Overexpression of Bcl-2 Protects from Ultraviolet B-Induced Apoptosis but Promotes Hair Follicle Regression and Chemotherapy-Induced Alopecia

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Hair follicle (HF) growth and regression is an exquisitely regulated process of cell proliferation followed by massive cell death and is accompanied by cyclical expression of the apoptosis regulatory gene pair, Bcl-2 and Bax. To further investigate the role of Bcl-2 expression in the control of hair growth and keratinocyte apoptosis, we have used transgenic mice that overexpress human Bcl-2 in basal epidermis and in the outer root sheath under the control of the human keratin-14 promoter (K14/Bcl-2). When irradiated with ultraviolet B (UVB) light, K14/Bcl-2 mice developed about 5–10-fold fewer sunburn cells (ie, apoptotic keratinocytes) in the basal layer of the epidermis, compared to wild-type mice, whereas cultures of primary keratinocytes from transgenic mice were completely resistant to UVB-induced histone formation, at doses that readily induced histone release from wild-type cells. K14/Bcl-2 mice show no alteration of neonatal hair follicle morphogenesis or of the onset of the first wave of HF regression (catagen). However, compared to wild-type controls, K14/Bcl-2 mice subsequently displayed a significant acceleration of spontaneous catagen progression. During chemotherapy-induced alopecia, follicular dystrophy was promoted in K14/Bcl-2 mice. Thus, although K14-driven overexpression of Bcl-2 protected murine epidermal keratinocytes from UVB-induced apoptosis, it surprisingly promoted catagen- and chemotherapy-associated keratinocyte apoptosis. (*Am J Pathol* 2000, 156:1395–1405)

Apoptosis, a morphologically and biochemically defined cell suicide program by which redundant or damaged cells are eliminated from an organism, plays a crucial role not only during the morphogenesis of embryonic tissues, but also in tissue homeostasis in the adult.^{1–5} One of the

most instructive models for neonatal tissue development and adolescent tissue remodeling is growth and regression of the hair follicle (HF).^{6,7} These processes reflect, to a large extent, the balance of intrafollicular keratinocyte proliferation and apoptosis: during morphogenesis and anagen, human and murine HFs are characterized by intense proliferation and substantial apoptotic activity,^{8,9} whereas HF regression is mainly driven by massive apoptosis of hair bulb keratinocytes.¹⁰ However, the molecular regulation of keratinocyte apoptosis in the HF, which is of pivotal clinical importance,⁷ is still largely obscure.

Bcl-2 is the founding member of a large family of highly homologous apoptosis regulatory proteins.^{2–5} The members most closely related to Bcl-2, such as Bcl-x_L, and Bcl-2 itself, promote cell survival, whereas more distant relatives, such as Bax, Bcl-x_S, or Bak, induce apoptosis.^{2–5} Pro- and antiapoptotic members of this family can heterodimerize with each other by virtue of conserved sequence motifs, thereby regulating each other's activity.^{2–5} One possible mechanism of action of the antiapoptotic proteins may involve their ability to inhibit the activation of caspases such as ICE (interleukin 1 β converting enzyme, caspase 1), the proteases that function in the execution phases of apoptosis (reviewed in refs. 2 and 11).

Bcl-2 expression in HF is strictly hair cycle-dependent,^{10,12} being more strongly expressed during phases of intense proliferative activity and down-regulated during HF regression. Although functional evidence has not been available to date, Bcl-2 has been suspected to play an important role in follicular keratinocyte regression and growth during the hair cycle.^{10,12} Disruption of the tight control of Bcl-2 expression might thus be expected to have a profound effect on hair follicle morphogenesis and cycling. Indeed, Bcl-2 α - and Bcl-2 $\alpha\beta$ -deficient mice reportedly show a substantial retardation of HF cycling

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compared to wild-type (WT) mice^{13–15}, and Bcl-2 $\alpha\beta$ null mutants display retarded anagen development after depilation.¹³

We describe here a new transgenic mouse line that overexpresses human Bcl-2 in the basal layer of epidermis and various components of the hair follicle, under the control of the human keratin-14 promoter. We have used this mouse model to investigate the effect of high-level expression of Bcl-2 on neonatal HF morphogenesis and adolescent HF cycling in mice, with particular emphasis on apoptosis-driven catagen development.¹⁰ Because we had previously shown that chemotherapy-induced alopecia in mice is associated with a massive increase in intrafollicular apoptosis,¹⁰ cyclophosphamide-induced alopecia¹⁶ was also compared between K14/Bcl-2 and WT mice. The effects of Bcl-2 overexpression on ultraviolet B (UVB)-induced apoptosis of murine epidermal keratinocytes *in situ* ("sunburn cells") and *in vitro* were examined to ensure that the transgenic Bcl-2 was functioning as expected. Taken together, these assays reveal surprisingly complex consequences of Bcl-2 overexpression for apoptosis-related phenomena in different epithelial tissue compartments of murine skin.

Materials and Methods

Mice

FVB/N and Swiss Webster mice (Taconic Farms, Germantown, NY) were used for the construction of the transgenic lines. These mice were housed in community cages, with 12-hour light periods, at the Harvard Skin Disease Research Center, Animal Facilities (Boston, MA), and were fed water and mouse chow *ad libitum*.

Reagents

The Cell Death Detection ELISA was purchased from Boehringer-Mannheim (Indianapolis, IN). All enzymes were purchased from Boehringer-Mannheim. Hamster anti-human-Bcl-2, clone 6C8, and anti-mouse-Bcl-2, clone 3F11, were purchased from Pharmingen (San Diego, CA). Polyclonal rabbit anti-K14 was a kind gift of Dennis Roop (Baylor College of Medicine, Houston, TX), and a mouse monoclonal anti-human CK14, which cross-reacts with mouse cytokeratin 14, was purchased from Neomarker (Fremont, CA). Horseradish peroxidase-conjugated goat anti-hamster IgG (for Western blotting) was from Southern Biotechnology (Birmingham, AL), and the appropriate secondary biotinylated reagents for mouse and hamster primary antibodies (for immunohistochemistry) were from Amersham (Little Chalfont, UK).

DNA Constructs and Transgenic Mice

The full-length cDNA for human Bcl-2 (1.9 kb) contained in the Bluescript-KS vector was a kind gift of Stanley Korsmeyer (Harvard, Boston, MA). To specifically direct expression of Bcl-2 to the basal layer of the epidermis, the insert was excised from this vector by *EcoRI* diges-

tion, modified with *Bgl*II linkers (New England Biolabs, Beverly, MA) and ligated into the single *Bam*HI site of the K14/human growth hormone (hGH) expression vector,¹⁷ kindly provided by Elaine Fuchs (University of Chicago, Chicago, IL). The original vector had been modified for these experiments so that it no longer supported transcription of active growth hormone.¹⁸ The completed construct was electroporated into the competent DH5a strain of *Escherichia coli*; colonies bearing the vector in the correct orientation were identified and amplified; and the 6.1-kb insert was recovered with a single *EcoRI* digestion, purified, and used for microinjection into the pronuclei of fertilized FVB/N ova.¹⁹ Surviving two-cell embryos were transferred into pseudopregnant Swiss Webster recipients, and the offspring were screened by polymerase chain reaction (PCR) amplification of genomic DNA obtained from ear punch biopsies, using primers directed at the hGH segment of the transgene.²⁰ Two founder mice were identified, and the presence of the transgene was confirmed by Southern blotting, using the complete cDNA excised with *EcoRI* from the Bluescript-KS plasmid as a radioactively labeled probe.

Western Blotting

Human Bcl-2

Ears from transgenic and wild-type mice were mechanically scraped with a scalpel, and the resulting crude cell suspension was immediately lysed in isotonic buffer with protease inhibitors. The lysates were cleared of cell debris by high-speed centrifugation, and 15 mg of protein was separated on a 15% polyacrylamide gel, under denaturing conditions, transferred electrophoretically to nitrocellulose membranes in ice-cold Towbin's buffer,²¹ probed with the hamster anti-human Bcl-2 monoclonal antibody, and developed with horseradish peroxidase-conjugated goat anti-hamster secondary reagent (1:2000) and an enhanced chemiluminescence (ECL) detection system (Amersham).

Murine Bcl-X(L)

Sixteen-day-old female Bcl-2 and wild-type mice were shaved, and full-thickness skin samples from one longitudinal half of the whole dorsal area were snap-frozen in liquid nitrogen and treated as described above. The lysates were probed with 0.5 μ g/ml polyclonal rabbit anti-Bcl-x (Transduction Laboratories) and developed with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Pierce) secondary reagent and the ECL detection system (Amersham). To quantitate the amount of loaded protein more accurately, the membranes were reblocked for an hour after the ECL reaction was completed and probed with a monoclonal anti-actin antibody (clone C4, ICN, 1:20,000). Actin antibody binding was detected with a sheep anti-mouse immunoglobulin horseradish peroxidase-conjugated secondary reagent and ECL as before. All membrane blocking, washing, and antibody dilutions were performed using the buffer systems developed by Niland et al.²²

Skin Harvesting and Skin Specimen Preparation

Sex- and age-matched, 1–20-day-old neonatal transgenic and wild-type littermates were used for the analysis of neonatal HF morphogenesis, postnatal HF regression (catagen), and the first anagen phase.^{23,24} and 6–9-week-old adolescent transgenic and wild-type mice were used for the analysis of cyclophosphamide-induced alopecia. These mice were housed in community cages, with 12-hour light periods, at the Harvard Skin Disease Research Center and were fed water and mouse chow *ad libitum*.

Using a special skin harvesting and processing technique for obtaining longitudinal cryosections through murine HF,^{24,25} we covered skin samples with embedding medium (GSV; SLEE Technik GmbH, Main, Germany). They were then snap-frozen in liquid nitrogen and stored at -80°C until cryosectioning ($86\ \mu\text{m}$). Sections were placed on poly-L-lysine-covered glass slides, air-dried for 1 hour, and fixed at -20°C in acetone for 10 minutes to be stored at -20°C until immunohistology was performed.

At least 20 HFs per mouse were examined: five mice on days 1 and 12 (neonatal morphogenesis) and days 18 and 19 after birth (first postnatal catagen and anagen), respectively. Thus, at least 60 to 100 different HFs obtained from three to five mice per stage of HF morphogenesis or cycling were analyzed by quantitative histomorphometry, as described.^{26–28}

Immunohistochemistry

Human and murine Bcl-2 immunoreactivity (IR) and murine Bax and Bcl-X(L) IR were detected immunohistologically by the avidin-biotin complex and alkaline phosphatase technique (Vecta-Stain Kit, Vector, Burlingame, CA). The specific monoclonal antibody against human Bcl-2 was used in a dilution of 1:50; staining for murine ICE, Bcl-2, Bax, and NT-3 and double-labeling of murine Bcl-2/Bax were performed as described.^{10,27}

Induction of Apoptosis by UVB

For the induction of apoptosis by UVB *in vivo*, groups of four or five mice aged 8 to 9 weeks were depilated on their dorsal surface with a commercial cream 1 to 2 days before irradiation. The mice were then anesthetized with a mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg) to immobilize them and irradiated in a Stratallinker 2400 (Stratagene, La Jolla, CA) fitted with lamps capable of delivering a chosen energy level of UVB at a wavelength of 312 nm. Either a single dose of $1250\ \text{J}/\text{m}^2$ or five consecutive doses of $500\ \text{J}/\text{m}^2/\text{day}$ were given. Twenty-four hours after the last irradiation, the mice were sacrificed, and the dorsal skin was harvested and immersed in 10% buffered formalin. After appropriate fixation and deparaffinization, $5\text{-}\mu\text{m}$ sections were stained with hematoxylin and eosin; sunburn cells, defined as cells with a shrunken, highly eosinophilic cytoplasm, and condensed

nuclei, were counted in both the basal and suprabasal layers of interfollicular epidermis.²⁹

For the *in vitro* induction of apoptosis by UVB irradiation, 1×10^5 freshly prepared epidermal cells from mouse ears in 1 ml of culture medium (Dulbecco's minimum essential medium without CaCl_2 , supplemented with glutamine and antibiotics (all Gibco, Paisley, Scotland), 5% chelex fetal calf serum, $0.1\ \text{mmol/L}\ \text{CaCl}_2$, $2.5\ \text{ng/ml}$ murine epidermal growth factor (EGF; Sigma)) were dispensed into Costar 24 wells and were allowed to adhere overnight. The medium was removed, the cells were washed with warm phosphate-buffered saline (PBS) containing $0.03\ \text{mmol/L}\ \text{CaCl}_2$ and $0.8\ \text{mmol/L}\ \text{MgCl}_2$ (PBS/Ca,Mg) and irradiated with a metal halide lamp (Mutzhas, Munich, Germany) filtered for emission of UVB light in the range of 290–330 nm. Power output was measured with an IL1700 International Light Research Radiometer (Newbury Port, MA). After irradiation, the PBS/Ca,Mg was replaced with culture medium, and the cells were incubated for a further 24 hours at 37°C . The degree of apoptosis was then quantitated using the Cell Death Detection ELISA, according to the manufacturer's instructions.

Cyclophosphamide-Induced Alopecia

For the study of chemotherapy-induced apoptosis we used the well-studied model of cyclophosphamide-induced alopecia.^{10,16} Briefly, 7-week-old TG and WT mice with all back skin HF in telogen were depilated to induce anagen VI HF.^{24,30} At day 9 after depilation, $120\ \text{mg/kg}$ cyclophosphamide was injected intraperitoneally, and 36 hours later, namely when the highest rate of apoptotic activity is found,¹⁰ the first group of TG and WT mice was harvested, and the second group was harvested after 5 days, when massive alopecia is visible macroscopically.¹⁰

Results

Bcl-2 Transgenic Mice Display an Apparently Normal Skin Phenotype

Pronuclear injection of fertilized ova with the construct described above yielded two founder mice, which were bred to establish hemizygous lines, designated bcl1 and bcl7. The founder mouse for the bcl7 line had only intermittent Bcl-2-positive cells in the epidermal basal layer, indicative of a mosaic pattern of expression (not shown). Both mice bred normally, and the bc17 founder was bred to give progeny with a complete expression pattern. The two transgenic lines appeared to have about equal gene copy numbers by Southern blot analysis (not shown); however, the probe used did not detect sufficient endogenous Bcl-2 to allow quantitative estimation of the absolute copy number.

As expected, the K14-driven expression of the transgene in the epidermis was restricted to the basal layer (Figure 1A). No transgenic Bcl-2 protein could be detected at all in control mice (Figure 1B), whereas K14 was

Figure 2. Western blot analysis of keratin-14-driven human Bcl-2 (K14/Bcl-2) expression in murine skin. Lysates of cells from mouse ear scrapings and from a Bcl-2 expressing tumor cell line (SY5Y) were separated on 15% acrylamide gels, and Bcl-2 protein was detected as described in Materials and Methods.

equally expressed in interfollicular epidermis in both transgenic and wild-type mice (not shown). Western blotting of lysates prepared from epidermal ear scrapings demonstrated that the Bcl-2 protein detected by this antibody had an expected size of about 26 kD (Figure 2).

The skin architecture of transgenic mice remained grossly and microscopically normal: there was no spontaneous epidermal hyperplasia, even in older mice, although we found increased BrdU incorporation in the interfollicular epidermis of transgenic mice (Rossiter et al, unpublished observation, manuscript in preparation). Wound healing did not show substantial irregularities (S. O'Kane, unpublished results), and macroscopically the mice did not show signs of spontaneous hair growth abnormality, up to an age of 24 months.

Intrafollicular Bcl-2 Transgene Expression Is Strictly Dependent on Hair Follicle Development and Cycling and Corresponds to K14 Immunoreactivity

The immunohistological analysis of K14-driven transgenic Bcl-2 IR in murine skin revealed that the human Bcl-2 protein in murine skin was not constitutively expressed in the hair follicle, in contrast to the expected expression patterns,¹⁷ but that the transgene expression was strictly dependent on the respective stage of hair follicle development and cycling. During early skin and HF development, transgenic Bcl-2 IR was found strongly on all keratinocytes of the basal epidermal layer and the developing hair germ (Figure 1C). Later, the developing

inner root sheath (IRS) showed decreasing K14/Bcl-2 IR (Figure 1C), and the entire outer root sheath (ORS) displayed strong K14/Bcl-2 IR (Figure 1C). In contrast, the entire proximal part of fully developed HF displayed only very faint K14/Bcl-2 IR (Figure 1D).

During catagen, on the other hand, strong transgene IR was found on all follicular keratinocytes and was particularly strong on the keratinocytes of the regressing epithelial strand (Figure 1E). Telogen HF displayed only some K14/Bcl-2 IR on keratinocytes of the proximal ORS and the secondary hair germ close to the dermal papilla (DP) (Figure 1F). During the first anagen after birth, the central ORS and the developing IRS displayed virtually no transgene IR, whereas the proximal ORS was strongly K14/Bcl-2-positive (Figure 1G). Thus this developmentally controlled and hair cycle-dependent transgene IR was found exactly in those regions that have been defined as "apoptosis hot spots" during murine HF morphogenesis and cycling (central IRS, distal ORS, epithelial strand),¹⁰ and a substantial decline of transgene IR was found on the entire proximal part of fully developed HF during morphogenesis (Figure 1E) and anagen (not shown).

To determine whether the hair cycle-dependent expression of the transgene was dependent on a hair growth-dependent expression of K14 or was the result of Bcl-2 function in the HF, we have analyzed the IR patterns of K14 during neonatal HF morphogenesis in wild-type mice. Similar to the transgene IR patterns shown in Figure 1, C–G, strong K14 IR was first found on all keratinocytes of the epidermis and stage 2–3 hair buds (Figure 1, H and I), as well as on the ORS of stage 4 HF (Figure 1, I and J), but not on their developing IRS (Figure 1, I and J). Fully developed stage 8 HFs display strong K14 IR on the distal and central ORS (Figure 1K, arrowhead), but not on the proximal parts (Figure 1K, arrows).

The Bcl-2 Transgene Protects against Sunburn Cell Formation by Epidermal Keratinocytes in Vivo and Prevents UVB-Induced Apoptosis in Vitro

Because the transgenic animals demonstrated no overt skin abnormalities, we first undertook to verify that the

Figure 1. Immunolocalization of keratin-14-driven human Bcl-2 (K14/Bcl-2) expression in murine skin. **A** and **B**: Strong K14/Bcl-2 expression on keratinocytes of the basal epidermal layer in transgenic mice (**A**) and no expression in wild-type littermates (**B**). **C–G**: Hair growth-dependent expression of K14/Bcl-2 in transgenic mice of different ages post partum (pp). **C**: Day 1 pp. Strong K14/Bcl-2 immunoreactivity (IR) is shown on keratinocytes of the basal epidermal layer (e) and hair germs (g) (stage 2); there is strong expression on hair matrix (arrowhead) and outer root sheath keratinocytes (arrow) of hair pegs (stages 3 and 4). **Asterisk**, dermal papilla. **D**: Day 3 pp. Strong K14/Bcl-2 IR is shown on keratinocytes of the basal epidermal layer (e), hair germs (g), and outer root sheath keratinocytes (arrow) of stage 5 hair follicles. Virtually no K14/Bcl-2 IR is apparent on keratinocytes of the hair matrix (arrowheads) and the developing inner root sheath (irs). **Asterisks**, dermal papilla. **E**: Day 18 pp. Strong K14/Bcl-2 IR is seen on keratinocytes of the basal epidermal layer (e), the outer root sheath (arrow), and the regressing epithelial strand (arrowhead) of a catagen VI hair follicle. **Asterisks**, dermal papilla; c: club hair; cts: trailing connective tissue sheath. **F**: Day 19 pp. Strong K14/Bcl-2 IR is seen on keratinocytes of the basal epidermal layer (e), the proximal outer root sheath (arrow), and the secondary hair germ (arrowhead) of a telogen hair follicle. **Asterisks**, dermal papilla; c: club hair; sg, sebaceous gland. **G**: Day 19 pp. Strong K14/Bcl-2 IR is found on keratinocytes of the basal epidermal layer (e) and the developing hair bud (stage 2) (arrow). **Asterisks**, dermal papilla. **H**: Day 1 pp. Stronger K14 IR is found on keratinocytes of the basal epidermal layer (e) and slightly weaker K14 IR on hair germs (stages 3 and 4) (arrows); strong K14 IR is found on the developing outer root sheath keratinocytes (arrowheads) but not on the developing inner root sheath (I). **Asterisk**, dermal papilla. **I**: Day 1 pp. Strong K14 IR is found on basal epidermal layer (e) keratinocytes and hair germs (stage 2) (arrow); strong K14 IR is found on the developing outer root sheath keratinocytes (arrowheads) but not on the developing inner root sheath (I) of stage 3 hair follicles. **Asterisk**, dermal papilla. **J**: Day 2 pp. Strong K14 IR is found on basal epidermal layer (e) keratinocytes and hair germs (stage 2) (arrow); strong K14 IR is found on the developing outer root sheath keratinocytes (arrowheads) but not on the proximal part (arrows) of stage 8 hair follicles. Scale bars: 100 μ m (**A** and **B**) and 50 μ m (**C–K**).

Figure 3. Transgenic Bcl-2 protects against UVB-induced apoptosis. **A:** Mice were irradiated with 1250 J/m² and sacrificed 24 hours later, and the sunburn cells in the basal layer of formalin-fixed, paraffin-embedded tissue sections of dorsal skin were counted. Two further experiments in which mice were irradiated with 5 × 500 J/m² gave similar results. Average numbers of sunburn cells for the respective groups were as follows: bcl-1, 0.269 ± 0.139; bcl-7, 0.380 ± 0.255; nontransgenic, 1.984 ± 1.199 cells/mm epidermis. *P* < 0.02 for both bcl-1 and bcl-7 groups compared to nontransgenic mice. Bcl-1, transgenic line 1; Bcl-7, transgenic line 7; WT, wild type. **B:** Freshly isolated ear keratinocytes were allowed to adhere in culture medium at 37°C overnight and were irradiated as described in Materials and Methods with the doses indicated, and histone formation in cell lysates was detected after 6 hours of further culture. The columns indicate the means of two independent experiments performed in duplicate. **P* < 0.05.

Bcl-2 transgene was active. One of the well-characterized apoptotic stimuli against which Bcl-2 protein protects is UVB radiation, which was first demonstrated for lymphoid cells.³¹ Skin irradiation with UVB induces the appearance of apoptotic ("sunburn") cells, mainly in the basal layer of the epidermis.³² Indeed, irradiation of both transgenic lines bcl1 and bcl7 with UVB resulted in a significant (*P* < 0.02) reduction in the numbers of sunburn cells in the interfollicular basal layer of epidermis of the transgenic strains compared to controls, 24 hours after a single dose of 1250 J/m², confirming the normal functioning of the transgene (Figure 3A). Similar results were obtained in mice up to 12 months of age (not shown) and in two further experiments in which mice from each of the other strains were irradiated on 5 consecutive days with 500 J/m² and sunburn cells were counted after 24 hours. The numbers of sunburn cells in the suprabasal layers were much lower than in the basal layers (not shown), and no significant difference was observed be-

tween transgenic and nontransgenic animals, as expected, because the Bcl-2 transgene was not expressed in this location.

One of the hallmarks of apoptosis is the endonuclease-mediated cleavage of DNA into histone-associated fragments,³³ which can be detected by specific antibodies. Figure 3B shows that cultured primary keratinocytes from normal FVB/N mice undergo UVB-induced apoptosis in a dose-dependent manner, allowing their histones to be detected in an ELISA-based assay. At the same doses, virtually no histones can be detected in keratinocytes derived from the transgenic strains. Histones were nevertheless detectable by ELISA in cultures of cells of both transgenic and nontransgenic origin when, in some experiments, there was some degree of spontaneous histone release by nonirradiated cells (not shown).

Bcl-2 Transgenic Mice Display No Alteration of Hair Follicle Morphogenesis and Exhibit Normal Onset but Accelerated Progression of Hair Follicle Regression (Catagen) and Premature Anagen Development

To precisely analyze the velocity of HF morphogenesis, the percentage of HFs in distinct stages of HF development was assessed by quantitative histomorphometry in TG and WT mice.²⁶ Compared to WT mice, the transgenic mice displayed no statistically significant difference in the velocity of early HF development (day 1 after birth) (Figure 4A) and—surprisingly—no significant difference in the onset of the first phase of HF regression (catagen) after birth (Figure 4B).

Quantitative histomorphometry of WT and TG dorsal skin revealed a statistically significant (*P* < 0.01) acceleration of catagen progression at day 18 after birth (Figure 5, A–C), and a statistically significant (*P* < 0.05) acceleration of the first anagen development at day 19 in TG mice compared to WT littermates (Figure 5, D–F). In addition, catagen development in the transgenic mice was significantly (*P* < 0.01) less well-synchronized than in age-matched WT controls, ie, the K14/Bcl-2 mice still showed catagen VIII alongside anagen III HF, whereas WT mice displayed only telogen and anagen I/II HF (Figure 5F). Furthermore, at day 18 after birth the transgenic mice showed a significant increase in TUNEL⁺ hair bulb keratinocytes (*P* < 0.05) compared to WT controls (Figure 6, A–C), also supporting an acceleration of catagen development.¹⁰ Thus, in contrast to our expectations, the Bcl-2 transgene seems to promote apoptosis of HF keratinocytes.

Immunohistological analysis of the endogenous Bcl-2/Bax ratio and the endogenous Bcl-2/Bcl-X_L ratio, NT-3 IR in the ORS, as well as the IR of ICE, as one representative member of the caspase family, revealed no substantial difference between wild-type and transgenic mice (not shown). In addition, Western blot analysis of full dorsal skin homogenates revealed no marked difference in Bcl-X_L expression between transgenic and wild-type littermates (not shown).

Figure 4. The difference in alteration of hair follicle morphogenesis and the onset of catagen in K14/Bcl-2-overexpressing mice is not statistically significant. **A:** Quantitative histomorphometry of age- and sex-matched transgenic mice and wild-type littermates harvested at day 1 pp revealed no statistically significant alteration of hair follicle morphogenesis. Stages of hair follicle morphogenesis are according to Paus et al.⁴³ **B:** Quantitative histomorphometry of age- and sex-matched transgenic mice and wild-type littermates harvested at day 14 pp revealed no statistically significant alteration of hair follicle morphogenesis. Note that anagen VI and catagen I hair follicles cannot be distinguished by morphological or immunohistological criteria.

Bcl-2 Transgenic Mice Displayed Increased Alopecia, Hair Follicle Dystrophy, and Apoptosis after Cyclophosphamide Injection

Consistent with the acceleration of spontaneous catagen development, the transgenic mice also showed a significant increase in TUNEL⁺ hair bulb keratinocytes ($P < 0.05$) compared to WT controls 36 hours after cyclophosphamide injection (Figure 6, D–F). Compared to WT mice, the K14/Bcl-2 mice displayed macroscopically visible hair loss 2 days earlier (not shown), and the wave of alopecia progressing from neck to tail was substantially accelerated (Figure 7A). In addition, 5 days after cyclophosphamide treatment, K14/Bcl-2 mice displayed a statistically significant acceleration of dystrophic catagen, ie, a significantly higher rate of shorter and deformed hair follicles, as well as more abnormally widened hair canals ($P < 0.05$) (Figure 7B).

Discussion

Apoptosis is a central feature of the developing and cycling hair follicle,^{8,10,34} and it may be expected that disruption of the exquisite intrafollicular controls of keratinocyte proliferation and apoptosis may lead to profound alterations of HF morphogenesis and cycling. Indeed, compared to wild-type (WT) mice, Bcl-2 $\alpha\beta$ and Bcl-2 α -deficient mice reportedly show a substantial retardation of the first anagen development and depilation-induced anagen.^{13–15,35} Although these observations were not supported by quantitative histomorphometry, our data complement in part the findings reported in these studies: our Bcl-2-overexpressing mice paradoxically showed a marked accelerated spontaneous and cyclophosphamide-induced catagen progression, as well as an accelerated first anagen development. Bcl-2-deficient mice should thus be tested by quantitative histomorphometry to determine whether they also consistently display a retardation of the first catagen progression.

The data presented in the present study imply that Bcl-2 *per se* cannot protect hair follicle keratinocytes against catagen-associated apoptosis. Furthermore, the induction and the progression of catagen seem to be two distinctly controlled processes, because the onset of catagen displayed no statistically significant differences between wild-type and transgenic mice, whereas subsequent catagen progression was markedly different (Figures 4B and 5).

In contrast to the expected expression patterns,¹⁷ we show here that the K14-driven transgene is not expressed constitutively in follicular keratinocytes, but that its expression is developmentally controlled and is strictly associated with distinct stages of HF morphogenesis and cycling (Figure 1).

These stage-dependent transgene expression patterns are consistent with the immunoreactivity patterns of K14 during murine HF morphogenesis and cycling (Figure 1, H–K). However, these findings have to be confirmed by additional methods, such as K14-driven β -galactosidase or green fluorescent protein expression, to exclude the possibility that cells are altered when they express K14-linked Bcl-2, leading indirectly to a hair growth-dependent expression pattern caused by Bcl-2 function. In addition, the stage-dependent transgene expression is also age-dependent, because skin and HF morphogenesis and cycling follow a precisely controlled timetable.³⁶ This finding has important implications for all studies performed with transgenes under the control of the K14 promoter. In particular, in the case of forced expression of secreted molecules, in light of our data, it would seem critical to analyze the stage/age-dependent level of the K14-driven transgene in various tissues and in the circulation, and to ensure that experimental comparisons between test and control animals are only performed between age-matched mice that are in precisely the same stage of the hair cycle, so that artifacts arising from hair cycle-associated differences in transgene expression are avoided.

It is intriguing that Bcl-2, a potent apoptosis inhibitory protein, when it is overexpressed in follicular keratino-

Figure 5. Accelerated catagen progression and anagen development in K14/Bcl-2-overexpressing mice. **A:** Representative micrograph of wild-type skin section (Giemsa staining) harvested at day 18 pp. Note catagen II-III hair follicles, which are characterized by a long or onion-shaped dermal papilla (asterisks) enclosed by hair matrix (m) keratinocytes deep in the subcutis. **B:** Representative micrograph of K14/Bcl-2 transgenic skin section (Giemsa staining) harvested at day 18 pp; note catagen VII hair follicles, which are characterized by a condensed dermal papilla (asterisk), a regressing epithelial strand (e), and a trailing connective tissue sheath (arrowhead). **C:** Quantitative histomorphometry of K14/Bcl-2 transgenic and wild-type skin harvested at day 18 pp revealed a statistically significant acceleration of catagen development. $^{**}P < 0.01$. **D:** Representative micrograph of wild-type skin section (Giemsa staining) harvested at day 19 pp; telogen hair follicles are characterized by a condensed dermal papilla (asterisk) lying entirely in the dermis. **E:** Representative micrograph of K14/Bcl-2 transgenic skin section (Giemsa staining) harvested at day 19 pp. Note anagen II-III hair follicles, which are characterized by an enlarged dermal papilla (asterisk) surrounded by keratinocytes of the developing hair matrix (m) lying at the border between the dermis and subcutis. **F:** Quantitative histomorphometry of K14/Bcl-2 transgenic and wild-type skin harvested at day 19 pp revealed a statistically significant acceleration of anagen development. Note that transgenic mice display a less synchronized hair cycle (range from catagen VIII to anagen III). cat, catagen; ana, anagen. $^{*}P < 0.05$; $^{**}P < 0.01$.

cytes, promotes accelerated spontaneous and cyclophosphamide-induced HF regression (catagen), processes that are driven mainly by apoptosis of HF keratinocytes.¹⁰ In interfollicular epidermal keratinocytes, the transgene itself was active as expected, because transgenic mice had a very marked reduction in UVB-induced sunburn cells, specifically in the basal keratinocytes, in which the transgene was expressed. A similar reduction in UVB-induced sunburn cells in mouse skin has been described for mice transgenic for another Bcl-2 family member, Bcl-xL, which also protects against UVB-induced apoptosis.³⁷ That the reduction of sunburn cells implies protection against UVB-induced apoptosis is supported by the finding that cultured keratinocytes from transgenic mice produce no histones, a hallmark of apoptotic cells, when irradiated with the same doses of UVB that elicited high histone release from nontransgenic cells. This suggests that Bcl-2 exerts different apoptosis-modulating functions in epidermal versus follicular keratinocytes.

In contrast to Rodriguez-Villanueva et al,³⁸ who overexpressed Bcl-2 ectopically in suprabasal epidermal lay-

ers by using a keratin-1 promoter, we have expressed Bcl-2 orthotopically in the basal epidermal layer and in the ORS of the hair follicle. Surprisingly, we did not find any of the changes reported by Rodriguez-Villanueva et al,³⁸ such as multifocal hyperplasia without associated hyperkeratosis. These data suggest that ectopic Bcl-2 expression and orthotopic Bcl-2 expression substantially differ in their effects on keratinocyte biology, depending on their location (differences in the local signaling milieu) and state of differentiation (proliferative versus terminally differentiating cell pool).

The suppression of UVB-induced apoptosis in our K14/bcl-2 mice reveals for the first time that orthotopic Bcl-2 overexpression *in vivo* indeed results in reduced UVB-induced apoptosis. This is suggested by previous evidence from *in vitro* experiments on lymphoid cells³¹ and ectopically Bcl-2-expressing transgenic mice.³⁸

The promotion of catagen- and chemotherapy-associated apoptosis in K14/bcl-2 transgenic mice is a very unexpected finding because the overexpression of an apoptosis inhibitor such as Bcl-2 should, in theory, re-

Figure 6. Increased rate of spontaneous or chemotherapy-induced apoptosis in K14/Bcl-2 transgenic mice. **A** and **B**: TUNEL staining. More TUNEL⁺ cells (arrowheads) are found in catagen II hair follicles of transgenic mice (**A**) than in wild-type littermates (**B**). **C**: TUNEL staining of K14/Bcl-2 and wild-type littermate skin sections harvested at day 18 pp revealed a significantly higher level of TUNEL⁺ cells in the hair follicle bulb of transgenic mice than in age-matched wild-type littermates. ** $P < 0.01$; tg, K14/Bcl-2 transgenic mice; wt, wild-type littermates. **D** and **E**: TUNEL staining revealed more TUNEL⁺ cells (arrowheads) in the regressing proximal region of dystrophic catagen hair follicles of transgenic mice (**D**) compared to wild-type littermates (**E**) harvested 5 days after cyclophosphamide application. **F**: TUNEL staining of K14/Bcl-2 and wild-type littermate skin sections harvested 5 days after cyclophosphamide application revealed a significantly higher level of TUNEL⁺ cells in the regressing epithelial strand of dystrophic catagen hair follicles of transgenic mice than in age-matched wild-type littermates. * $P < 0.05$; tg, K14/Bcl-2 transgenic mice; wt, wild-type littermates.

duce follicular apoptosis and should delay spontaneous or dystrophic catagen development. Arriola et al³⁹ demonstrated in another system (testicular germ cell tumors) that the overexpression of Bcl-2 reciprocally down-regu-

lates Bcl-X(L), another antiapoptotic member of the Bcl-2 family, and leads to a higher susceptibility of these cells to chemotherapy-induced apoptosis. In addition, compared to Bcl-2, Bcl-X(L) appears to play a far more im-

Figure 7. Accelerated progression of dystrophic catagen development and hair loss in K14/Bcl-2-overexpressing mice. **A:** Quantitative histomorphometry of K14/Bcl-2 transgenic and wild-type skin harvested 5 days after cyclophosphamide application revealed a statistically significant acceleration of dystrophic catagen development. early, early dystrophic catagen; mid, middle dystrophic catagen; late, late dystrophic catagen. * $P < 0.05$. **B:** Cyclophosphamide-treated K14/Bcl-2 transgenic mice and wild-type littermates display substantial hair loss compared to untreated sex- and age-matched wild-type controls. The wave of hair loss progressing from head to tail is substantially advanced in K14/Bcl-2 transgenic mice. **Black bars,** end of visible hair loss progression.

portant role in chemotherapy-induced apoptosis in these cells. However, we did not detect any marked difference in the ratio of Bcl-2 and Bcl-X(L) in K14/bcl-2 mouse skin compared to controls, by immunohistology or Western blotting of full dorsal skin homogenates (not shown).

Because the overexpression of Bcl-2 might have been counterregulated by up-regulation of proapoptotic Bcl-2 family members such as Bax or counterregulatory activity of caspases that are directly upstream of DNA fragmentation, we have analyzed in the transgenic *versus* WT mice, for example, the ratio of endogenous Bcl-2/Bax IR as well as caspase 1 (ICE) IR as one key member of the large caspase family. However, no significant alteration between the two mouse lines was found.

Furthermore, K14/Bcl-2 may prolong the survival of the HF keratinocyte subpopulation in the ORS otherwise destined to undergo apoptosis, which now continue to secrete catagen-promoting factors such as NT-3. We have recently demonstrated that NT-3 promotes catagen development

and that many NT-3⁺ keratinocytes can be found in the ORS of late anagen VI HF.²⁶ Thus K14/bcl-2 overexpression of ORS keratinocytes in late anagen may retain such NT-3⁺ ORS keratinocytes inside the HF for a longer time than normal and consequently accelerate catagen progression. However, additional immunohistological analyses of NT-3 expression in transgenic *versus* control mice did not reveal any substantial differences in any HF compartment between the two strains (not shown).

Interestingly, during the revision of this paper, Pena et al⁴⁰ presented an explanation for a skin phenotype similar to the one observed by us in a transgenic mouse line overexpressing Bcl-X(L) under control of the K14 promoter (K14/BclX(L) mice). As in K14/Bcl-2 mice, the transgene was expressed on basal layer keratinocytes of the epidermis and the ORS, leading to accelerated catagen development. These authors have suggested the plausible argument that accelerated catagen development in K14/Bcl-X(L) mice might be explained, at least in part, by prolonged FGF-5 production of ORS keratinocytes surviving longer than normal, because the skin phenotype was substantially reversed in FGF-5-deficient mice, given that FGF-5 is an important catagen-promoting factor⁴¹ and that FGF-5 mRNA steady-state levels are maximally high during catagen in mice.⁴²

Therefore, K14/Bcl-2 overexpression in the ORS of transgenic mice may well lead to the survival of ORS keratinocytes that elaborate catagen-promoting factors such as FGF-5 or NT-3. Unfortunately, however, with the methodology we have used we have failed to detect significant expression differences in this respect between transgenic and WT mice (our attempts to obtain reliable FGF-5 IR patterns well above background were frustrated).

Nevertheless, in concert with the publication by Pena et al,⁴⁰ our findings in K14/Bcl-2 mice suggest that Bcl-2 plays a crucial role in regulating death and survival of ORS keratinocytes and invite one to exploit in future studies the novel transgenic mouse strain reported here as an instructive model for further exploration of the role of selected ORS keratinocytes in the control of catagen development.

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References

1. Miller LJ, Marx J: Apoptosis. *Science* 1998, 281:1301
2. Adams JM, Cory S: The bcl-2 protein family: arbiters of cell survival. *Science* 1998, 281:1322–1326
3. Chinnaiyan AM, Orth K, Rourke K, Duan H, Poirier GG, Dixit VM: Molecular ordering of the cell death pathway. Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. *J Biol Chem* 1996, 271:4573–4576
4. White E: Life, death, and the pursuit of apoptosis. *Genes Dev* 1996, 10:1–15

5. Chao DT, Korsmeyer SJ: BCL-2 family: regulators of cell death. *Annu Rev Immunol* 1998, 16:395-419
6. Paus R: Principles of hair cycle control. *J Dermatol (Tokyo)* 1998, 25:793-802
7. Paus R: Control of the hair cycle and hair diseases as cycling disorders. *Curr Opin Dermatol* 1996, 3:248-258
8. Polakowska RR, Piacentini M, Bartlett R, Goldsmith LA, Haake AR: Apoptosis in human skin development: morphogenesis, periderm, and stem cells. *Dev Dyn* 1994, 199:176-188
9. Magerl M: Patterns of proliferation and apoptosis during hair follicle morphogenesis. *J Invest Dermatol* 1998, 110:532a (abstr.)
10. Lindner G, Botchkarev VA, Botchkarev NV, Ling G, van der Veen C, Paus R: Analysis of apoptosis during murine hair follicle regression (catagen). *Am J Pathol* 1997, 151:1601-1617
11. Thornberry NA, Lazebnik Y: Caspases: enemies within. *Science* 1998, 281:1312-1316
12. Stenn KS, Lawrence L, Veis D, Korsmeyer S, Seiberg M: Expression of the bcl-2 protooncogene in the cycling adult mouse hair follicle. *J Invest Dermatol* 1994, 103:107-111
13. Yamamura K, Kamada S, Ito S, Nakagawa K, Ichihashi M, Tsujimoto Y: Accelerated disappearance of melanocytes in bcl-2-deficient mice. *Cancer Res* 1996, 56:3546-3550
14. Kamada S, Shimono A, Shinto Y, Tsujimura T, Takahashi T, Noda T, Kitamura Y, Kondoh H, Tsujimoto Y: Bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. *Cancer Res* 1995, 55:354-359
15. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ: Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 1993, 75:229-240
16. Paus R, Handjiski B, Eichmüller S, Czarnetzki BM: Chemotherapy-induced alopecia in mice. Induction by cyclophosphamide, inhibition by cyclosporine A, and modulation by dexamethasone. *Am J Pathol* 1994, 144:719-734
17. Vassar R, Rosenberg M, Ross S, Tyner A, Fuchs E: Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. *Proc Natl Acad Sci USA* 1989, 86:1563-1567
18. Williams IR, Rawson EA, Manning L, Karaoli T, Rich BE, Kupper TS: IL-7 overexpression in transgenic mouse keratinocytes causes a lymphoproliferative skin disease dominated by intermediate TCR cells—evidence for a hierarchy in IL-7 responsiveness among cutaneous T cells. *J Immunol* 1997, 159:3044-3056
19. Hogan B, Constantini F, Lacy E: Manipulation of the Mouse Embryo: A Laboratory Manual. Plainview, NY, Cold Spring Harbor Laboratory Press, 1986
20. Williams IR, Kupper TS: Epidermal expression of intercellular adhesion molecule 1 is not a primary producer of cutaneous inflammation in transgenic mice. *Proc Natl Acad Sci USA* 1994, 91:9710-9714
21. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979, 76:4350-4354
22. Niland S, Cremer A, Krieg T: Apoptosis of primary human fibroblasts seeded in three-dimensional collagen matrices: differential expression of bcl-2 and bax. *Arch Dermatol Res* 1999, 291:133-134
23. Vielkind U, Sebzda MK, Gibson IR, Hardy MH: Dynamics of Merkel cell patterns in developing hair follicles in the dorsal skin of mice, demonstrated by a monoclonal antibody to mouse keratin 8. *Acta Anat* 1995, 152:93-109
24. Paus R, Hofmann U, Eichmüller S, Czarnetzki BM: Distribution and changing density of gamma-delta T cells in murine skin during the induced hair cycle. *Br J Dermatol* 1994, 130:281-289
25. Paus R, Eichmüller S, Hofmann U, Czarnetzki BM: Expression of classical and non-classical MHC class I antigens in murine hair follicles. *Br J Dermatol* 1994, 131:177-183
26. Botchkarev V, Botchkareva N, Albers K, van der Veen C, Lewin G, Paus R: Neutrophin-3 involvement in the regulation of hair follicle morphogenesis. *J Invest Dermatol* 1998, 111:279-285
27. Botchkarev V, Welker P, Albers K, Botchkareva N, Metz M, Lewin G, Bulfone-Paus S, Peters E, Lindner G, Paus R: A new role for neurotrophin-3: involvement in the regulation of hair follicle regression (catagen). *Am J Pathol* 1998, 153:279-285
28. Botchkarev VA, Lewin GR, Subramaniam A, Botchkareva NV, Welker P, Metz M, Bulfone-Paus S, Paus R: New role for neurotrophins: involvement of brain-derived neurotrophic factor and neurotrophin-4 in hair growth control. *FASEB J* 1998, 13:395-410
29. Schwarz ARB, Aragane Y, Mahnke K, Riemann H, Metz D, Luger T, Schwarz T: Ultra-violet-B-induced apoptosis of keratinocytes: evidence for partial involvement of tumor necrosis factor-alpha in the formation of sunburn cells. *J Invest Dermatol* 1995, 104:922-927
30. Paus R, Foitzik K, Welker P, Bulfone-Paus S, Eichmüller S: Transforming growth factor-beta receptor type I and type II expression during murine hair follicle development and cycling. *J Invest Dermatol* 1997, 109:518-526
31. Strasser A, Harris AW, Jacks T, Cory S: DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by bcl-2. *Cell* 1994, 79:329-339
32. Young C: The sunburn cell. *Photodermatology* 1987, 4:127-134
33. Arends MJ, Morris RG, Wyllie AH: Apoptosis: the role of the endonuclease. *Am J Pathol* 1990, 136:593-608
34. Soma T, Ogo M, Suzuki J, Takahashi T, Hibino T: Analysis of apoptotic cell death in human hair follicles in vivo and in vitro. *J Invest Dermatol* 1998, 111:948-954
35. Nakayama K, Nakayama K, Negishi I, Kuida K, Sawa H, Loh DY: Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. *Proc Natl Acad Sci USA* 1994, 91:3700-3704
36. Tokura Y, Hofmann U, Müller-Röver S, Paus R, Wakita H, Yagi H, Seo N, Furukawa F, Takigawa M: Spontaneous hair follicle cycling may influence the development of murine contact photosensitivity by modulating keratinocyte cytokine production. *Cell Immunol* 1997, 178:172-179
37. Pena JC, Rudin CM, Thompson CB: A bcl-xL transgene promotes malignant conversion of chemically initiated skin papillomas. *Cancer Res* 1998, 58:2111-2116
38. Rodriguez-Villanueva J, Greenhalgh D, Wang XJ, Bundman D, Cho S, Delehedde M, Roop D, McDonnell TJ: Human keratin-1.bcl-2 transgenic mice aberrantly express keratin 6, exhibit reduced sensitivity to keratinocyte cell death induction, and are susceptible to skin tumor formation. *Oncogene* 1998, 16:853-863
39. Arriola EL, Rodriguez-Lopez AM, Hickman JA, Chresta CM: Bcl-2 overexpression results in reciprocal downregulation of Bcl-X(L) and sensitizes human testicular germ cell tumours to chemotherapy-induced apoptosis. *Oncogene* 1999, 18:1457-1464
40. Pena JC, Kelekar A, Fuchs EV, Thompson CB: Manipulation of outer root sheath cell survival perturbs the hair-growth cycle. *EMBO J* 1999, 18:3596-3603
41. Hebert JM, Rosenquist T, Gotz J, Martin GR: FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell* 1994, 78:1017-1025
42. Pethö-Schramm A, Müller HJ, Paus R: FGF5 and the murine hair cycle. *Arch Dermatol Res* 1996, 288:264-266
43. Paus R, Müller-Röver S, Van Der Veen C, Maurer M, Eichmüller S, Ling G, Hofmann U, Foitzik K, Mecklenburg L, Handjiski B: A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. *J Invest Dermatol* 1999, 113:523-532