Suprabasal BCL-2 Expression Does Not Sensitize to Chemically-induced Skin Cancer in Transgenic Mice

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Abstract. *Background: BCL-2 overexpression is frequently detected in nonmelanoma skin cancer. In normal skin, BCL-2 expression is restricted to the basal cell layer and the hair follicle bulge. Both contain stem cells targeted by carcinogens upon initiation of mouse skin carcinogenesis. It is unknown whether the anti-apoptotic activity of BCL-2 is involved in the susceptibility of this cell type to malignant transformation. If so, extending the pool of BCL-2-expressing cells to suprabasal skin layers should increase the likelihood of skin tumour formation. Materials and Methods: To resolve this issue, we generated a novel transgenic mouse line overexpressing BCL-2 in suprabasal layers of the epidermis. The influence of suprabasal BCL-2 on tumour formation was then tested by chemically inducing skin cancer using the two-stage initiation-promotion protocol. Results: Bcl-2 expression neither influenced the incidence nor the multiplicity of papillomas upon chemical tumour induction with 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), nor their progression to carcinomas. Conclusion: Suprabasal expression of BCL-2 in skin does not increase the formation of papillomas or their malignant progression to squamous cell carcinomas in two-stage mouse skin carcinogenesis.*

The 26 kDa-sized protein BCL-2 is the founding member of a family of related proteins known to control apoptotic cell death (1). Initially identified due to its involvement in follicular lymphoma, BCL-2 turned out to play a role in epithelial malignancies as well (2). BCL-2 expression is frequently detected in nonmelanoma skin cancer and is suspected to contribute to disease (3). In normal skin, BCL-2 expression is restricted to the basal cell layer (4) and hair follicle bulge (5), which both contain stem cells targeted by chemicals inducing skin cancer (6). It is unknown whether the antiapoptotic activity of BCL-2 is involved in the susceptibility of this cell type to malignant transformation. Overexpression of BCL-2 in both basal and suprabasal layers of the skin led to an increased propensity to develop chemically induced skin cancer in *HK1.bcl-2*-transgenic mice (7). In contrast, a transgenic mouse line overexpressing BCL-2 only in basal cells displayed retarded tumour formation (8), suggesting that BCL-2 expression in suprabasal cells might be responsible for the increased tumour susceptibility of *HK1.bcl-2* transgenic mice. A possible explanation is that suprabasally expressed BCL-2 may increase the pool of cells susceptible to transformation by chemical carcinogens.

To resolve this issue a novel transgenic mouse model was generated expressing BCL-2 under control of a 3.7 kb fragment of the human *involucrin* gene promoter. This promoter sequence is known to harbour all information necessary for a strong transgene expression specifically in suprabasal layers of stratified squamous epithelia (9). The influence of suprabasal BCL-2 expression on tumour formation was then tested by chemical induction of skin cancer using the two-stage initiation-promotion protocol.

Materials and Methods

Generation of transgenic mice. The expression plasmid pH3700-pL2 (9) containing 3.7 kb of the 5' regulatory region of the human *involucrin* gene was digested with Not I to remove the β-galactosidase encoding fragment located between the SV40 splice donor/acceptor and the SV40 polyadenylation sequences. The *BCL-2α* cDNA was then inserted into this Not I site after purification from *BCL-2/PURO* plasmid (10) by EcoR I digestion and ligation of EcoR I/Not I adaptors.

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The 5.2 kb expression cassette of the resulting *iv-bcl-2* plasmid was separated from vector sequences by Sal I restriction and subsequent agarose gel electrophoresis. The DNA was purified by electro-elution and prepared for microinjection as described previously (11). Pronucleus injections were performed into $B_6D_2F_2$ zygotes resulting from $B_6D_2F_1$ x DBA/2 hybrid breeding.

Genotyping of the offspring was performed as described previously (12). In brief, tail biopsies of 3- to 4-week-old mice were taken and the DNA was extracted upon digestion of the proteins with proteinase K. Twenty μg of genomic DNA were BamH I-restricted and subjected to Southern blot analysis as described previously using ^{32}P-radiolabelled full-length *BCL-2α* cDNA as probe (12). The identified transgenic founder mice were mated with DBA/2 mice. In the subsequent breeding transgenic animals were routinely identified by PCR using primers 5'-ACTTGTGGCCCAGATAGGCACCCAG-3' and 5'-CGACTTCGCCGAGATGTCCAGCCAG-3' leading to an amplification product of 381 nucleotides in length (13). Non-transgenic litter mates served as control animals for all analyses. All mice were obtained from Charles River Germany, Sulzfeld.

Culture and transfection of PAM212 cells. The mouse keratinocyte cell line PAM212 (14) was cultivated in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) containing 10% (v/v) foetal calf serum (Invitrogen) in a humidified atmosphere at 37˚C and 5% CO_2. For transfection, 5×10^5 cells were seeded on a 10 cm culture dish. The medium was replaced by 3 ml medium containing 20 μg Polybrene (Sigma-Aldrich) and 20 μg *iv-bcl-2* plasmid DNA. Following incubation for 6 h, the medium was replaced by fresh medium containing 20% (v/v) dimethylsulfoxide (Sigma-Aldrich, München, Germany) for 4 min, washed in serum-free medium and kept for 42 h in normal growth medium for the subsequent extraction of proteins.

Protein analyses. Tissues were snap frozen in liquid nitrogen, pulverized and suspended in protein buffer [50 mM Tris-HC1 pH 6.8; 2.5% (w/v) SDS]. To prepare proteins from cultured cells, cells were washed with phosphate-buffered saline (PBS), pelleted and lysed in protein buffer. After 10 min incubation at 100˚C, the samples were sonicated and centrifuged. Supernatants were taken and protein concentrations determined spectrophotometrically. After adding 1/10 volume of sample buffer [600 mM Tris pH 6.8, 8% (w/v) SDS, 20% (v/v) 2-mercapto-ethanol] and heating to 100˚C for 10 min, 50 μg of proteins were separated on an 8% SDS-polyacrylamide gel (15) and afterwards electrotransferred to a PVDF membrane. Following blocking in 5% (w/v) dry milk and 0.3% (v/v) Tween 20 dissolved in PBS the membrane was exposed to the monoclonal mouse anti-human BCL-2 antibody clone 124 (16) and afterwards a peroxidase-conjugated anti-mouse antibody. Reactivity was detected using the Amersham-ECL system (GE Healthcare, München, Germany).

Treatments. For epicutaneous applications, the dorsal skin was shaved with an electric clipper 3 or 7 days before treatment. For initiation–promotion experiments, groups of 20 7-week-old female transgenic mice or non-transgenic litter mates were initiated by a single epicutaneous application of 0.1 μmol 7,12-dimethylbenz(a)anthracene (DMBA; Sigma-Aldrich) in 0.1 ml acetone. Beginning 2 weeks later, the mice were treated each twice weekly with 10 nmol 12-*O*-tetradecanoylphorbyl-13-acetate (TPA; Sigma-Aldrich) in 0.1 ml acetone for 28 weeks. The tumour incidence (tumour bearers/survivors as percentage) and multiplicity (number of tumours/survivors) were recorded weekly. Tumours were identified in a first step macroscopically and afterwards by histological diagnosis.

All animal experiments were permitted by the animal welfare department of the Regierungspräsidium Karlsruhe, Germany and were under the surveillance of the intramural animal welfare committee.

Statistical analysis. The data analysis is based on weekly recorded individual papilloma counts using a statistical analysis procedure described elsewhere (17). Papilloma incidence data were compared with the log-rank test. For the analysis of papilloma multiplicity, the maximum number of papillomas was determined for each animal. Comparisons between the two groups were performed by Wilcoxon rank sum test.

Results

To overexpress BCL-2 in suprabasal skin layers we placed the human *BCL-2α* cDNA under control of the human *involucrin* gene promoter (Figure 1A). To make sure that this construct qualifies for BCL-2 expression in mouse keratinocytes, it was first transiently transfected into the murine keratinocyte cell line PAM212. Subsequently, gene expression was assessed by Western blot analysis. As shown in Figure 1B, an antibody directed against the human BCL-2 protein detected a protein of 26 kDa specifically in transfected cells. Serving as control, a protein of the same size was detected in nontransfected primary human keratinocytes which are described as expressing BCL-2 endogenously.

After pronucleus injection of the DNA construct into mouse zygotes and their subsequent transfer into oviducts of pseudo-pregnant foster mice, six founder mice were identified carrying the transgene as revealed by Southern blot analysis (Figure 1C). One of these founder mice (# 3446) transmitted the transgene to progeny resulting in a transgenic founder line termed *iv-bcl-2* and used for the subsequent experiments.

Western blot analyses revealed strong transgene expression in stratified epithelia such as the skin and fore-stomach of *iv-bcl-2* transgenic mice (Figure 2B). Transgene expression neither led to altered tissue architecture nor to the occurrence of spontaneous skin tumours (data not shown). To assess the effect of BCL-2 overexpression on chemically induced tumourigenesis of the skin we applied the classical DMBA/TPA protocol of tumour initiation and promotion. *iv-bcl-2* transgenic mice were used and their non-transgenic litter mates served as controls. Both groups were treated with DMBA and subsequently with TPA in weekly intervals. The percentage of animals bearing papillomas was monitored over a time course of 48 weeks, and the number of papillomas per animal was determined. As shown in Figure 2A, suprabasal BCL-2 expression did not influence the incidence of papilloma significantly. The

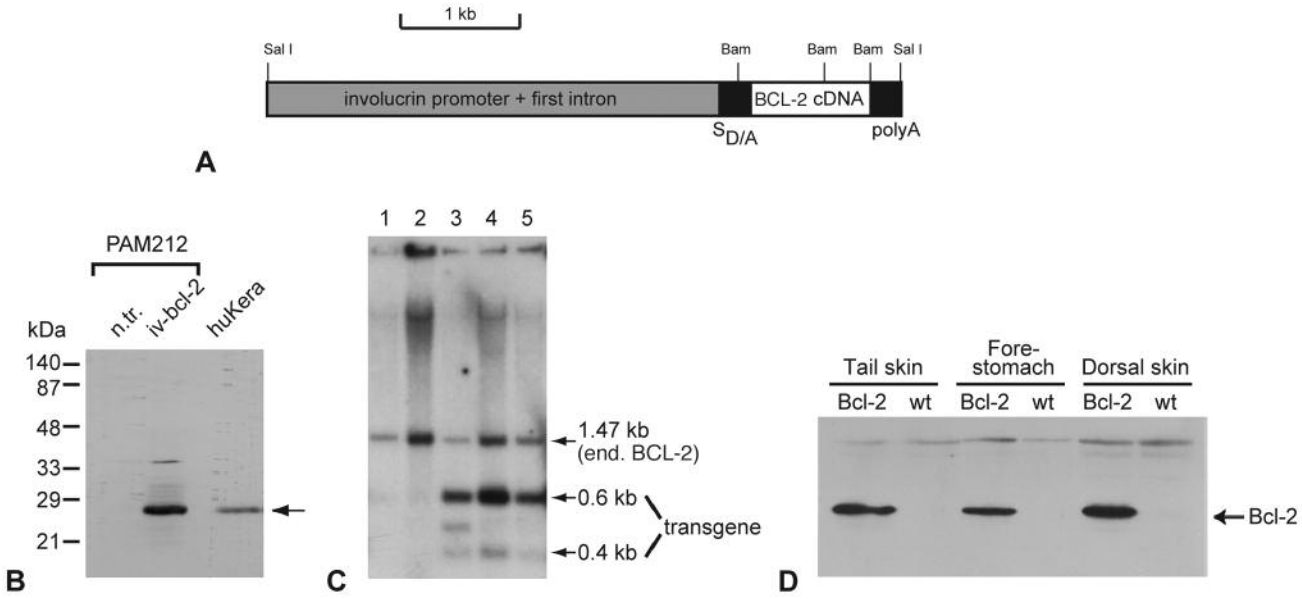

Figure 1. *Transgene and transgene expression. A, Scheme of the transgene construct used to express BCL-2 in skin. The locations of the regulatory region of the human involucrin gene (grey rectangle), the splice donor/acceptor ($S_{D/A}$) and polyandenylation (polyA) sequences of SV40 (black rectangles) and the human BCL-2 cDNA (white rectangle) are depicted. Sal I restriction sites used to remove the vector backbone as well as BamH I restriction sites relevant for Southern blot analysis are indicated. B, Test of the transgene construct by Western blot analysis of protein extracts of PAM212 cells either not transfected (n.tr.) or transfected with the iv-bcl-2 construct. An antibody specific to human BCL-2 detects a protein of the appropriate size in transfected cells (arrow). huKera: primary human keratinocytes used as a positive control. C, Detection of the transgene by Southern blot analysis of BamH I-restricted genomic tail DNA. 1, 2: non-transgenic mice; 3-5: independent transgenic founder mice. The founder line # 3446 used in the subsequent experiments is represented by lane 5. The ^{32}P-labelled full-length BCL-2 cDNA served as hybridization probe. D. Expression of the transgene-derived BCL-2 protein in stratified epithelia of transgenic mice (Bcl-2) or wild-type controls (wt) as revealed by Western Blot analysis using a human BCL-2-specific antibody.*

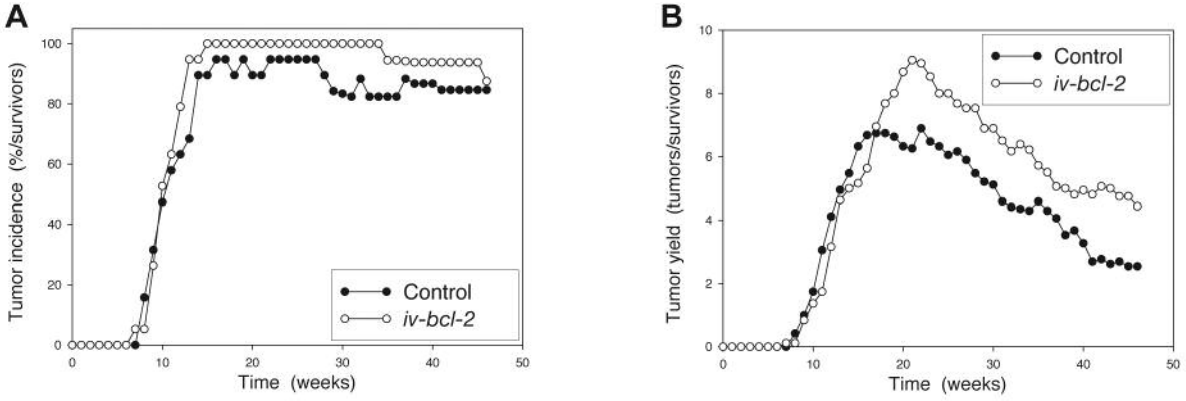

Figure 2. *Skin tumour formation in DMBA/TPA-treated mice. A, Kinetics of tumour development of control (filled circles) versus transgenic mice (open circles). The percentage of tumour-bearing mice is plotted over the time in weeks. B, Kinetics of tumour yield of control (filled circles) versus transgenic mice (open circles). The average number of tumours per animal is plotted over time in weeks.*

tumour yield per animal remained statistically unaffected, although a tendency towards higher tumour numbers was evident in the transgenic group (Figure 2B). Maximum papilloma count and the respective time required to reach maximum papilloma count were not different between the two groups of mice (Table I). Histological analyses revealed that most tumours were benign papillomas, and the occurrence of squamous cell carcinomas was not significantly different between both groups of mice (data not shown). Therefore, suprabasal expression of BCL-2 did not influence papilloma development or their progression to squamous cell carcinomas.

Table I. *Papilloma counts of DMBA/TPA-treated iv-bcl-2 mice (transgenic) and control mice (non-transgenic).*

	Maximum papilloma count		Time to maximum papilloma count (weeks)	
Mouse group	No. of mice	Mean±SD	No. of mice	Mean±SD
Transgenic	20	9.65±5.14	19	20.79±3.43
Nontransgenic	19	9.79±5.6	18	21.94±6.26

SD: standard deviation.

Discussion

In this report we describe a novel transgenic mouse model expressing BCL-2 protein in skin and other stratified squamous epithelia under control of the *involucrin* gene promoter that had previously been shown to confer transgene expression to suprabasal layers of mouse skin. In this *iv-bcl-2*-transgenic mouse line BCL-2 expression did not lead to an altered susceptibility to chemically induced skin cancer. Although transgenic mice showed a trend towards a higher number of tumours, the difference in mean tumour number of tumour-bearing animals was not statistically significant at any time point due to the high variability between the animals.

The rationale of our experiments has been the location of keratinocyte stem cells within the skin compartment expressing BCL-2, *i.e.* the basal layer of the interfollicular epidermis and the hair follicle bulge (4, 5, 18). These stem cells have been shown to represent the target cells of chemicals inducing skin cancer (6). In fact, BCL-2 is frequently overexpressed in human non-melanoma skin cancer, suggesting that its antiapoptotic activity may account for the susceptibility of the stem cells to cellular transformation (3). A strong argument in favour of this hypothesis has been the finding that transgenic mice bearing only one copy of the *Bcl-2* gene displayed a reduced propensity to develop skin cancer (19). Therefore, we speculated that overexpression of BCL-2 in skin cells (normally not expressing Bcl-2) would increase the pool of cells susceptible to chemically induced skin cancer. If this hypothesis applies, a deregulation of *BCL-2* gene expression would likely be involved in human skin cancer, too.

To date, the published studies analysing transgenic mice overexpressing BCL-2 in skin have led to conflicting results. BCL-2 overexpression in both basal and suprabasal skin under control of the human *keratin-1* gene promoter led to an increased susceptibility to develop skin cancer in *HK1.bcl-2* transgenic mice (7). In this study, BCL-2 was shown to suppress apoptosis elicited by DMBA or TPA in keratinocytes *in vitro*, suggesting that BCL-2 promotes tumourigenesis by suppressing apoptosis (7). In another study, however, transgenic mice overexpressing BCL-2 under control of the *keratin-14* gene promoter in basal keratinocytes displayed a reduced tumour susceptibility (8). This finding may be due to an antiproliferative activity of high BCL-2 levels as was described for transgenic mice overexpressing BCL-2 in the mammary gland (20). Moreover, these mice displayed a reduced incidence of developing breast cancer after DMBA treatment. The controversial results of Bcl-2 overexpression in skin may in part be due to differences in the experimental designs of the particular studies, such as usage of mouse strains exhibiting different tumour-susceptibilities (21). They could, however, also be interpreted as being caused by an increased pool of cells susceptible to transformation beyond the basal cell layer in the *HK1.bcl-2* transgenic mice. The experiments described in our study do, however, not support this hypothesis since *iv-bcl-2* transgenic mice were not sensitized to chemically induced skin cancer.

In *iv-bcl-2* transgenic mice the progression of papillomas to carcinomas was not altered. In another study, overexpression of the BCL-2 family member BCL-XL increased tumour progression (22). Again, different cell types expressing the transgenes may account for these contradictory results. In addition, they may reflect different properties of the particular BCL-2 family members which have specific cellular interaction partners mediating their activity (23).

In conclusion, we were unable to demonstrate any effect of suprabasal BCL-2 expression on chemically induced skin cancer. Overexpression of the *BCL-2* gene in human skin cancer is, therefore, likely not a reflection of a deregulation of its promoter but rather a property of the cell type from which the tumour originated.

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