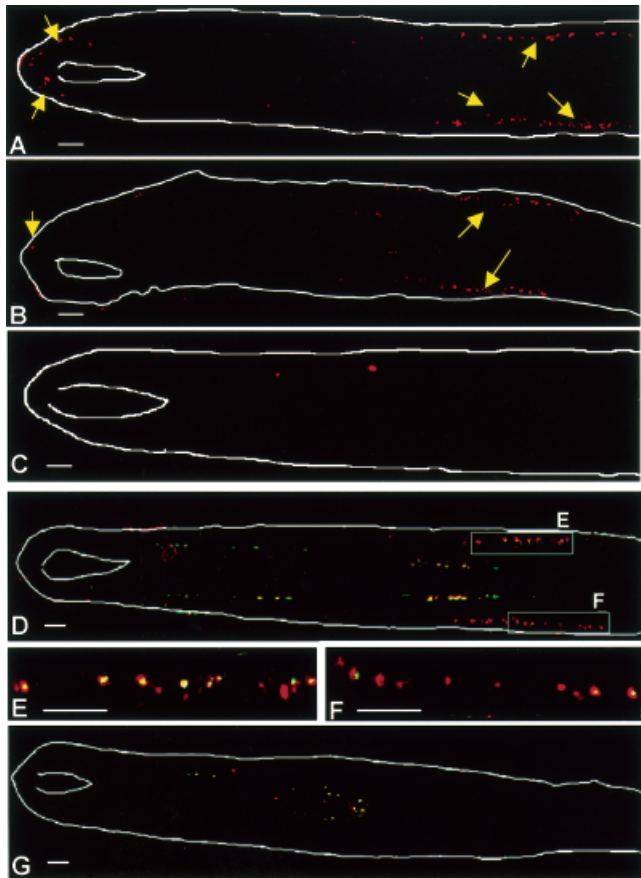


Figure 1. Caspase activation in hair follicles treated with TGF- β 2. Hair follicles were cultured in the presence (A, B, D E and F) or absence (C and G) of TGF- β 2 and immuno-stained with the cleavage site-directed antibody to caspase-3 (A) or caspase-9 (B). Caspase activation occurred in the lower part of germinative matrix cells and outer layers of outer root sheath cells (arrow) in the hair follicles treated with TGF- β 2, compared with the control (C). Dual staining for active caspase-3 and TUNEL demonstrated colocalization of these cells (D-F). E and F show magnified views. The anagen hair follicle in the absence of TGF- β 2 showed positive signals around hair cortex, which are not related to TGF- β 2 (G). scale bars: 100 μ m

showed the TUNEL-positive cells consistently overlapped with caspase-3-positive cells (Fig 1D-F). Some cells were only positive for active caspase-3 (Fig 1E,F). These cells probably lacked nuclear material in the thin section. Control hair follicles (Fig 1G) showed several positive cells around the cortex, suggesting that these are not related to activity of exogenous TGF- β .

Hydrangea macrophylla, a potential TGF- β 2 antagonist, caused elongation of hair follicles Suppression of TGF- β was hypothesized to prevent apoptosis and to delay catagen entry. Therefore, we sought a natural inhibitor of TGF- β action. We developed a screening method for TGF- β suppression by monitoring changes in PAI-1, a TGF- β responsive gene product. (Zhang *et al*, 1998). Over 400 plant extracts were screened. Among these, the extract from *Hydrangea macrophylla* (tea of Heaven) was most effective in suppressing PAI-1 synthesis (Fig 2A). Using the organ culture system, the effect of the *H. macrophylla* extract on hair follicle elongation was tested. Human hair follicles were cultured in the presence of the extract for 7 d. The resulting follicles showed significant elongation (Fig 2B). Treated follicles showed an average of 2.25 mm elongation, compared to an average of 2.0 mm for the controls. The difference is significant to a p-value of <0.05 as assessed by the one-way ANOVA and Dunnett's posthoc procedure.

H. macrophylla delayed catagen progression in vivo To confirm the effect of the *H. macrophylla* extract on catagen progression, an *in vivo* test was performed using C57BL/6 mice. A new, synchronized hair cycle was induced by wax depilation. The plant extract was topically applied continuously on the back for 10 d from day 12 after hair induction. Skin sections were prepared, stained with HE, and the catagen stage of each hair follicle was scored. Stages of catagen were determined according to the method of Muller-Rover *et al* (2001). Figure 3A and 3B show the typical features of histochemical analyzes. Figure 3C summarizes the ratio of each catagen stage for each group. Over half of the hair follicles treated with 0.2% *H. macrophylla* extract were in earlier catagen stages than in the control skin. Two percent extract showed a slightly more potent effect on catagen progression than 0.2%.

DISCUSSION

TGF- β is known to regulate various physiological reactions including apoptotic cell death (Oberhammer *et al*, 1992; Ohta *et al*, 1994). Previously we have shown the spatio-temporal localization of TGF- β isoforms during the human hair cycle, and we suggested that up-regulation and specific localization of TGF- β 2 in the anagen-catagen transition may initiate the process of catagen (Soma *et al*, submitted). In this report, we analyzed activation of a caspase network by TGF- β 2 in human hair follicles. We focused on the initiator caspase-9 and the effector caspase-3. Our results clearly demonstrated that TGF- β 2 can activate these two caspases in an organ culture system. This is the first evidence that TGF- β can elicit apoptotic cell death through the activation of a caspase network in human hair follicles. We still do not know all the molecules that may play roles in this network activation by TGF- β . Recently it was suggested that caspase-8 is located upstream of caspase-9 (Li *et al*, 1998; Pan *et al*, 2001). Caspase-8 could also be included in apoptosis induced by TGF- β 2.

Our preliminary data indicate that caspase activation may not be an immediate early response to TGF- β (unpublished data). Well-known mediators for TGF- β signal transduction include members of the Smad family (Hoodless and Wrana, 1998). Smad2 can specifically mediate TGF- β /activin signal transduction. After TGF- β stimulation, phosphorylated Smad2 binds with Smad4 and the complex translocates to the nucleus. We speculate that the mechanism of caspase activation by TGF- β includes the induction of other functional molecules. TSC-22, TGF- β stimulated clone-22, is a TGF- β responsive gene that was suggested to

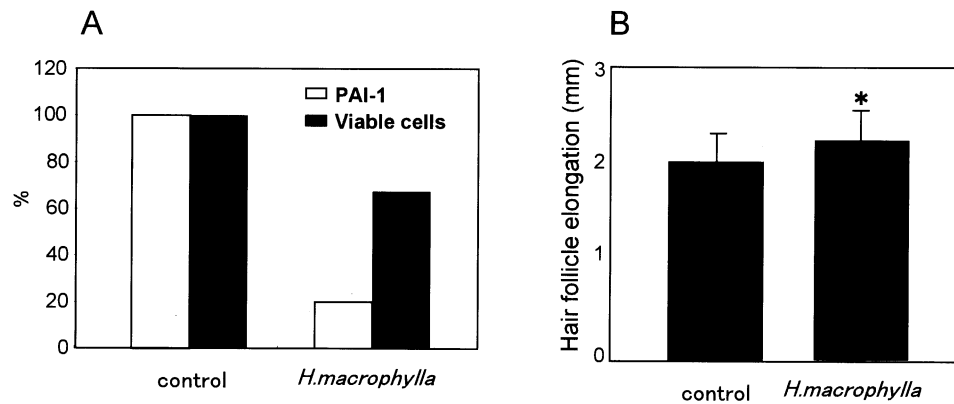


Figure 2. Effect of *H. macrophylla* on TGF- β suppression and hair elongation A: Suppression of PAI-1 production by *H. macrophylla* extract. Dermal papilla cells were incubated in the presence or absence of the extract of *H. macrophylla* (5 μ g/ml). The amount of PAI-1 culture medium and cytotoxicity of the extract was analyzed simultaneously. The relative values were calculated and compared to control. (B) Effect of the extract on the hair elongation. Hair follicles were incubated in the presence of *H. macrophylla* (5 μ g/ml). Results are expressed as the mean \pm SD * p < 0.05 (one-way ANOVA and Dunnett's posthoc procedure).

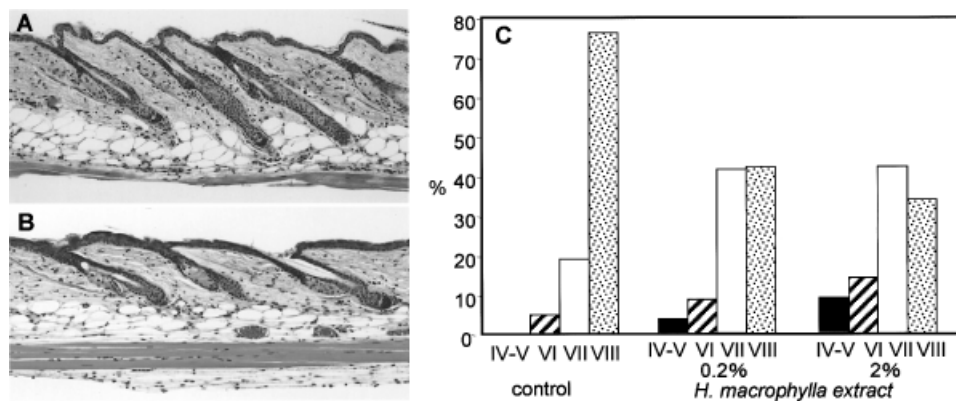


Figure 3. Delay of catagen progression by the extract of *H. macrophylla*. The extract of *H. macrophylla* (0.2% or 2%) was topically applied on the back of C57BL/6 mice after hair induction. Skin sections were prepared and stained with hematoxyline and eosin. (A) Morphology of the skin treated with 2% *H. macrophylla* extract, (B) vehicle treated skin. Catagen stages of individual hair follicle were scored (C). A hundred hair follicles identified on sections were graded for each mouse and 5 mice were used for each group.

promote apoptotic cell death induced by TGF- β (Ohta *et al*, 1997). Our experiments indicate that TSC-22 is present in the epithelial component of hair follicles *in vivo* (Dohrmann *et al*, 1999). TSC-22 may be a candidate molecule in this process.

Involvement of TGF- β in catagen induction is also suggested in mice. TGF- β 1 levels increase in late anagen and remain at maximal throughout catagen (Foitzik *et al*, 2000). TGF- β type II receptor was restricted in hair follicles and was up-regulated in anagen-catagen transition phase (Paus *et al*, 1997).

Our next goal was to test whether suppression of TGF- β 2 could delay catagen entry. In this study, we identified an *H. macrophylla* extract as a potential suppressor of TGF- β 2 action, because it could reduce PAI-1 production from dermal papilla cells. Although PAI-1 transcription requires both TGF- β activated Smad proteins and independently activated AP-1 (Zhang *et al*, 1998), we favor the model that this extract blocks TGF- β function because of its effect on hair follicles. The *H. macrophylla* extract was effective on hair elongation in organ culture system. Furthermore, using the *in vivo* mouse model, we showed that topical application of the extract leads to delay of catagen progression. Suppression of TGF- β is expected to provide a novel and efficient tool for hair cycle regulation.

The authors would like to thank Dr Tetsuo Ezaki for his cooperation in obtaining materials and Miss Hitomi Uegaki for her assistance in immunohistochemical analysis.

REFERENCES

- Dohrmann CE, Belaousoff M, Raftery LA: Dynamic Expression of TSC-22 at Sites of Epithelial-Mesenchymal Interactions During Mouse Development. *Mech. Dev* 84:147-151, 1999
- Foitzik K, Linder G, Mueller-Roeber S, *et al*: Control of murine hair cycle regression (catagen) by TGF- β *in vivo*. *FASEB J* 14:752-760, 2000
- Fujita E, Urase K, Egashira J, *et al*: Detection of caspase-9 activation in the cell death of the Bcl-x-deficient mouse embryo nervous system by cleavage sites-directed antisera. *Brain Res Dev Brain Res* 122:135-147, 2000
- Galbraith GM, Thiers BH, Vasily DB, Fudenberg HH: Immunological profiles in alopecia areata. *Br J Dermatol* 110:163-170, 1984
- Hoodless PA, Wrana JL: Mechanism and function of signaling by the TGF- β Superfamily. *Curr Top Microbiol Immunol* 228:235-272, 1998
- Itami S, Kurata S, Takayasu S: 5 alpha-reductase activity in cultured human dermal papilla cells from beard compared with reticular dermal fibroblasts. *J Invest Dermatol* 94:150-152, 1990
- Jahoda CAB: Cellular and development aspects of androgenetic alopecia. *Exp Dermatol* 7:235-248, 1998
- Kligman AM: The human hair cycle. *J Invest Dermatol* 33:307-316, 1959
- Kouroku Y, Urase K, Fujita E, *et al*: Detection of activated Caspase-3 by a cleavage site-directed antiserum during naturally occurring DRG neurons apoptosis. *Biochem Biophys Res Commun* 247:780-784, 1998
- Kumar S: Mechanisms mediating caspase activation in cell death. *Cell Death Differ* 6:1060-1066, 1999
- Li H, Zhu H, Xu CJ, Yuan J: Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 21:491-501, 1998
- Muller-Rover S, Handjiski B, van der Veen C, *et al*: A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *J Invest Dermatol* 117:3-15, 2001
- Oberhammer FA, Pavelka M, Sharman S, Tiefenbacher R, Purchio A, Bursch W, Schulte-Hermann R: Induction of apoptosis in cultured hepatocytes and in

- regressing liver by transforming growth factor- β 1. *Proceedings of the Natl Acad Sci USA* 89:5408–5412, 1992
- Ohta S, Yanagihara K, Nagata K: Mechanism of apoptotic cell death of human gastric carcinoma cells mediated by transforming growth factor beta. *Biochem J* 15:777–782, 1994
- Pan J, Xu G, Yeung SC: Cytochrome c release is upstream to activation of caspase-9, caspase-8 and caspase-3 in the enhanced apoptosis of anaplastic thyroid cancer cells induced by manumycin and paclitaxel. *J Clin Endocrinol Metab* 86:4731–4740, 2001
- Paus R, Foitzik K, Welker P, Bulfone-Paus S, Eichmuller S: Transforming growth factor- β receptor type I and type II expression during murine hair follicle development and cycling. *J Invest Dermatol* 109:518–26, 1997
- Paus R, Handjiski B, Eichmuller S, Czarnetzki BM: Chemotherapy-induced alopecia in mice. Induction by cyclophosphamide, inhibition by cyclosporin A, and modulation by dexamethasone. *Am J Pathol* 144:719–734, 1994
- Philpot MP, Green MR, Kealey T: Human hair growth in vitro. *J Cell Sci* 97:463–471, 1990
- Soma T, Ogo M, Suzuki J, Takahasi T, Hibino T: Analysis of apoptotic cell death in human hair follicles. *J Invest Dermatol* 112:518–526, 1998
- Zhang Y, Feng XH, Derynk R: Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF α -induced transcription. *Nature* 394:909–913, 1998

