Analysis of Apoptotic Cell Death in Human Hair Follicles *In Vivo* and *In Vitro*

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We analyzed changes of growth and apoptotic cell death in human hair follicles. In anagen hair follicles, terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick labeling-positive cells were observed in the keratogenous zone of the upper bulb matrix, the inner root sheath, and the companion layer of the outer root sheath. DNA ladder formation was also detected in anagen hair follicles. In catagen hair follicles, the lower bulb matrix cells around the dermal papilla and the outer layer cells of the outer root sheath became strongly positive, showing that apoptosis in catagen hair is distinct from that in anagen hair. We also confirmed the mRNA expression of four caspases (caspase-1, caspase-3, caspase-4, and caspase-7) in anagen hair follicles by reverse transcriptase-polymerase chain reaction and *in situ* hybridization. When human anagen hair follicles were cultured in the presence of transforming growth factor-β or tumor necrosis factor-α in the serum-free medium, transforming growth factor-β but not tumor necrosis factor-α induced catagen-like morphologic changes, which were indistinguishable from normal catagen hair follicles. Tumor necrosis factor-α, however, strongly inhibited the elongation of the hair shaft in a dose-dependent manner, accompanied by abnormal morphology and increased cell death in the bulb matrix cells. Our results suggest that apoptosis in hair follicles involves two different types. One is related to the terminal differentiation of follicular epithelial cells in anagen hair. The other occurs as a major driving force to eliminate the distinct portion of epithelial components in catagen hair. Furthermore, this study strongly indicates that the transforming growth factor-β pathway is involved in the induction of catagen phase in human hair cycle. ***Key words: hair regression/organ culture/terminal differentiation/TGF-β. J Invest Dermatol 111:948–954, 1998***

Apoptosis or programmed cell death, which is distinguished from necrosis, is involved not only in normal embryonic development (Polakowska and Haake, 1994; Jacobson *et al*, 1997) but also homeostatic mechanisms in various tissues including normal skin (Polakowska and Haake, 1994).

The process of apoptosis is controlled through the activation of many factors, including some cytokines (Bellomo *et al*, 1992; Oberhammer *et al*, 1992), tumor suppressor gene products (Symonds *et al*, 1994), bcl-2 family gene products (Vaux *et al*, 1988; Hockenbery *et al*, 1990), and ICE family proteases (Miura *et al*, 1993; Yuan *et al*, 1993). The importance of the ICE family proteases as the executor of apoptosis is now widely accepted. Horvitz *et al* (Ellis and Horvitz, 1986; Ellis and Horvitz, 1991) demonstrated the molecular mechanism of apoptosis in *Caenorhabditis elegans*. Among the molecules they discovered, ced-3 plays a key role in the process of apoptosis (Miura *et al*, 1993). As soon as the ced-3 gene was cloned, it revealed a striking homology to a mammalian protease, interleukine-1β converting enzyme, known as ICE (Yuan *et al*, 1993). Moreover, overexpression of ICE or ced-3 was enough to induce apoptotic cell death in Rat-1 cells (Miura *et al*, 1993). Mammalian homolog of the suppressor of apoptosis, ced-9, was also identified to be bcl-2, formally thought of as an oncogene (Hengartner and Horvitz, 1994). It is now clear that essentially the same mechanism is used through nematode to mammals. Human ICE homologs have been discovered one after another and there are now at least 10. Recently the name caspase was given to this family (Alnemri *et al*, 1996). Although apoptosis can be induced by a variety of stimuli, growing evidence indicates that caspase activation is required at some point of the apoptotic process (Salvesen *et al*, 1997). ICE (caspase-1) knockout mouse was produced and revealed that ICE is essential in T cell apoptosis (Kuida *et al*, 1995; Li *et al*, 1995). Deficiency of CPP32 (caspase-3) caused decreased apoptosis in brain (Kuida *et al*, 1996). These data suggest that the process of apoptosis is a cell-type-specific event and the execution of apoptosis is probably dependent on the activation of certain types of caspases. In other words, regulation of apoptosis can be accomplished by controlling a cell-specific caspase.

Hair growth is a highly regulated cyclical process. Three distinct phases have been defined for the mammalian cycle: anagen (growing phase), catagen (regressing phase), and telogen (resting phase) (Kligman, 1959). Little is known about the mechanism that regulates the hair cycle. The transition from anagen to catagen has been thought to be an apoptotic process, followed by matrix remodeling. In this process, caspase must be proteolytically activated to cause apoptotic morphologic changes and DNA fragmentation. Recently some growth factors such as fibroblast growth factor-5 (Hébert *et al*, 1994) and transforming growth factor-β (TGF-β) (Seiberg *et al*, 1995; Welker *et al*, 1997) were suggested to participate in the induction of the catagen phase in mice; however, how the mechanism of apoptosis, including caspase cascade, works in human hair cycle is still not known.

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Abbreviations: DIG, digoxigenin; DP, dermal papilla; KZ, keratogenous zone; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick labeling.

In this study, we analyzed growth and apoptotic cell death in human hair cycle using anti-proliferating cell nuclear antigen (PCNA) antibody and the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick labeling (TUNEL) method. The mRNA expression of several caspase family proteases in hair follicles were also investigated by reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization. Furthermore, using the *in vitro* organ culture system, we examined the effects of TGF-β and tumor necrosis factor (TNF)-α on morphology, growth, and cell death in human hair follicles in order to assess whether these cytokines are involved in the induction of catagen phase.

MATERIALS AND METHODS

Materials Williams E medium, Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, streptomycine, and fungizone were supplied by Life Technologies (Rockville, MD). All other tissue culture reagents including TGF-β2 and TNF-α were purchased from Sigma (St. Louis, MO).

Tissue preparation Human scalp skin specimens were obtained from plastic surgery. Skin pieces were fixed with 4% paraformaldehyde in phosphate-buffer (pH 7.4) at 4°C for 4 h and embedded in paraffin wax. Hair follicles, which were isolated from the subcutaneous fat portion of human scalp skin, were embedded in HISTORESIN (Lica, Nussloch, Germany) or paraffin wax. Serial sections of 3–5 μm were cut and mounted on slides precoated with silane (Matsunami, Tokyo, Japan).

Immunohistochemistry Tissue sections were incubated with mouse monoclonal anti-human PCNA antibody (PC10: DAKO, Glostrup, Denmark) or anti-human caspase-3 antibody (clone 19: Immunotech, Marseille Cedex, France) in a moisture chamber at room temperature for 1 h. A biotinylated rabbit anti-mouse IgG (Nichirei, Tokyo, Japan) was used as secondary antibody, followed by the reaction with peroxidase-cojugated streptavidin (Nichirei, Tokyo, Japan). Diaminobenzidine or tetramethylbenzidine (Kirkegaard & Perry Laboratories, 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 first red (Sigma). 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay was performed using the cell proliferation kit (Amersham, Bucks, U.K.).

TUNEL method TUNEL reaction was performed as described by Gavriel *et al* (1992) with minor modifications using the TACS 2 terminal deoxynucleotidyltransferase *in situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Tissue sections were treated with 20 μg proteinase K per ml for 10 min at room temperature. After rinsing with phosphate-buffered saline, sections were incubated in a reaction buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium chloride, 1 mM cobalt chloride), containing terminal deoxynucleotidyltransferase (0.3 U per ml) and 10 mM biotinylated deoxyuridine triphosphate in a humidity chamber at 37°C for 1 h. Reaction was terminated by transferring the slides in a stop buffer (300 mM sodium chloride, 30 mM sodium citrate), nonspecific binding was blocked by 10% normal serum. After washing, sections were covered with peroxidase-conjugated streptavidin and incubated for 10 min at room temperature. Color was developed using tetramethylbenzidine for about 5 min, followed by countersatining with nuclear first red.

Detection of fragmented DNA in anagen hair follicle Isolated anagen hair follicles were homogenized in a lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1 mM ethylenediamine tetraacetic acid, 0.1% sodium dodecyl sulfate, 200 μg proteinase K per ml, pH 8.0) with a glass homogenizer (Wheaton, Millville, NJ) and were incubated at 37°C for 15 min. After being treated with RNase A (Boehringer, Mannheim, Germany), total DNA was extracted twice with phenol/chloroform, followed by isopropanol precipitation. For positive control of DNA ladder formation, Pam 212 cells were grown to subconfluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After washing with phosphate-buffered saline, the culture medium was changed to 10% fetal bovine serum/Dulbecco's modified Eagle's medium containing 10 ng TNF-α per ml and 10 μg staurosporine per ml, followed by a incubation for 16 h. The cells floated in the medium were collected and resuspended in a lysis buffer. Then, total DNA was extracted as described above. DNA fragmentation was analyzed by 2% agarose gel electrophoresis (McCall and Cohen, 1991).

RT-PCR and *in situ* hybridization analysis of caspases Total RNA were extracted from anagen hair follicles isolated from the subcutaneous fat portion, followed by the synthesis of cDNA using oligo (dT) and superscript II (Life Technologies). The reverse-transcribed products were PCR amplified using the specific primers of six caspase family proteases. Primers were designed based on the known sequences to produce cDNA frangments as directly applicable for *in situ* hybridization. The following primers were used: for caspase-1, 5′-TTGATTGACTCCGTTATTC-3′ and 5′-CTCTGCCGACTTTTGTTTC-3′ (Thornberry *et al*, 1992) (285 bp); for caspase-2, 5′-ATGGCCGCTGACAGGGG-3′ and 5′-GAACAGAAACCGTGCAT-3′ (Wang *et al*, 1994) (1080 bp); for caspase-3, 5′-ATACTCCTTCCATCAAATAG-3′ and 5′-AACATCACAAAACCATAATC-3′ (Fernandes-Alnemri *et al*, 1994) (410 bp); for caspase-4, 5′-GCTGTTTACAAGACCCACGTGG-3′ and 5′-GTGGCTTCCATTTTCAA-3′ (Munday *et al*, 1995) (280 bp); for caspase-5, 5′-CGGATGTGCTGCTTTATGAC-3′ and 5′-AGGTTGCTCGTTCTATGGTG-3′ (Munday *et al*, 1995) (423 bp); for caspase-7, 5′-CAAAGCCACTGACTGAGATG-3′ and 5′-CAACCCAATGAATAAATGAT-3′ (Fernandes-Alnemri *et al*, 1995) (258 bp); for G3PDH, 5′-TGAAGGTCGGTGTCAACGGA-3′ and 5′-GATGGCATGGACTGTGGTCA-3′ (Tso *et al*, 1985) (533 bp). Predicted sizes were shown in parentheses. PCR products were analyzed on a 2% agarose gel. The PCR products of caspase-1, caspase-3, caspase-4, and caspase-7 were cloned into the pGEM-T vector (Promega, Madison, WI). Sense and anti-sense RNA probes of each caspase were synthesized by using the digoxigenin (DIG) RNA labeling kit (SP6/T7) (Boehringer) according to the manufacturer's instructions. For *in situ* hybridization, tissue sections were treated with 10 μg proteinase K (Nacali Tesque, Kyoto, Japan) per ml in phosphate-buffered saline for 30 min at 37°C and fixed again in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 15 min. After incubation in 0.2 N HCl for 10 min, the sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer for 10 min. Hybridization was performed with the DIG-labeled RNA probes (0.5 μg per ml) in the hybridization buffer [50% deionized formamide, 5 × sodium citrate/chloride buffer (15 mM sodium citrate, 150 mM sodium chloride) and 5×Denhardt's solution] at 53°C for about 16 h in a humidified chamber. The slides were washed in 2 × sodium citrate/chloride buffer containing 0.1% sodium dodecyl sulfate and in 0.1 × sodium citrate/chloride buffer containing 0.1% sodium dodecyl sulfate at 53°C for 30 min, respectively. After washing and blocking, the sections were covered with anti-DIG alkaline phosphatase conjugate (Boehringer) diluted to 300 mU per ml in the DIG blocking solution and incubated at room temperature for 30 min. Color was developed with the Vector Red (Vector Laboratories, Burlingame, CA) in Tris-HCl buffer (pH 8.2).

Isolation and culture of human hair follicles Human hair follicles were isolated and cultured by the method described by Philpott *et al* (1990) with minor modifications. Subcutaneous fat portion of human scalp skin including lower hair follicles were dissected from epidermis and dermis. Then anagen hair follicles were isolated under a binocular dissecting microscope with fine forceps. Isolated anagen follicles were maintained in either 1 ml of Williams E medium containing 100 U penicillin per ml, 10 μg streptomycine per ml, and 2.5 μg fungizone (basal medium) per ml or the basal medium supplemented with 10 μg insulin per ml, 10 ng hydrocortisone per ml, 10 μg transferrin per ml, and 10 ng sodium selenite (plus medium) per ml at 37°C in 5% CO_2/95% air. In separate experiments, 10 hair follicles for each dose were incubated in the basal medium in the presence or absence of TGF-β or TNF-α. Culture medium was replaced every 3 d unless mentioned. For the analysis of DNA synthesis, 3 μg BrdU per ml or 0.5 μCi of [methyl-^{3}H]thymidine (Amersham) was added to the culture medium, followed by a incubation for 16 h.

Measurement of DNA synthesis and DNA content in hair follicles The amounts of [methyl-^{3}H]thymidine incorporated into hair follicles were measured by the method described by Philpott *et al* (1990). DNA contents in hair follicles were determined using the Fluorometer (DyNA Quant 200: Hoefer Pharmacia Biothech, San Francisco, CA) according to the manufacturer's instructions.

RESULTS

Apoptotic cell death in anagen hair follicle **Figure 1**(***a***) shows gross morphology of a human hair follicle in anagen phase. To investigate proliferation and cell death pattern of anagen hair follicles, horizontal sections were carefully prepared and stained with conventional hematoxylin and eosin (**Fig 1*b***), anti-PCNA antibody (**Fig 1*c***), and TUNEL method (**Fig 1*d***). In anagen hair follicles, bulb matrix cells around the dermal papilla (DP) and the outer layer cells of the outer root sheath (ORS) were positively stained by anti-PCNA antibody, indicating that these cells were in the growing phase (**Fig 1*c***). On the other hand, the nuclei of the Henle's layer cells, the Huxley's layer cells, and the cuticle cells of the inner root sheath (IRS) in the upper part of the keratogenous zone of the hair bulb, were positively stained by TUNEL method, where a large number of the hair cortex cells showed apoptotic cell death (**Fig 1*d***). The cells of the companion layer underwent apoptosis in the area where keratinization and terminal differentiation occurred. The nuclei of DP cells and dermal sheath

Figure 1. Growth and apoptotic cell death in anagen hair follicle. The lower portion of hair follicles, which were embedded in the subcutaneous fat portion, were isolated from normal scalp (*a*). Sections of anagen hair follicles were stained with hematoxylin and eosin (*b*), anti-PCNA antibody (*c*), and TUNEL method (*d*). The nuclei of the IRS cells and the bulb matrix cells (bulb) in the keratogenous zone (KZ) were positively stained with TUNEL method (*d*, indicated by *arrows*). Positive staining was also observed at the companion layer (*d*, indicated by *arrowheads*). On the other hand, TUNEL positive cells were not observed in the bulb matrix and the outer layers of the ORS that were stained by anti-PCNA antibody (*c*, indicated by *arrows*). *Scale bars*: 100 μm.

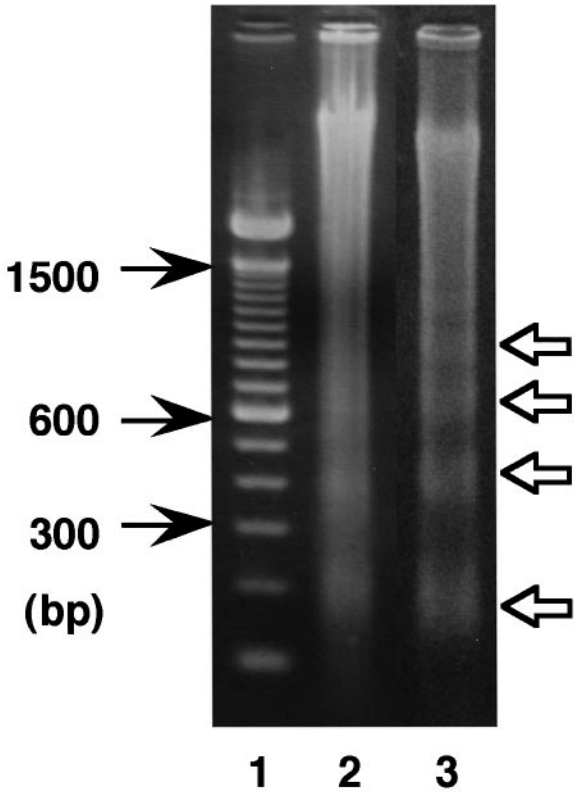

Figure 2. Detection of fragmented DNA in anagen hair follicle. DNA extracted from 10 anagen hair follicles was run on a 2% agarose gel, and analyzed for DNA fragmentation. As shown in this figure, even in anagen hair follicles, characteristic smear and DNA ladder formation (indicated by *open arrows*) were detected (*lane 2*). *Lane 1*, DNA molecular weight markers (100 bp ladder marker); *lane 3*, positive control of DNA ladder (Pam 212 treated with 10 ng TNF-α per ml and 10 μg staurosporine per ml).

cells remained intact and did not show any positive staining by either anti-PCNA antibody or TUNEL method. To confirm whether the TUNEL-positive staining occurred in anagen hair follicles is actually the result of apoptotic cell death, DNA extracted from anagen hair follicles was analyzed by 2% agarose gel to detect DNA fragmentation. As shown in **Fig 2**, even in anagen hair follicles, characteristic DNA "ladder" formation was observed. These results clearly showed that apoptosis also takes place in anagen hair.

Apoptotic cell death in catagen hair follicle **Figure 3**(***a***) shows gross morphologic changes in a mid-catagen hair follicle. In the matrix region of the hair bulb, arrest of melanogenesis and an upward movement of the hair shaft away from the DP were the characteristic features of the catagen phase. Formation of a club structure in the upper hair bulb was also obvious. Histologic study of catagen hair follicles demonstrated the condensation of the DP, the disappearance of the bulb matrix cells around the DP, and the indistinctness of the IRS in the hair bulb (**Fig 3*b***). PCNA positive cells were no longer observed in the follicular epithelial cells (**Fig 3*c***). Intense and abundant staining for TUNEL reaction was observed in the broad area of the ORS, the epithelial strand, and the epithelial cells surrounding the DP (**Fig 3*d***). The DP cells never showed positive staining either for PCNA or for TUNEL (**Fig 3*c*, *d***).

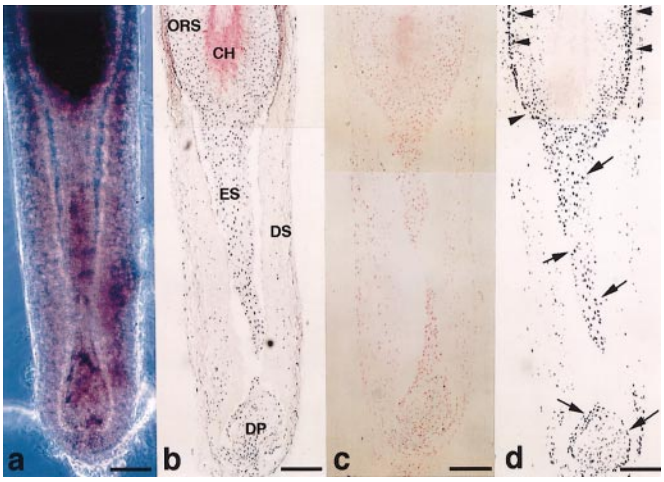

Figure 3. Growth and apoptotic cell death in catagen hair follicle. A catagen hair follicle (*a*) that was characterized with the long epithelial strand (ES) between the club hair and the DP was stained with hematoxylin and eosin (*b*), anti-PCNA antibody (*c*), and TUNEL method (*d*). The expression of PCNA were decreased in the bulb matrix cells and the ORS cells (*c*). Positive staining for TUNEL reaction was observed in the epithelial strand, the epithelial cells around the DP (indicated by *arrows*), and the outer layers of the ORS (indicated by *arrowheads*) (*d*). This apoptosis pattern is distinct from that seen in anagen hair follicles. *Scale bars*: 100 μm.

Expression of caspases The amplified PCR products were visualized on an agarose gel (**Fig 4**). The PCR products, which were consistent with predicted sizes shown in parentheses, were observed for caspase-1 (285 bp), caspase-3 (410 bp), caspase-4 (280 bp), and caspase-7 (258 bp). The restriction digestion was performed using Hind III for caspase-1, Afl II for caspase-3, Ssp I for caspase-4, and Stu I for caspase-7. All showed the expected fragments. Interestingly, the gene expression of all these caspases were also confirmed in cultured human keratinocytes (data not shown).

Using these PCR products as probes, *in situ* hybridization was carried out in anagen hair follicle. Among four anti-sense caspase probes tested, results of caspase-3 were shown in **Fig 5**(***a***). The bulb matrix cells and the IRS cells in the keratogenous zone were stained with the anti-sense RNA probe to caspase-3, but the DP cells and the dermal sheath cells were almost negative. Caspase-3 anti-sense probe did not hybridize to the outermost layers of the ORS. The caspase-3 mRNA also showed some specific expression at the companion layer

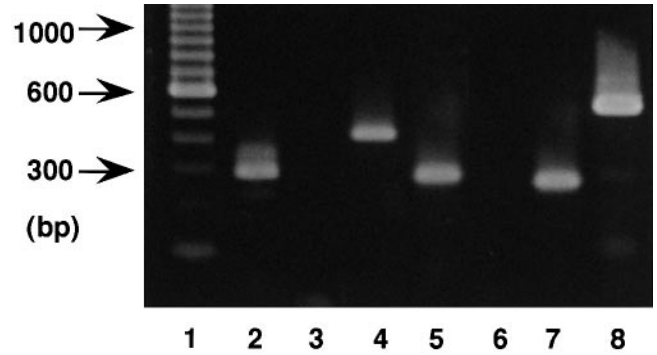

Figure 4. RT-PCR analysis of caspases expression in anagen hair follicles. Total RNA was extracted from five anagen hair follicles. Expression of six caspase family members was tested in isolated anagen hair follicles by RT-PCR. mRNA of four caspase proteases [*lane 2*, caspase-1 (285 bp); *lane 4*, caspase-3 (410 bp); *lane 5*, caspase-(280 bp); *lane 7*, caspase-7 (258 bp)] were confirmed in anagen hair follicles. *Lanes 3* and *6* show results of caspase-2 and -5, respectively. No band was detected. *Lane 1*, DNA molecular weight markers (100 bp ladder marker); *lane 8*, G3PDH (533 bp).

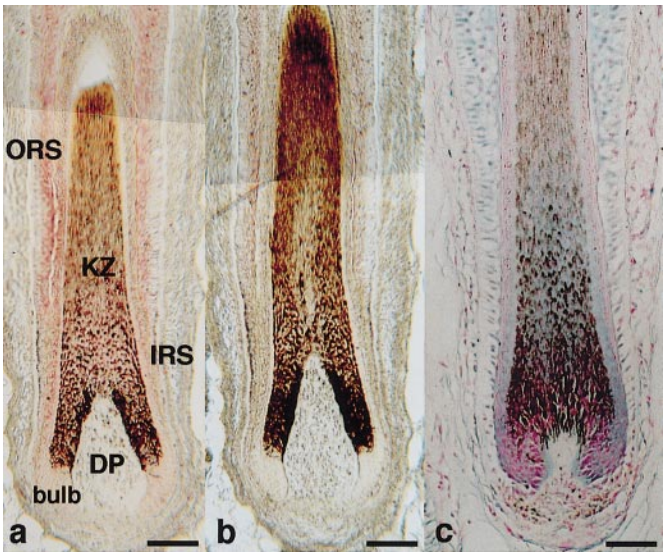

Figure 5. *In situ* hybridization and immunohistochmical detection of caspase-3 in anagen hair follicles. Anti-sense caspase-3 RNA probe showed specific hybridization indicated with red color in the bulb matrix cells (bulb), the IRS cells, and the ORS cells, but not in the DP cells (*a*). No hybridization was observed if sense caspase-3 RNA probe was used (*b*). Caspase-3 protein (blue color) was detected essentially in the same area (*c*). *Scale bars*: 100 μm.

cells of the ORS and the sebaceous gland cells whose nuclei were positively stained with TUNEL method (data not shown). In the epidermis, TUNEL-positive cells were detected in the uppermost layer of the granular layer, whereas the anti-sense probe to caspase-3 hybridized to the spinous and the basal layers (data not shown). The sense probe to caspase-3 did not show any significant hybridization in hair follicles (**Fig 5*b***) or in epidermis (data not shown). We also examined mRNA expression of caspase-1, caspase-4, and caspase-7. The mRNA expression patterns of these caspases were essentially the same and were not different from that of caspase-3 (data not shown). Immunohistochemical study demonstrated that caspase-3 localized in the lower hair follicles, similar to areas where caspase-3 mRNA were detected (**Fig 5*c***).

Establishment of an organ culture system Human anagen hair follicles isolated from scalp skin were allowed to grow in the serum-free medium. Growth rate was calculated to be ≈0.3 mm per d (**Fig 6**), when they were cultured in the plus medium containing insulin and other supplements. In this organ culture system, it was confirmed that isolated human hair follicles retained their growth rate

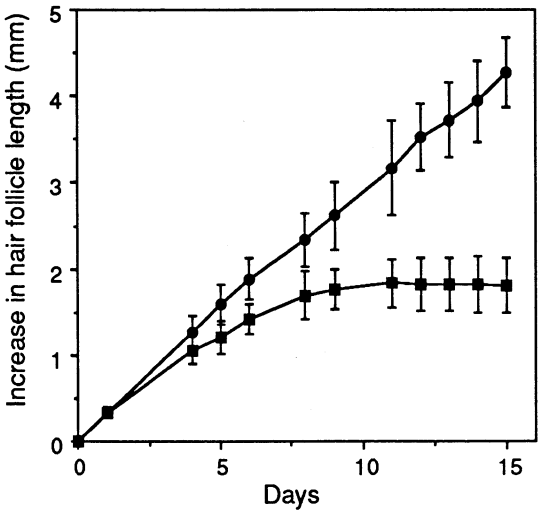

Figure 6. Hair growth in organ culture. In order to evaluate the effect of various factors, we established an organ culture system for human hair follicles. In this system, we were able to culture human anagen hair follicles isolated from scalp for more than 2 wk at the elongation rate of 0.3 mm per day in the plus medium (●), which was comparable with the *in vivo* growth rate. When hair follicles were cultured in the basal medium (■), the growth rate was reduced and the elongation was no longer observed after 10 d. Each point represents the mean ± SD of 10 follicles.

more than 2 wk. After cultivation in the plus medium for 7 d, strong uptake of BrdU was still obvious in the lower matrix cells and some of the ORS cells. TUNEL-positive staining distributed only in the keratogenous zone of hair follicles (data not shown). When hair follicles were maintained in the basal medium, their growth lasted about 1 wk and the growth rate was considerably lower compared with the hair follicles in the plus medium (**Fig 6**). In addition, they showed catagen-like morphologic changes after 5–6 d. We detected very few cells that incorporated BrdU in the ORS and the bulb matrix. Furthermore, as hair follicles were continued to culture, a short epithelial strand was formed between the DP and the club-like structure, and TUNEL-positive cells appeared in the bulb matrix around the DP (data not shown).

Effects of TGF-β and TNF-α on hair follicle growth and morphology *in vitro* Now we obtained a very reliable organ culture system for human hair follicles. We then tested the effects of TGF-β, a suppressor of epitheliala cell growth, and TNF-α, a well-known apoptosis inducer. In the presence of TGF-β2, the elongation of hair follicles was not suppressed significantly in the first 6 d (**Fig 7*a***). In contrast to TGF-β2, TNF-α strongly inhibited hair follicle elongation in a dose–response manner (**Fig 7*b***). Approximately 70% of hair follicles (seven of 10) treated with TGF-β2 entered into a catagen-like state after 4 d in culture, whereas only one of 10 nontreated hair follicles showed some morphologic changes. After 6 d of cultivation, ≈80% of hair follicles were affected and showed early catagen-like changes in the basal medium (**Fig 8*a***). On the other hand, all the hair follicles demonstrated catagen-like features and it was far more profound in the presence of TGF-β2 (**Fig 8*b***). Although TGF-β2 did not show statistically significant suppression in hair growth, it inhibited [methyl-^{3}H]thymidine incorporation by hair follicles strongly in a dose–response manner (**Fig 9**). Morphologic changes were further analyzed for cell proliferation and cell death (**Fig 10*a***). In the presence of TGF-β2, hematoxylin and eosin staining showed characteristic features of catagen phase. BrdU incorporation was dramatically decreased (**Fig 10*b***), and apoptotic cell death was strongly induced to the epithelial cells derived from the bulb matrix cells or the ORS cells (**Fig 10*c***). TNF-α gave unusual effects on hair follicle morphology, which was characterized by the distortion of the DP, the abnormal keratinization of the bulb matrix cells and the IRS cells, the aggregation of melanin or melanocyte, and the vacuolation of the ORS cells (**Fig 10*d***). BrdU was still taken into the ORS cells of hair follicles cultured with TNF-α (**Fig 10*e***)

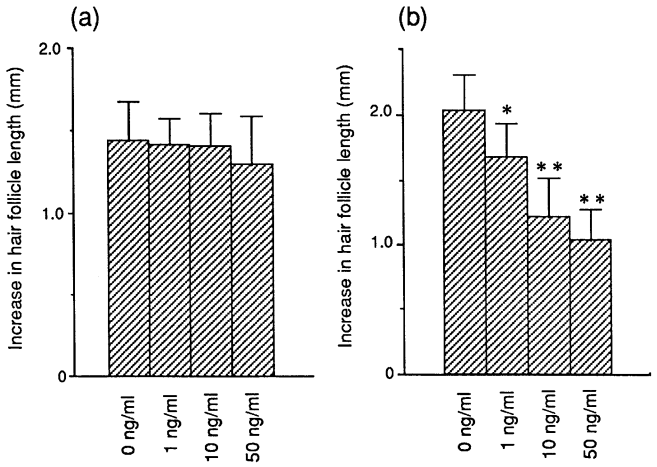

Figure 7. Effect of TGF-β2 and TNF-α on the elongation of hair follicles cultured *in vitro*. In the presence of TGF-β2, elongation of hair follicles was not affected significantly in the first 6 d (*a*). On the other hand, TNF-α strongly suppressed the growth of hair follicles cultured for 7 d in a dose-dependent manner (*b*). Results were expressed as the mean ± SD of 10 follicles. Statistical analysis was carried out using one-way analysis of variance and Dunnett's post-hoc procedure; *$p < 0.05$, **$p < 0.01$.

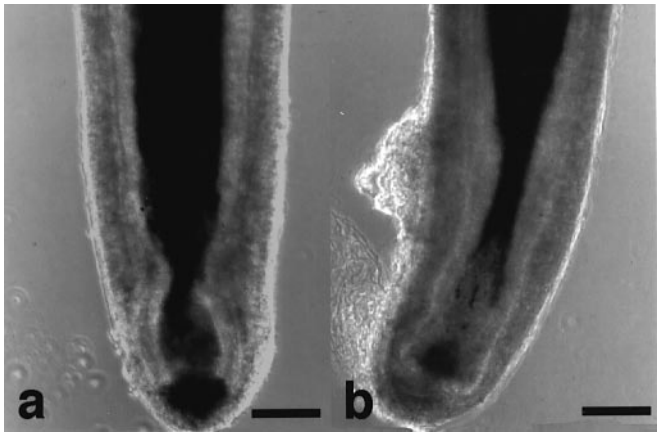

Figure 8. Gross morphology of hair follicles treated with TGF-β2. When anagen hair follicles were cultured with 50 ng TGF-β2 per ml in the basal medium, a catagen-like morphologic change was markedly accelerated (*b*) compared with that in the absence of TGF-β2 (*a*). *Scale bars*: 100 μm.

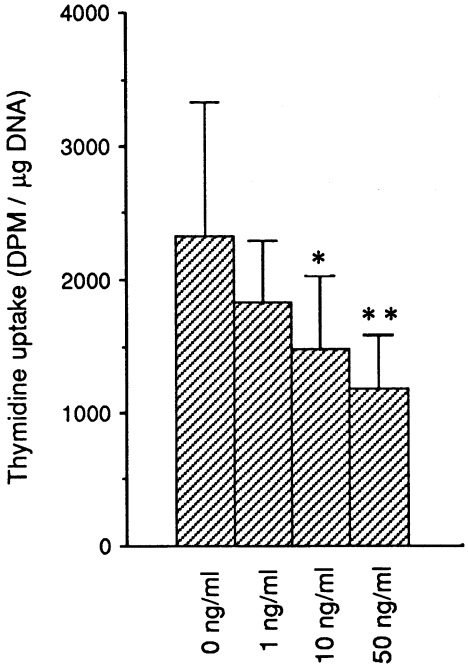

Figure 9. Thymidine incorporation of hair follicles treated with TGF-β2. TGF-β2 significantly inhibited the [methyl-^{3}H]thymidine incorporation of hair follicles at day 6 in a dose-dependent manner. Results were expressed as the mean ± SD of eight follicles. Statistical analysis of the data were carried out using one-way analysis of variance and Dunnett's post-hoc procedure; *$p < 0.05$, **$p < 0.01$.

after the cultivation for 7 d. TUNEL reaction demonstrated that most of the bulb matrix cells above the DP and some of the ORS cells were strongly positive (**Fig 10*f***).

DISCUSSION

By comparing PCNA staining and TUNEL reaction using serial resin-embedded sections, we analyzed proliferation and apoptotic cell death in human hair follicles in anagen and catagen phases. Immunoreactivity of anti-PCNA antibody in anagen hair follicles was observed in the hair matrix and ORS cells. This finding is in accordance with the work by Miyauchi *et al* using anti-Ki-67 antibody (Miyauchi *et al*, 1990), demonstrating the proliferation of the epithelial component in anagen. Interestingly, we demonstrated positive staining for TUNEL reaction in the keratogenous area of the IRS, the hair cortex, and the ORS. Moreover, we detected characteristic DNA ladder formation. In the epidermis, some of the uppermost layer of granular cells were always positive for TUNEL reaction. Our results clearly showed that apoptotic cell death takes place even in the growing phase of hair follicles; however, it was restricted in the area of the terminal differentiation of epithelial cells. Some groups also reported the presence of apoptotic nuclei in the granular layer of normal epidermis (McCall and Cohen, 1991; Tamada *et al*, 1994). Although TUNEL reactivity do not necessary fulfil the requirement of apoptosis, morphologic changes seen in the denuclearization process of keratinocytes suggest that at least similar mechanisms would be used in the terminal differentiation. Indeed we showed caspase activities and isolated epidermal caspases including a caspase-3-like enzyme from human cornified cells extract (Takahashi *et al*, 1998)

In catagen hair follicles, PCNA positive cells were no longer observed in follicular epithelial cells. Instead, TUNEL-positive cells increased dramatically in the outer layer of the ORS, in the area of epithelial strand, and in the epithelial cells surrounding the DP. It is noteworthy that the increase of TUNEL-positive cells is due to the occurrence of apoptotic cells in the area where terminal differentiation never takes place. Rather, apoptosis seemed to happen in the cells where PCNA-positive cells were located in the anagen hair. From our results it is clear that apoptosis in catagen hair follicles is distinct from that in anagen, suggesting that there are two types of apoptosis-like cell death in the epithelial component of the hair follicles, depending on the hair cycle. One is apparently related to terminal differentiation and is found even in anagen hair. The other was seen in catagen hair follicles and seems to fit more adequately to the definition of apoptosis.

Caspases play essential roles in the execution of apoptosis. We demonstrated that at least four species of caspases were expressed in human hair follicles by RT-PCR and *in situ* hybridization. The mRNA expression of caspases was observed only in epithelial cells and the localization of each caspase was essentially the same in anagen hair follicles. Our study revealed that caspases were synthesized in almost all the living epithelial cells in hair follicles and it was not limited to the cells where apoptotic cell death occurred. This clearly indicates that induction of catagen is independent on the production of caspases but is dependent on the activation of certain caspases. Enari *et al* demonstrated that during Fas-mediated apoptosis, sequential activation of caspases occurs in a cell-free system (Enari *et al*, 1996). Specific inhibitors of caspase-1 or caspase-3 can inhibit Fas-mediated apoptosis, confirming that the activation process is most important in this apoptotic process. Involvement of sequential activation of caspases was also confirmed by other investigators (Fernandes-Alnemri *et al*, 1996; Srinivasula *et al*, 1996; Li *et al*, 1997). We still do not know how many

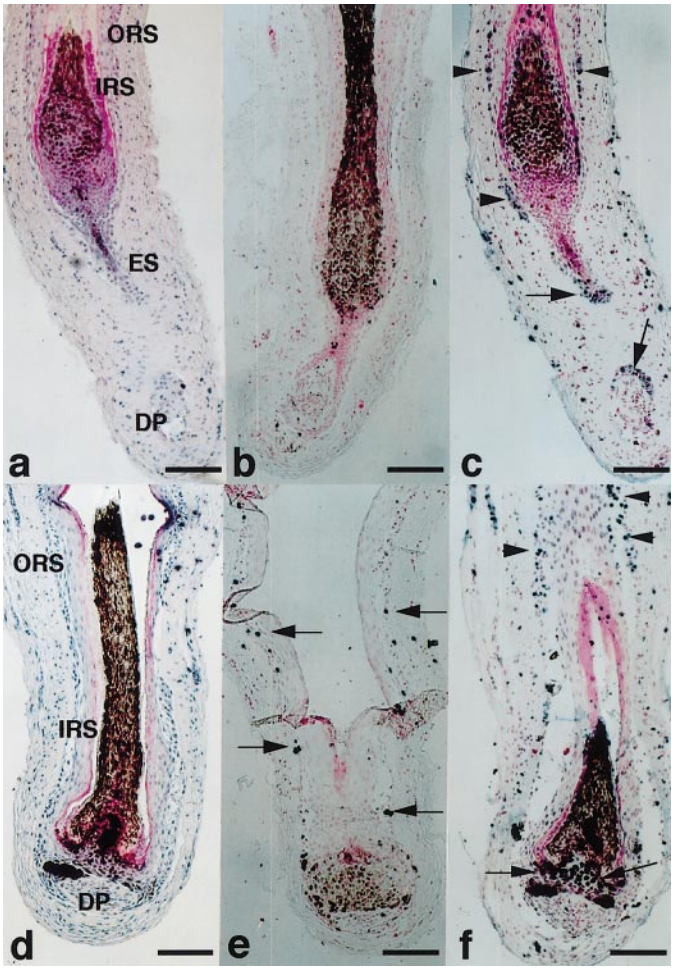

Figure 10. Histologic analysis for the effects of TGF-β2 and TNF-α. Morphologic changes, growth, and apoptotic cell death of hair follicles cultured for 7 d in the presence of 50 ng TGF-β2 per ml (*a–c*) or 50 ng TNF-α per ml (*d–f*) were shown. When anagen hair follicles were cultured in the basal medium with TGF-β2, similar catagen-like morphology (*a*) was induced much earlier than that in the basal medium alone. Little uptake of BrdU was seen in the bulb matrix cells and the ORS cells (*b*). Moreover, the pattern of characteristic cell death in the bulb matrix cells around the DP (indicated by *arrows*) and the ORS cells (indicated by *arrowheads*) was induced as seen in catagen (*c*). On the other hand, hair follicles cultured with TNF-α showed abnormal morphologic changes that were distinct from those observed in *in vivo* catagen phase (*d*). Marked BrdU uptakes into some ORS cells were observed (*e*, indicated by *arrows*), although TUNEL positive cells were increased in the bulb matrix (*f*, indicated by *arrows*) and in the ORS (*f*, indicated by *arrowheads*). *Scale bars*: 100 μm.

caspases are required to reach the final step of execution. It could be possible that there are multiple lines of activation processes in hair follicles, since we have detected at least four kinds of caspases. Responding to some specific signals, particular enzymes among four or more caspases are activated in the different portion of the hair follicle, or the different stage of the hair cycle, which leads to the initiation of the catagen phase. In contrast to apoptosis of epithelial cells in the hair follicle, no apoptotic cell was observed in the dermal papilla and dermal sheath mesenchymal cells. This finding, consistent with previous reports (Stenn *et al*, 1994; Lindner *et al*, 1997), suggests that the dermal papilla cells were prevented from apoptosis through the hair cycle probably by expressing bcl-2 gene product.

Although we showed a distinct cell death pattern in catagen hair follicles, little is known about what kind of molecules are involved in the initiation of this process. We chose TGF-β and TNF-α for possible candidates, because TGF-β is a potent negative growth regulator of most epithelial cells, and TNF-α is a well-known apoptosis inducer in many cell types (Bellomo *et al*, 1992; Nagata, 1997). In order to analyze the effect of these molecules on the human hair cycle, we employed the organ culture method described by Philpott *et al* (1990). Our results clearly demonstrated the difference between TGF-β and TNF-α. In the presence of TGF-β2, [methyl-^{3}H]thymidine incorporation in hair follicles was strongly inhibited; however, the elongation of the hair shaft was not affected. The result probably reflected that the hair shaft continued to move upward during catagen, giving the illusion of the hair elongation. TGF-β treatment also changed the time course of catagen induction in basal medium. In addition, we performed a similar experiment using 50 ng TGF-β per ml in the supplemented medium containing insulin as a survival factor (data not shown). It took longer culture days (day 7–8) to start catagen-like morphologic changes. Time course study showed significant inhibition of hair follicle growth at day 10. All of our results suggested that TGF-β could work in growth factor-deficient as well as sufficient conditions to induce catagen. TGF-β family members play critical roles in the morphogenesis of skin and its appendages (Lyons *et al*, 1990; Blessing *et al*, 1993; Matzuk *et al*, 1995). Transgenic mouse studies that were targeted to TGF-β or its receptor (Sellheyer *et al*, 1993; Cui *et al*, 1995; Wang *et al*, 1997) revealed inhibition of normal development and/or suppression of epithelial cell proliferation in epidermis and hair follicles. In adult skin tissue, suprabasal keratinocytes express and secrete anti-proliferative factors including TGF-β (Münger *et al*, 1992). In anagen rat hair follicles, mRNA and protein of TGF-β were detected (Little *et al*, 1994). During mouse hair cycle, mRNA levels of TGF-β1 and β3 in back skin were increased in the late anagen, and dramatically decreased between late catagen to telogen (Welker *et al*, 1997). Paus *et al* investigated the localization and the expression of TGF-β receptors in mouse hair cycle (Paus *et al*, 1997). They showed hair cycle-dependent expression of transforming growth factor-β type II receptor protein in the bulb matrix cells and the ORS cells in mouse hair follicles. All of these results, including ours, indicate that the TGF-β family would be a strong candidate in the initiation of catagen phase.

TNF-α induced drastic morphologic changes to the hair bulb due to the marked condensation of the DP, abnormal differentiation, and keratinization of the bulb matrix cells and the IRS cells. We detected apoptotic cell death not only in the ORS cells but also in the precortical cells in the bulb matrix that were never stained with TUNEL method in normal anagen hair follicles. Interestingly TNF-α stimulated proliferation of some ORS cells as shown by BrdU incorporation, whereas it inhibited hair follicle growth in a dose-dependent manner. These results strongly suggest that TNF-α may not be a major driving force for catagen induction. Alopecia areata is known to be caused by autoimmune mechanisms and is shown to have linkages with ICAM-1 and HLA class II antigens (Swerlick *et al*, 1991; Trefzer *et al*, 1991; McDonagh *et al*, 1993). Expression of these molecules was induced by several cytokines including TNF-α. TNF-α secreted from activated T cells around the hair bulb may lead to the apoptosis of the matrix cells, resulting in the abnormal morphology of hair follicles in this disease.

We demonstrated that in catagen hair follicles, apoptosis in the epithelial component exactly reflects the loss of epithelial cells of this stage. In addition, TGF-β would be involved in the initiation of catagen phase. We still do not know whether TGF-β can activate certain caspase directly or indirectly. Further study is necessary to understand the catagen induction mechanism concerning the activation of the caspase network. This study provides the basis for the elucidation of the apoptotic mechanism of catagen induction in human hair cycle.

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