Cyclosporin A-induced hair growth in mice is associated with inhibition of calcineurin-dependent activation of NFAT in follicular keratinocytes

Anat Gafter-Gvili,1 Benjamin Sredni,2 Rivka Gal,1 Uzi Gafter,1 and Yona Kalechman2

1Departments of Nephrology and Pathology, Rabin Medical Center, Petah Tikva, 49372; Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978; and 2Cancer and AIDS Research Institute, Faculty of Life Sciences, Bar Ilan University, Ramat Gan, 52900 Israel

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Gafter-Gvili, Anat, Benjamin Sredni, Rivka Gal, Uzi Gafter, and Yona Kalechman. Cyclosporin A-induced hair growth in mice is associated with inhibition of calcineurin-dependent activation of NFAT in follicular keratinocytes. Am J Physiol Cell Physiol 284: C1593–C1603, 2003; 10.1152/ajpcell.00537.2002.—One of the most common side effects of treatment with cyclosporin A (CsA) is hypertrichosis. This study shows that calcineurin activity is associated with hair keratinocyte differentiation in vivo, affecting nuclear factor of activated T cells (NFAT1) activity in these cells. Treatment of nude or C57BL/6 depilated normal mice with CsA inhibited the expression of keratinocyte terminal differentiation markers associated with catagen, along with the inhibition of calcineurin and NFAT1 nuclear translocation. This was associated with induction of hair growth in nude mice and retardation of spontaneous catagen induction in depilated normal mice. Furthermore, calcineurin inhibition blocked the expression of p21wafr1 and p27kip1, which are usually induced with differentiation. This was also associated with an increase in interleukin-1α expression (nude mice), a decrease in transforming growth factor-β (nude and normal mice), and no change in keratinocyte growth factor expression in the skin. Retardation of catagen in CsA-treated mice was accompanied by significant alterations in apoptosis-related gene product expression in hair follicle keratinocytes. The ratio of the anti-apoptotic Bcl-2 to proapoptotic Bax expression increased, and expression of p53 and interleukin-1β converting enzyme activity decreased. These data provide the first evidence that calcineurin is functionally active in follicular keratinocytes and that inhibition of the calcineurin-NFAT1 pathway in these cells in vivo by CsA enhances hair growth.

catagen; epithelial cells; terminal differentiation; apoptosis; hypertrichosis; nuclear factor of activated T cells

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) (20). The hair follicle regression during catagen reflects a tightly coordinated process characterized by apoptosis and terminal differentiation of the proximal epithelial hair bulb, perifollicular proteolysis, and matrix remodeling, as well as termination of follicular melanogenesis (22). Although the morphological changes associated with the hair cycle are well described, the underlying molecular controls that terminate anagen and initiate catagen are still poorly understood (28, 39, 42).

Immunosuppressive immunophilin ligands such as cyclosporin A (CsA) and FK506 are known as potent hair growth modulatory agents in rodents and humans that induce active hair growth (anagen) (18, 24, 34) and inhibit hair follicle regression (catagen) (24, 32). The immunosuppressive effectiveness of these drugs has been generally attributed to inhibition of T cell activation through well-characterized pathways. Specifically, CsA and FK506 bind to intracellular proteins, principally cyclophilin A and FKBP12, respectively, and thereby inhibit the phosphatase calcineurin (35). This leads to an increased phosphorylation of calcineurin substrates, particularly the family of nuclear factor of activated T cells (NFAT) nuclear transcription factors, preventing translocation of NFAT to the nucleus, resulting in decreased IL-2 production and T cell activation (35). Interestingly, calcineurin and members of the NFAT family are also expressed in a variety of nonimmune cells including heart, muscle, and brain (11, 26, 35) and exert diverse effects such as regulation of cardiac hypertrophy, blood vessel assembly, muscle differentiation, and memory formation (11, 13, 26, 35). In addition to the well-recognized CsA target in signal transduction, cyclophilin-calcineurin-NFAT route, other signaling routes have been suggested as the targets of CsA as the phospholipids metabolism-PKC signal transduction route (44), resulting in the inhibition of the release of arachidonic acid, prostaglandin, leukotrienes, and specific cytokines (14). Other regulatory molecules such as nuclear factor-κB (NFκB) (17) and nitric oxide (16) have been reported to be affected by CsA. Recently, calcineurin/NFAT have been localized in mouse keratinocytes (37) and in human skin (2). Moreover, these have been shown to be involved in the control of keratinocyte terminal differentiation in vitro (37).
A common side effect of both CsA and FK506 in the skin is induction of hair growth. This side effect is probably independent of the immune system because it occurs also in T cell-deficient nude mice (49). The spontaneously occurring nude mouse mutation has pleotropic effects that result in the abnormal development of the skin, hair follicles, and thymus (9). Although the nude mouse appears hairless, its dermis contains a substantial number of follicles. However, these are aberrant and incompletely developed (29, 49). The abnormal keratinization of the hair follicles is characterized by short, bent hair shafts that rarely protrude from the follicle. Keratinization of the epidermis is also aberrant in that the stratum corneum contains highly irregular piles of cornified debris. The nude mouse phenotype results from loss-of-function mutations in Whn, a winged-helix transcription factor, resulting in the disruption of the balance between growth and differentiation in self-renewing epidermal tissue (4, 27). One major effect of the winged-helix nude (whn) gene mutations is the impairment of cutaneous terminal differentiation reflected in premature termination of follicle growth and induction of catagen. As a result, in nude hair follicles, the inner root sheath and hair shaft exhibit structural abnormalities, the most striking of which is the absence of the hair cortex. Moreover, nude epidermis displays irregular formation of the stratum corneum (21). Whn-mutated keratinocytes have a greater propensity for expressing markers of differentiation under basal proliferating conditions, producing lower amounts of the early marker keratin 1 and higher levels of the late markers involucrin and filagrin (4).

Growth and development of hair follicles are influenced by a variety of growth factors and cytokines, the most widely described being keratinocyte growth factor (KGF), interleukin-1 (IL-1), and transforming growth factor β (TGFβ) (5, 6, 10). Recently, TGFβ1 has been reported to control murine hair follicle regression (catagen) in vivo (10). At least in vitro, CsA has been previously described to modulate the production of these cytokines (1, 7, 23).

Although CsA has been previously reported to induce anagen and to inhibit massive catagen development, few data are currently available on the in vivo mechanism of action of these effects. The aim of the present study is to obtain insight into the mechanism of CsA-induced hair growth. For this purpose, we used two models: 1) the nu/nu mouse model in which premature termination of follicle growth and induction of catagen occurs, and 2) depilated C57BL/6 mice. This study addressed the questions as to the role of calcineurin/NFAT1 in the in vivo control of keratinocyte premature differentiation in nude mice. In addition, the control by CsA of hair keratinocyte apoptosis in vivo regarding caspases activity and expression of apoptotic and anti-apoptotic products was explored. Furthermore, the in vivo effect on the production of cytokines known to modulate hair growth and regression was examined.

**MATERIALS AND METHODS**

**Reagents and Antibodies**

Anti-p21\(^{\text{wat}}\) (mouse IgG2b), anti-p27\(^{\text{kap1}}\) (rabbit IgG), and anti-involucrin (goat IgG) were from Santa Cruz Biotechnolog (Santa Cruz, CA). Anti-calcineurin/PP2B A and B (rabbit IgG) and anti-NFAT1 (rabbit antiserum) were from Upstate Biotech. The immunogen used for its production is a glutathione S-transferase (GST)-fusion protein corresponding to residues 1–297 of murine NFAT1. Anti-KGF (donkey IgG), anti-IL-1α (goat IgG), anti-TGFβ3 (rabbit IgG), anti-p53 (goat IgG), anti-Bcl2 (mouse IgG1), anti-Bax (rabbit IgG), and IL-1β converting enzyme (ICE) (rabbit IgG) were also from Santa Cruz Biotechnology. Anti-loricrin (rabbit IgG) was from Covance (Richmond, CA), and CsA was from Sandimmune Novartis (Basel, Switzerland).

**Treatment Protocol**

**Animals.** Athymic male nude (nu/nu) mice of Balb/c origin and normal female C57BL/6 mice, 7 wk old, were obtained from Charles River Laboratories (Wilmington, MA). Animal experiments were performed in accordance with approved institutional protocols and approved by the Institutional Animal Care and Use Committee.

Specific concentrations of CsA in soy oil or soy oil vehicle control were daily administered orally by cannulation to nude mice. On day 16, after daily treatments with CsA, nude mice were evaluated for hair density and hair coverage. At this time, gross photographs were taken and skin sections from each mouse were harvested and fixed in buffered formalin for routine hematoxylin and eosin (H and E)-stained histology.

Spontaneous catagen development was induced as previously described (31). In brief, C57BL/6 mice in telogen (pink skin) were induced to enter anagen by depilation. After 16–19 days, these follicles entered spontaneously into catagen, as can be appreciated from the conversion of their skin color from black (anagen) to gray (catagen) and finally to pink (telogen). Mice were fed daily with CsA on days 8–12 after depilation. Inhibition of catagen was recorded on days 16–18. Skin sections were taken from standardized back skin regions on days 16–18 after depilation for the isolation of hair keratinocytes and for preparing whole skin lysates.

The oral doses of CsA (10–100 mg/kg) we used have been previously shown to be the effective doses of CsA for inducing hair growth (31, 32). To assess the blood levels of CsA-treated mice, we fed mice with the highest experimental dose (100 mg/kg) of CsA. Blood levels were measured 12 h after feeding (similar to humans) in pooled blood from six pairs of mice. The mean blood level was 821 ± 91 ng/ml, slightly higher than in human kidney recipients treated with CsA during the first year of transplantation.

**Histological Assessment of Hair Growth**

Paraflin-embedded 5-μm sections were stained with H and E. Hair growth was evaluated microscopically in these stained sections of skin taken from the dorsum and abdomen. The following were assessed: 1) analysis of follicles that contain hairs that are birefringent under polarized light; the number of follicles was expressed per the entire slide; and 2) evaluation of birefringent hairs outside the skin; the number of birefringent hairs was expressed per the entire slide.

Some substances with well-organized and -oriented structures exhibit double refraction under polarized light. The hair fiber is made of keratin, which is organized into fila-
ments. These structures, as well as crystals, collagen, silica, etc., give typical diffraction patterns (birefringence). Examination of the hair shaft under polarized light is therefore a precise method that allows us to see the slightest structural abnormalities of hair.

Isolation of Hair Epithelial Cells

Epidermis was dissected from the dorsal skin of mice as previously reported (31). The dermis was dispersed in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.25% collagenase at 37°C for 1 h, stirred occasionally. The dermis suspension was filtered through a 212-µm nylon mesh, and the filtrate was centrifuged at 1,400 rpm. The pellet was resuspended in phosphate-buffered saline (PBS), and the suspension was left to stand for 15 min, allowing the hair follicle tissue to precipitate due to the difference in specific gravity, after which the supernatant was removed by using an aspirator. The hair follicle tissue was resuspended and precipitated. This precipitation process was repeated three times. Finally, the hair follicle tissue was incubated in 0.05% EDTA-0.25% trypsin in Hanks’ balanced calcium and magnesium-free salt solution at 37°C for 5 min. The suspension was filtered via a 212-µm nylon mesh, the cells were centrifuged, and the pellet was resuspended in DMEM supplemented with 10% FBS. Cells were plated on collagen-coated dishes and incubated at 37°C. Twenty-four hours after seeding, the medium was discarded and the cells were washed with 0.02% EDTA and treated with 0.15% trypsin at 37°C for 5 min to remove contaminated dermal fibroblasts. The remaining cells were subjected to lysis for the preparation of whole or nuclear cell lysates. This isolated population represents hair tissue keratinocytes and is not contaminated with mesenchymal cells. Fluorescence-activated cell sorting (FACS) analysis shows 97% positive staining for anti-pan keratin and negative staining for anti-vimentin. The ultrastructure of cells similarly isolated has been previously characterized, resembling hair matrix cells (45).

Preparation of Cytoplasmic and Nuclear Cell Lysates

Cells were washed in cold PBS. Cell membranes were disrupted by repeated freezing and thawing in liquid nitrogen. The mixture was suspended in buffer A [10 mM NaCl, 10 mM HEPES buffer, pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, 5% (vol/vol) glycerol, 0.5 mM PMSF, 50 mM NaF, 0.1 mM sodium vanadate, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 2 µg/ml chymostatin, and 1.5 µg/ml pepstatin] and was then centrifuged at 10,000 rpm. The supernatant (cytoplasmic cell lysate) was collected and microscopically visualized for lack of nuclei. The pellet, containing nuclei clearly microscopically visualized, was suspended in buffer B (identical to buffer A except for the NaCl concentration [400 mM]) and centrifuged at 10,000 rpm for removal of debris. The supernatant (nuclear cell lysate) was collected and stored at −80°C. Western blot analysis with anti-Histone 1 used as the positive control for the nuclear extraction and GAPDH served as the negative control. Alternatively, Histone 1 and GAPDH served as negative and positive controls for the cytoplasmic lysates (data not shown).

Western Blot Analysis

Extracts from whole skin (KGF, IL-1α, and TGFβ) or from epithelial cells from dissected hair follicles (p21вал, p27kip1, involucrin, loricrin, K1, NFAT and calcineurin, Bax, Bel-2, P53, and ICE) were prepared by fractionation of whole, nuclear, and cytoplasmatic cell lysates. Whole cell lysates were prepared by suspension in ice-cold radioimmunoprotective assay (RIPA) buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, 0.5% deoxytartaricosterone, 0.2 mM PMSF, 50 mM NaF, 200 µM NaVO3, 20% glycerol, 5 µg/ml aprotinin, and 5 µg/ml leupeptin). Samples were immunoprecipitated using anti-mouse KGF. Immune complexes were precipitated with protein A-Sepharose. Immune precipitates were blotted with specific antibodies. Blots were developed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection system (Amersham Pharma Biotech, Uppsala, Sweden).

Statistical Analysis

Data are presented as means ± SE. Multiple comparisons between groups treated with different doses of CsA were performed with the Kruskal-Wallis one-way analysis of variance. The Mann Whitney U-test was used for comparisons between two groups. Spearman’s correlation was used to evaluate correlations between the different groups.

RESULTS

Induction of Hair Growth by CsA

Nude mice. CsA induced a dose-dependent hair growth, 100 mg/kg being the optimal dose (Fig. 1). In all mice, the dorsal hair was more prominent than the abdominal hair. Hair growth was evident as early as 7 days after commencing CsA treatment, and continuous administration of CsA was mandatory for sustaining hair growth. Discontinuation of CsA treatment resulted in the total loss of the newly grown hair as early as 7 days after treatment stopped. Readministration of CsA for 16 days after termination of treatment induced hair to grow again, reaching a similar level as observed in the uninterrupted treatment. Histological analysis of skin specimens in control nude mice revealed dystrophic follicles that contained fragmented hair shafts that did not emerge from the hair follicles and barely showed any birefringence under polarized light (Fig. 2A). Treatment with CsA resulted in relatively normal follicles containing well-differentiated straight hair shafts that usually emerged from hair follicles reaching the skin’s surface and showing clear birefringence of the cuticle (Fig. 2B) as in normal hair. Moreover, the number of follicles that contain hairs that are birefringent under polarized light was also significantly increased in both areas of CsA-treated mice. In the dorsum, it rose from 5.83 ± 1.96 to 25 ± 6.15/field at 100 mg/kg CsA (P < 0.02) with a positive correlation between the number of hair follicles with birefringent hairs and the dose of CsA (r = 0.725; P < 0.001) (Table 1). Likewise, in the abdomen, the number of hair follicles with birefringent hairs rose from 1 ± 0.37 to 6.8 ± 2.35/field at 50 mg/kg CsA (P < 0.02). Furthermore, the number of birefringent hairs outside the skin was also significantly elevated in CsA-treated mice both in the dorsum and in the abdomen (Table 1).

C57BL/6 mice. Hair growth control by CsA in normal mice was assessed by studying its ability to delay the
spontaneous catagen in the C57BL/6 mouse model by assessing defined, hair cycle-dependent, cutaneous color changes that reliably indicate hair cycling in the back skin of mice after repetitive feeding of CsA. Inhibition of catagen was studied in mice that had been induced to enter anagen by depilation. Figure 3 shows that 18 days after depilation, 86.6% of vehicle-fed mice reentered telogen via catagen as opposed to 26.6% of mice fed with 10 mg/kg CsA and 6.66% of those fed with 50 mg/kg CsA. The delay in catagen was prominent at all days tested and was CsA-dose-dependent.

In Vivo Modulation of Cytokine Production by CsA in Nude Mice

We evaluated the protein expression of three cytokines known to modulate hair growth and regression. KGF expression in the skin of CsA-treated nude and C57BL/6 mice was not different from that of control mice at all CsA doses tested. However, the expression of IL-1α in nude, but not in C57BL/6 mice, significantly increased at 50 and 100 mg/kg CsA, and at 10 mg/kg it
Table 1. Histologic analysis of hair growth

<table>
<thead>
<tr>
<th>CsA, mg/kg</th>
<th>Hair follicles with birefringent hair</th>
<th>Birefringent hair outside skin</th>
<th>Hair follicles with birefringent hair</th>
<th>Birefringent hair outside skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.83 ± 1.96</td>
<td>2.67 ± 0.98</td>
<td>1.0 ± 0.37</td>
<td>1.5 ± 0.62</td>
</tr>
<tr>
<td>10</td>
<td>20.0 ± 4.2*</td>
<td>7.00 ± 2.22</td>
<td>4.0 ± 3.37</td>
<td>2.5 ± 0.96</td>
</tr>
<tr>
<td>50</td>
<td>23.0 ± 2.6*</td>
<td>8.40 ± 7.17</td>
<td>6.8 ± 2.35†</td>
<td>12.0 ± 1.7†</td>
</tr>
<tr>
<td>100</td>
<td>25.0 ± 6.15†</td>
<td>14.75 ± 3.07†</td>
<td>2.0 ± 1.68</td>
<td>6.7 ± 4.5</td>
</tr>
</tbody>
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Soy oil or cyclosporin A (CsA) at various concentrations was orally administered daily to nude mice. On day 16, sections of skin taken from the dorsal and abdomen were fixed in formalin and stained in hematoxylin and eosin. Sections were examined under polarized light for hairs with birefringence within follicles and for birefringent hairs outside the skin. The results represent means ± SE of 7 mice/group (vehicle control) or 5 mice/group (CsA). *P < 0.01 increase vs. control; †P < 0.02 increase vs. control.

was already noticeable. Assessment of the levels of TGFβ expression in the skin of both types of treated mice revealed a dose-dependent decrease in protein expression of this catenin-associated cytokine (Fig. 4).

In nude mice, keratinocyte premature differentiation occurs. In general, catagen represents terminal differentiation of follicular keratinocytes. Because p21^{waf1/cip1} and p27^{kip1} are usually induced with differentiation of both CDK inhibitors. In the C57BL/6 model, a less, treatment with CsA almost eliminated the expression of both proteins were found at all days in CsA-treated mice. This decrease was dose-dependent (Fig. 5).

Inhibition of Calcineurin-Dependent NFAT1 Nuclear Translocation in Follicular Keratinocytes In Vivo Is Associated with Delayed Expression of Terminal Differentiation Markers

Recently, Santini et al. (37) showed that calcineurin regulates the expression of mouse keratinocyte differentiation markers in vitro. We therefore wished to determine whether induction of hair growth by CsA in both mice models is indeed associated with delayed catagen, a stage that reflects a tightly coordinated process characterized by apoptosis and terminal differentiation of the proximal epithelial hair bulb, proteolysis, and matrix remodeling, as well as termination of follicular melanogenesis (22, 33, 42). Figure 6 shows that follicular keratinocytes from control nude mice express high levels of the differentiation markers involucrin and loricrin. These were considerably decreased after treatment with CsA in a dose-dependent manner. In the C57BL/6 model, the terminal of terminally differentiated markers expression gradually increased in control mice from day 16 to day 18 after depilation. In the CsA-treated mice, the protein expression of
these markers was extensively decreased at day 16 and did not change any further (Fig. 6).

Recently, Santini et al. (37) and Al-Daraji et al. (2) showed activation of the calcineurin-dependent transcription factor NFAT in cultured keratinocytes induced to differentiate in vitro. We therefore wished to ascertain whether the premature differentiation of follicular keratinocytes of nude mice and the terminal differentiation of hair epithelial cells in normal spontaneous regression (catagen) in normal mice is associated with the activation of NFAT. As depicted in Fig. 7, high levels of nuclear NFAT were expressed in follicular keratinocytes of control nude mice. In the depilated C57BL/6 mice, a gradual translocation of NFAT1 from the cytoplasm to the nucleus occurred in a time-dependent manner from day 16 to day 18. Treatment with CsA prevented NFAT1 nuclear translocation at day 16, and the high protein level of this transcription factor in the cytoplasm did not change during the overall catagen period. In nude mice, nuclear NFAT1 was abundant in follicular keratinocytes of control mice,
whereas in CsA-treated mice it was expressed predominantly in the cytoplasm (Fig. 7). Expression of NFAT1 in whole cell lysates showed a lower mobility form (higher molecular mass) in samples originating from both CsA-treated nude and depilated mice, suggesting prevention of calcineurin-dependent dephosphorylation of NFAT1 by CsA (Fig. 7).

Comigration of calcineurin to the nucleus is required to maintain NFAT in a dephosphorylated state, prevent export of NFAT from the nucleus, and sustain activation of NFAT target genes (38). It can be clearly seen that expression of terminal differentiation markers in epidermal keratinocytes of both nude mice and normal mice in catagen is associated with both nuclear translocation of NFAT1 and comigration of calcinurin A and B subunits. The level of nuclear calcineurin expression, however, is significantly decreased in CsA-treated mice (Fig. 8).

Role of CsA in the Inhibition of Apoptosis-Associated Gene Products in Hair Epithelial Cells

Keratinocyte apoptosis is a central element in the regulation of hair follicle regression (catagen). In this study, we show that the ratio of the antiapoptotic Bcl-2 and proapoptotic Bax expression, considered to be one critical factor in apoptotic control, is increased in follicular keratinocytes of CsA-treated nude mice compared with control mice. Specifically, the level of Bcl-2 expression did not change after treatment, but that of Bax clearly decreased. In normal mice, high levels of Bax expression (higher than that of nude mice) are seen 16–18 days after depilation; the level of this proapoptotic protein extensively decreased after CsA treatment. Like in nude mice, no alterations in protein expression of Bcl-2 was noticed in follicular keratinocytes of all groups (Fig. 9). Recently, p53 was shown to control apoptosis-driven physiological hair follicle regression (3). In our study, the expression of p53 was abundant both in nude mice and in catagen-induced follicular epithelial cells. Nevertheless, CsA significantly decreased the level of this transcription factor (Fig. 9). We then evaluated the level of ICE in catagen and its response to CsA, because activation of members of the ICE-like cysteine protease family is thought to represent a fairly late, irreversible key event in the apoptosis control machinery. Strong ICE activity was expressed by follicular epithelial cells of C57BL/6 mice throughout catagen development, which was consistently decreased in CsA-treated mice. Nevertheless, minimal levels of ICE were found in follicular keratinocytes of control and CsA-treated nude mice (Fig. 9).

DISCUSSION

One of the most common side effects of treatment with CsA is hypertrichosis, which affects 50–80% of treated transplant recipients and is reversible (19). CsA has been also previously shown to induce hair growth both in nude (48) and normal mice (31, 34). Our experimental data support these reports. Daily oral administration of CsA to nude mice resulted in a dose-dependent pronounced hair growth. Histologically, abundant follicles containing straight hair shafts that usually emerge from hair follicles reaching the skin’s surface and showing clear birefringence were seen. In the depilated C57BL/6 model, we show that CsA treat-
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CALCINEURIN-NFAT IN KERATINOCYTES AFFECTS HAIR GROWTH

C57BL/6 mice were orally treated with 100 mg/kg CsA or soy oil. C57BL/6 mice were fed daily with 100 mg/kg CsA or soy oil. Nude mice were fed daily with 100 mg/kg CsA or soy oil. C57BL/6 mice were orally treated with 100 mg/kg CsA on days 8–12 after depilation. Protein expression of Bcl-2, Bax, p53, and ICE was assessed in whole cell lysates of hair epithelial cells from nude (day 16) or C57BL/6 mice (days 16–18 after depilation). Results are representative of 3 mice/group.

Fig. 9. Role of CsA in the inhibition of apoptosis-associated gene products in hair epithelial cells. Nude mice were fed daily with 100 mg/kg CsA or soy oil. C57BL/6 mice were orally treated with 100 mg/kg CsA on days 8–12 after depilation. Protein expression of Bcl-2, Bax, p53, and ICE was assessed in whole cell lysates of hair epithelial cells from nude (day 16) or C57BL/6 mice (days 16–18 after depilation). Results are representative of 3 mice/group.

C57BL/6 mice

NUDE MICE

DAYS AFTER DEPILATION

CsA(mg/kg)

16 17 18

0 100

Bcl-2

26kDa

BAX

23kDa

p53

53kDa

ICE

20kDa

ACTIN

42kDa

Normal hair growth requires a balance between keratinocyte growth and differentiation in the hair follicle (12). The hair follicle regression during catagen reflects a tightly coordinated process characterized by apoptosis and terminal differentiation of the proximal epithelial hair bulb, proteolysis, and matrix remodeling, as well as termination of follicular melanogenesis (22, 33, 42). The control of hair follicle regression (catagen) is still obscure. This hair cycle stage is clinically important because most cases of hair loss seen in clinical practice involve premature termination of follicle growth (anagen) and induction of catagen. Classically, this is seen in alopecia areata, chemotherapy-induced alopecia, and telogen effluvium but also in androgenic alopecia, in which progressive shortening of the anagen phase results in the vellus transformation of terminal hair follicles (30, 42). Similarly, in the nu/nu mouse, one major effect of the whn gene mutations is the impairment of cutaneous terminal differentiation reflected in premature termination of follicle growth and induction of catagen. Moreover, Whn-mutated keratinocytes have a greater propensity for expressing markers of differentiation under basal proliferating conditions (4). Thus, in nude mice, an alteration in the balance of keratinocyte growth and differentiation occurs.

In this study, we show for the first time that in the skin, the calcineurin/NFAT1 pathway is associated with hair keratinocyte differentiation in vivo. Treatment of nude or normal mice with CsA inhibited the expression of terminal differentiation markers associated with catagen, along with the inhibition of calcineurin and NFAT1 nuclear translocation. This was correlated with the induction of hair growth in nude mice and the retardation of spontaneous catagen induction in depilated normal mice. Furthermore, calcineurin inhibition by CsA treatment blocked the expression of p21waf/cip1 and p27kip1, two cyclin-depen-
dent kinase inhibitors that are usually induced with differentiation. Our results are in line with those of Santini et al. (37) who recently showed the involvement of the calcium/calmodulin-dependent phosphatase calcineurin in the switch between epithelial cell growth and differentiation in vitro through a mechanism that appears to involve an interaction between NFAT1/NFAT2 and the Sp1/Sp3 transcription factors (37). Coordinated expression of p21waf protein and mRNA in differentiating follicular epithelial cells suggests that p21 is involved in the differentiation process of hair matrix cells. Therefore, the results of Santini et al. (37) showing that calcineurin induces the activity of the p21waf promoter in cultured keratinocytes along with our data showing decreased p21waf/cip1 expression in hair keratinocytes of CsA-treated mice suggest that CsA prevents or retards premature and spontaneous terminal differentiation of hair keratinocytes in vivo via inhibition of calcineurin-dependent expression of p21waf/cip1 and possibly also p27kip1. Nevertheless, the importance of p21waf/cip1, but not that of p27kip1, in keratinocyte differentiation was previously reported by Missero et al. (25), who showed profound differences in the differentiation behavior of p21 vs. p27 knockout keratinocytes, with p21 (but not p27) deficiency causing a drastic downmodulation of differentiation markers linked with the late stages of the keratinocyte terminal differentiation program. Our results are also in line with those of Al-Daraji et al. (2), who recently showed that calcineurin and NFAT are localized in human skin particularly in keratinocytes (2).

Hair follicle transition from anagen to catagen is a physiological process of programmed organ involution that is characterized by tightly coordinated apoptosis in the cyclic portion of the hair follicle epithelium (22). The process of apoptosis is controlled through the activation of many factors, including some cytokines, tumor suppressor gene products, Bcl-2 family gene products, and ICE family proteases. In anagen hair follicles, dermal papilla fibroblasts secrete numerous
growth factors that maintain active proliferation and differentiation of keratinocytes of the proximal hair bulb leading to the hair fiber formation (43). Hair follicle transition from anagen to catagen is associated with a sudden decline in secretion of growth factors by the dermal papilla, resulting in the dramatic reduction of proliferation of hair matrix keratinocytes with cessation of hair shaft production. This is accompanied by massive apoptosis. We therefore evaluated whether prevention or retardation of catagen after treatment with CsA is associated with alterations in cytokines known to control hair growth. We chose to evaluate protein expression of three cytokines, KGF, previously described to stimulate hair growth in nude mice by stimulating follicular proliferation and inducing normalization of the nu/nu follicular keratin differentiation defect (6). In addition, KGF mRNAs have been previously localized in the dermal papilla of hair follicles (36). Nevertheless, hair growth induced by CsA was not associated with changes in KGF protein expression in both models. Interestingly, higher KGF transcripts were recently found in CsA-induced gingival outgrowth tissue (7). IL-1α has been described both as a potent stimulant of follicular keratinocytes (5) and an inhibitor of human hair follicle growth in whole organ cultures (15). CsA was shown to induce secretion of IL-1α and IL-β from human keratinocytes in culture (23). In our study, induction of hair growth by CsA in nude mice was associated with increased expression of IL-1α. More importantly, the expression of TGFβ, recently shown to control murine and human hair follicle regression in vivo (10, 40), was significantly decreased in CsA-treated mice in both models. It is noteworthy that CsA has been shown to stimulate the synthesis of TGFβ in a variety of cells (1, 46). Our results do not necessarily imply that CsA directly decreases TGFβ expression. Alternatively, it is conceivable that CsA delays hair follicle keratinocyte terminal differentiation via inhibition of calcineurin-dependent activation of NFAT1, resulting in retardation of catagen induction. This alteration may result in epithelial-mesenchymal interactions, keratinocytes sending in turn signals to the papilla to stop producing catagen-inducing cytokines. The papilla is an inductive structure that sends and receives signals, its effect depending on continuous and intimate interaction with the hair matrix epithelium (43). For example, keratinocytes have been reported to produce a specific factor, which stimulates the growth of human scalp papilla cells in vitro (47).

Apart from alterations in cytokine expression, we show that retardation of catagen in CsA-treated mice is accompanied by a notable decrease in both p53 and the ratio of Bax/Bcl-2-proteins that are encoded by p53 target genes and are implicated in the control of catagen.

Recently, the transcription factor p53 has been shown to be involved in the control of murine hair follicle regression. p53 was strongly expressed and colocalized with apoptotic markers in the regressing hair follicle compartments during catagen (3). Furthermore, p53 knockout mice show significant retardation of catagen accompanied by significant decrease in the number of apoptotic cells in the hair matrix (3). It has been speculated that p53 may serve as a molecule mediating apoptosis in hair matrix keratinocytes after growth factor withdrawal, because hair follicle transition from anagen to catagen is characterized by the decline in secretion of growth factors by dermal papilla for hair matrix keratinocytes.

We show that activated ICE is expressed in hair keratinocytes in the stage of catagen, being substantially decreased after treatment with CsA.

The importance of ICE family proteases as the executor of apoptosis is now widely accepted. 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. Soma et al. (39) showed that the induction of catagen is independent on the production of caspases but is dependent on the activation of certain caspases. Involvement of sequential activation of caspases in the apoptotic process has been reported and confirmed by various investigators (8, 41). It could be possible that there are multiple lines of activation processes in hair follicles, because Soma et al. (39) have detected at least four kinds of caspases (including ICE) in different portions of the hair follicle.

Collectively, our data reveal a unique cross talk in vivo among calcineurin, NFAT1, and upregulation of p21\(^{waf}\) affecting terminal differentiation of hair keratinocytes and induction of catagen, associated with alterations in cytokine expression, apoptosis-related suppressor genes, and activated ICE. Treatment with CsA reverses these processes, resulting in catagen retardation and induction of hair growth, one of the most common side effects affecting 50–80% of treated transplant recipients. In addition, given the crucial role of catagen control for most hair disorders seen in humans (alopecia, effluvium, hirsutism), these results may have important clinical implications. CsA has been shown to induce hair growth in nude mice and to retard physiological catagen when applied topically. Inhibiting premature catagen by delaying terminal differentiation via inhibition of NFAT activation of keratinocytes may represent a novel strategy for the management of various forms of alopecia and effluvium by topical treatment with CsA. Alternatively, activation of NFAT may serve for the management of hirsutism.

REFERENCES


