Survivin Expression in Mouse Skin Prevents Papilloma Regression and Promotes Chemical-induced Tumor Progression


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Survivin Expression in Mouse Skin Prevents Papilloma Regression and Promotes Chemical-induced Tumor Progression

Sarah M. Allen, Scott R. Florell, Adrienne N. Hanks, April Alexander, Miyoun J. Diedrich, Dario C. Altieri, and Douglas Grossman

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ABSTRACT

Induction of cutaneous squamous cell carcinoma (SCC) in mice, by topical chemical [9,10-dimethylbenzanthracene (DMBA) and phorbol 12-myristate 13-acetate (PMA)] application, is a multistep process involving papilloma formation and progression to carcinoma. We have generated a transgenic (Tg) mouse [keratin-14 (K14)-survivin] with skin expression of survivin, an inhibitor of apoptosis expressed in most human skin cancers and premalignant lesions. K14-survivin mice were resistant to DMBA-induced keratinocyte apoptosis. To investigate the role of survivin and apoptosis in cutaneous carcinogenesis, mice were treated once topically with DMBA followed by twice weekly with PMA for 32 weeks. Surprisingly, tumor formation was less frequent (31% versus 43%) and significantly delayed (P = 0.01) in K14-survivin mice compared with non-Tg littermates. On the other hand, papilloma regression was not observed in Tg mice, whereas 20% of papillomas regressed in non-Tgs; one SCC was generated in Tg mice, whereas none were seen in non-Tgs. To increase tumor formation and SCC in particular, a second experiment was performed with mice on a p53+/−/− background. Again, DMBA/PMA-induced tumor formation was less (71% versus 89%) and significantly delayed (P = 0.02) in K14-survivin p53+/−/− animals compared with p53+/−/− non-Tgs. Papilloma regression was also not observed in Tg p53+/−/− mice, whereas 10% of papillomas regressed in p53+/−/− non-Tgs. The rate of papilloma progression to SCC was 21% in Tg p53+/−/− mice compared with 12% in p53+/−/− non-Tgs. Papillomas did not reveal significant differences in mitotic or apoptotic indices. Survivin expression was detected in all of the tumors. These results indicate that despite a paradoxical negative effect on tumor formation, survivin expression prevents papilloma regression and promotes conversion to SCC, consistent with its expression in most skin cancers and their precursors.

INTRODUCTION

Skin cancer development represents a multistage process. In patients, benign precursor lesions termed actinic keratoses arise that may either regress or ultimately progress to invasive SCC1 (1). Chemical carcinogenesis in the mouse, yielding predominantly papillomas and occasionally SCC, has proven a useful biological model for these clinical phenomena (2). Experimental protocols generally involve two steps: initiation and promotion. Tumor initiation can be accomplished through a single topical application with carcinogen, such as DMBA. The critical molecular event associated with initiation appears to be mutation of the H-ras proto-oncogene (3, 4). In the tumor promotion stage, clonal expansion of initiated keratinocytes can be triggered by repeated application of PMA, ultimately producing a squamous papilloma (2). Papillomas exhibit an increased proliferative rate and delayed differentiation (2), although regression is a common occurrence (5). Progression to SCC in this model is an uncommon event, occurring in <5% of papillomas (2). A second experimental carcinogenesis model involves chronic UVB radiation, with UV treatment serving both as tumor initiator and promoter (6).

Apoptosis of keratinocytes that have sustained UVB-induced DNA damage, termed “sunburn cells,” requires p53 and represents a key protective mechanism against skin cancer by removing premalignant cells that have acquired mutations (7). Compromise of p53 function undermines this apoptosis-based defense mechanism, giving UVB-damaged cells a selective advantage to survive additional cycles of UVB exposure (8). Additional impairment of p53 and other genes through additional UVB-induced mutations may then lead to even greater resistance to apoptosis, increased proliferation, and ultimately development of SCC. Consistent with its protective role, mutations in p53 are a common finding in human SCC (9), and p53-knockout mice are highly susceptible to both UVB- (10) and chemical-induced (11) SCC. p53 mutations have also been found in SCC precursors or actinic keratoses (12, 13), suggesting that p53 mutation is an early event in SCC development.

Another potential modulator of keratinocyte apoptosis that is also dysregulated in SCC development is survivin (14), a structurally unique member of the inhibitor of apoptosis gene family (15). In contrast with Bcl-2 proteins that block apoptosis by interfering with mitochondrial release of cytochrome c (16), certain inhibitors of apoptosis molecules act by binding to caspases and inhibiting their proteolytic activity (15). In the case of survivin, its antiapoptotic function is mediated by phosphorylation-dependent interaction with caspase-9 (17). Distinguished from other apoptotic regulators by its absence in most normal tissues (14), survivin is overexpressed in most transformed cell lines (18) and human cancers examined (19). Consistent with these findings, survivin is undetectable in normal skin but abundantly expressed in actinic keratoses and most malignant keratinocytic neoplasms including SCC (20). Survivin expression has proven to be a poor prognostic factor in many cancers (19). In a large panel of SCC, there was a positive correlation between survivin expression, and increased aggression and invasion (21). As its name suggests, survivin is a survival factor for transformed cells, and interference with its expression or function by transfection of antisense or a dominant-negative mutant caused spontaneous apoptosis in both melanoma (22, 23) and keratinocyte (20) cell lines. The susceptibility of malignant cells to survivin inhibition (24) and its high selectivity of expression in cancers compared with normal tissues (25) makes it a particularly promising target for cancer therapy (26).

We developed recently a Tg mouse to study survivin function in vivo, and found that skin expression of survivin conferred resistance to apoptosis without affecting keratinocyte proliferation or differentiation (27). Here we investigate the role of survivin in skin cancer development using this mouse in an established experimental model of cutaneous carcinogenesis.
CHEMICAL CARCINOGENESIS IN K14-SURVIVIN MICE

MATERIALS AND METHODS

Mice. The generation and preliminary characterization of K14-survivin Tg mice is described elsewhere (27). Expression of the transgene in normal skin was demonstrated previously by reverse transcription-PCR, Western blot, and immunohistochemistry (27). K14-survivin animals from second and third generation backcrosses with C57BL/6NCr mice (National Cancer Institute, Bethesda, MD) were used for these studies. To generate animals on a p53+/– background, female K14-survivin mice were crossed with male p53-deficient mice (C57BL/6TacBRKOP3N4; Taconic Farms, Germantown, NY). All of the littermates were genotyped for the presence of the survivin transgene by PCR as described (27) and used at 7–9 weeks of age. Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Keratinocyte Apoptosis. After shaving (27), 0.2-ml acetone or acetone containing 2 μmol of DMBA (Sigma Chemical Co., St. Louis, MO) was applied to dorsal skin. After 48 h, treated skin was excised, fixed in buffered formalin, paraffin-embedded, and sections cut for routine staining with H&E. Apoptotic epidermal keratinocytes were identified microscopically as described previously (27).

Chemical Carcinogenesis. A single application of DMBA (200 nmol in 0.2-ml acetone) was applied to shaved dorsal skin. This high dose was used because these mice were generated on a C57BL/6 background, which has been reported to be relatively resistant to chemical carcinogenesis (28). After 2 weeks, 6.8 nmol of PMA (Sigma) was similarly applied twice weekly (Monday and Thursday). Animals were terminated after 32 weeks of PMA treatment or sooner if they developed an ulcerated tumor, because we found spontaneous ulceration to be a marker for SCC conversion.4 Tumor size and location was documented weekly. Animals were shaved periodically to maintain visualization of the dorsal skin.

Histological Analysis and Immunohistochemistry. Animals were killed by cervical dislocation, and all tumors 2 mm or greater in diameter were excised, fixed in 10% formalin, and embedded in paraffin. Tumor sections were visualized by routine staining with H&E. All of the slides were reviewed twice in blinded fashion by a dermatopathologist (S. R. F.), and assessed for tumor architecture, keratinocyte differentiation, cytoplastic atypia, and inflammation. Tumors exhibiting papillomatous architecture and comprised of well-differentiated keratinocytes without cytoplastic atypia, with mitotic figures limited to the basal and lower epidermis, were classified as typical papillomas. Tumors with similar architecture, but comprised of keratinocytes with cytoplastic atypia and increased mitotic figures not limited to the lower epidermis, were classified as atypical papillomas. Tumors demonstrating keratinocyte cytoplastic atypia and mitotic figures involving the entire epidermis were termed SCC-in situ; in some cases there were isolated atypical keratinocytes not confined by the epidermal-dermal junction, representing focal invasion. The classification of invasive SCC was reserved for tumors in which there was clear penetration or disruption of the basement membrane by malignant keratinocytes. Tumors <2 mm in diameter proved too small for reliable histological analysis and were assumed to be papillomas.

A subset of tumors with sufficient residual tissue remaining in the paraffin block were subjected to additional analysis. Five-μm tissue sections were cut, and slides were baked in a 58°C oven overnight. Sections were deparaffinized in xylene (Fisher Scientific, Pittsburgh, PA) for 45 min, washed twice in ethanol, and endogenous peroxidase activity was quenched with 3% hydrogen peroxide (Fisher) in methanol for 10 min. After washing in ethanol and then water, sections were subjected to antigen retrieval using a pressure cooker as described previously (22). Proliferating cells were stained with 0.5 μg/ml PCNA antibody (BD Transduction Laboratories, Palo Alto, CA) in dilution buffer (Zymed Laboratories, San Francisco, CA) overnight at 4°C, and sections were developed using a Histostain-Plus Broad Spectrum kit (Zymed) with 3-amino-9-ethylcarbazole as the peroxidase substrate. Mitotic index was assessed as described previously (22). Apoptotic index was assessed by TUNEL staining using the ApopTag kit (Intergen, Purchase, NY) as described previously (22).

Although we have detected Tg survivin in frozen sections previously (27), we found that staining of paraffin-embedded mouse tissues using antibodies characterized previously is problematic. However the antibody 60.11 described recently (29), generated against survivin residues 57–67 (identical sequence in human and mouse survivin) and now commercially available (Novus Biologicals, Littleton, CO), yielded weak cytoplasmic staining in tumors. Sections were incubated at 1:1000 to 1:500 dilution of ascites overnight at 4°C and developed as above. Mouse IgG (Sigma) served as a negative control.

Statistics. Data derived from multiple animals were subjected to survival analysis and unpaired t tests with Welch’s correction using Prism (Graphpad Software, San Diego, CA). Ps < 0.05 were considered statistically significant.

RESULTS

K14-Survivin Mice Are Resistant to DMBA-induced Apoptosis. In addition to acting as a tumor initiator, DMBA has also been shown to induce keratinocyte apoptosis in vitro (30). We found that 48 h after application of DMBA to mouse skin, apoptotic keratinocytes could optimally be detected in situ. However, compared with non-Tg littermates, K14-survivin mice were strikingly resistant (P < 0.001) to DMBA-induced keratinocyte apoptosis (Fig. 1). Apoptotic cells were not detected in untreated skin of K14-survivin or non-Tg animals (data not shown).

Chemical Carcinogenesis in K14-survivin Mice. To investigate the role of survivin and apoptosis in cutaneous carcinogenesis, we subjected these mice and non-Tg littermates to an established topical regimen using DMBA and PMA (2). Untreated K14-survivin mice did not spontaneously form skin tumors over a 1-year observation period. Treated mice were examined weekly for tumor formation, and tumor regression or growth. Animals developing ulcerated tumors or after 32 weeks of tumor promotion (end point) were euthanized, and tumors ≥2 mm in diameter were analyzed histologically. Tumors <2 mm in diameter were assumed to be papillomas. Given the selective expression of survivin in skin tumors (20), we were surprised to find K14-survivin mice were significantly more resistant to tumor formation (P = 0.01) than non-Tg littermates (Fig. 2). Whereas the median time to tumor formation was 23 weeks for the non-Tg animals (Fig. 2) and 43% had tumors at the 32-week end point, only 31% of K14-survivin mice ultimately formed tumors (Table 1). Moreover, the tumor burden for animals that formed tumors was also less in K14-survivin mice that formed a total of 12 tumors with a mean of 1.5 tumors per animal compared with 20 tumors and a mean of 2.0 tumors in non-Tg animals, although there was not a significant difference (P = 0.45) in mean individual tumor size or percentage of large (≥3 mm) tumors formed (Table 1). In the non-Tg group, there were fewer tumors at end point than suggested by the survival curve (Fig. 2), because 5 tumors (20% of tumors formed) underwent regression (Table 1). By contrast, no tumor regression was observed in K14-survivin mice. One tumor, that arose in a K14-survivin mouse after only 7 weeks of PMA treatment grew rapidly and ulcerated (Fig. 3D).

Histological analysis revealed it to be a poorly differentiated invasive SCC (Fig. 3D). By contrast, no SCC developed in non-Tg animals.

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4 D. Grossman, unpublished observations.
All of the tumors examined from both K14-survivin (11 of 11 papillomas and 6 of 6 SCCs) and non-Tg (12 of 12 papillomas and 6 of 6 SCCs) mice (Fig. 3, E–G). We did not appreciate consistent differences in intensity of survivin staining between histologically similar tumors in K14-survivin and non-Tg p53+/− mice. Tumor cell apoptosis was measured by TUNEL staining. Apoptotic cells were extremely rare in both typical and atypical papillomas, with only isolated or in many cases no TUNEL-positive cells detected in tumor sections, and no difference was noted between tumor types or genotypes (data not shown).

**DISCUSSION**

In this study, we have used a Tg mouse model to investigate the effects of constitutive survivin expression on cutaneous carcinogenesis. We found that survivin expression in mouse skin impacts multiple steps in the formation of chemical-induced skin tumors. First, K14-survivin mice were unexpectedly less susceptible to tumor induction than non-Tg littermates. Second, survivin expression prevented papilloma regression. Finally, the rate of papilloma conversion to SCC was enhanced in K14-survivin mice.

Apoptosis, or more precisely apoptosis resistance, is believed to be an independent factor in the development of most cancers (31). The selective expression of survivin in cancers compared with normal tissues makes it among the most cancer-specific of all genes (25). Recent development of the K14-survivin mouse provides the first opportunity to investigate the function of survivin in skin tumor formation and progression in vivo. This Tg model is particularly well suited to address the role of apoptosis inhibition per se, as our

Table 1. Summary of chemical-induced tumors in K14-survivin mice

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<th>Non-Tg</th>
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<td>Mice with tumors*</td>
<td>8/26 (31%)</td>
<td>10/23 (43%)</td>
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<tr>
<td>Total tumor number</td>
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<td>Tumor density:</td>
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<tr>
<td>Tumor size</td>
<td>1.50 (±0.19)</td>
<td>2.00 (±0.26)</td>
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<tr>
<td>Tumors ≥3 mm (%)</td>
<td>3 (25%)</td>
<td>6 (30%)</td>
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<tr>
<td>Tumors regressed (%)</td>
<td>0</td>
<td>5 (20%)</td>
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<tr>
<td>SCC (%) (progression)</td>
<td>1 (8.35%)</td>
<td>0</td>
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* Fifty mice (26 K14-survivin and 24 non-Tg littermates) were treated once topically with DMBA, followed by twice weekly with PMA for 32 weeks. One non-Tg animal died at 13 weeks (without tumors).

Fraction of mice with tumors at end points of 32 weeks or tumor ulceration. Tumors that regressed before 32 weeks not included.

Total number of tumors formed at end point. Tumors that regressed before 32 weeks not included.

Mean number of tumors per animal with tumors at end point. SE in parentheses. P = 0.07. Tumors that regressed before 32 weeks not included.

Mean tumor size in mm. SE in parentheses. P = 0.45. Tumors that regressed before 32 weeks not included.
previous studies (27) have shown that epidermal expression of survivin conferred resistance to apoptosis without impacting keratinocyte proliferation or differentiation. Experimental cutaneous carcinogenesis in the mouse involves distinct observable steps: papilloma formation, papilloma growth, conversion to carcinoma-\textit{in situ}, and finally invasive SCC. In this study, we have been able to assess the impact of survivin expression on each of these critical steps in the skin carcinogenesis pathway.

Our initial hypothesis was that survivin expression in the skin would promote tumor formation, given its presence in SCC and actinic keratosis precursors (20). Thus, we were surprised to observe both a delay in time to tumor formation and a negative effect on tumor number in K14-survivin mice compared with non-Tg littermates. This effect was seen both on wild-type and p53\(^{+/+}\)/H11001/H11002 backgrounds. These findings contrast with those from other Tg mouse models expressing negative regulators of apoptosis in the skin. For example, skin expression of Bcl-2 family members Bcl-2 (30) and Bcl-xL (32), and the p53 regulator MDM-2 (33) were all associated with increased susceptibility to chemical carcinogenesis. These effects may be promoter-specific, as expression of MDM2 (34) and Bcl-2 (35) using different promoters than in the studies cited above did not result in increased skin tumor susceptibility. Promoter effects may relate to strength of transgene expression or the state of keratinocyte differentiation, \textit{i.e.}, the epidermal compartment targeted, such as stem cell or more differentiated keratinocyte. In addition, individual apoptotic inhibitors may have different activities in the same promoter system, as expression of Bcl-xL (32) and Bcl-2 (35) in mice with contrasting phenotypes was driven by the same K14 promoter (36) used in the K14-survivin mice described here.

The negative effects on tumor formation suggest that survivin inhibitors may have different activities in the same promoter system, as expression of Bcl-xL (32) and Bcl-2 (35) in mice with contrasting phenotypes was driven by the same K14 promoter (36) used in the K14-survivin mice described here.

The negative effects on tumor formation suggest that survivin...
Table 2. Summary of chemical-induced tumors in p53+/− mice

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<th>K14-survivin p53+/−</th>
<th>Non-Tg p53+/−</th>
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<tr>
<td>Mice with tumorsa</td>
<td>17/24 (71%)</td>
<td>31/35 (89%)</td>
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<tr>
<td>Total tumor numberb</td>
<td>39</td>
<td>90</td>
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<td>Tumor densityc</td>
<td>2.29 ±0.33</td>
<td>3.00 ±0.42</td>
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<tr>
<td>Tumor sizef</td>
<td>2.44 ±0.27</td>
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<td>Tumors ≤3 mm (%)</td>
<td>11 (28%)</td>
<td>29 (32%)</td>
</tr>
<tr>
<td>Tumors regressed (%)</td>
<td>6 (10%)</td>
<td>10 (10%)</td>
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<tr>
<td>SCC (% progression)</td>
<td>8 (21%)</td>
<td>11 (12%)</td>
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* Fifty-nine p53+/− mice (24 K14-survivin and 35 non-Tg littermates) were treated once topically with DMBA, followed by twice weekly with PMA for 32 weeks.

** Fraction of mice with tumors at end points of 32 weeks or tumor ulceration. Tumors that regressed before 32 weeks not included.

† Total number of tumors formed at end point. Tumors that regressed before 32 weeks not included.

‡ Mean number of tumors per animal that formed tumors. SE in parentheses. P = 0.09. Tumors that regressed before 32 weeks not included.

§ Mean tumor size in mm. SE in parentheses. P = 0.30. Tumors that regressed before 32 weeks not included.

expression reduces the frequency of tumorigenic events and increases the time required for tumor development. The mechanism for this negative effect on tumor formation is unclear, and there is no apparent marker to visualize early events in chemical carcinogenesis as with p53-mutated clones in UVB-induced skin tumors (37). Nevertheless, we speculate that early cellular events in chemical-induced tumorigenesis mimic those in the UVB system, i.e., clone formation and clonal expansion of mutated (or initiated) keratinocytes, and that there may be a paradoxical requirement for apoptosis. It is intriguing that agents used for induction of skin carcinogenesis, namely UVB (7) and DMBA/PMA (30), are also potent inducers of apoptosis in mouse keratinocytes. Thus, tumor initiation by these agents, in addition to causing mutations that could lead to apoptosis resistance and promote tumor growth, would also be expected to cause apoptosis in some neighboring keratinocytes. This cell loss or drop-out within a fixed epidermal compartment may be required to provide space to accommodate the formation and expansion of adjacent tumor-forming clones. In the absence of this effect, clonal formation and expansion might be physically restrained and thereby limit tumor formation. Indeed, Zhang et al. (38) working in the UVB system reported recently that continued UVB was required to maintain p53-mutant clone growth, and if discontinued led to the development of “imprisoned clones” with increased cell number without increased area. We additionally speculate that early clonal events may precede survivin expression in the natural development of skin tumors and that constitutive skin expression of survivin as occurs in the K14-survivin mouse may prevent adequate induction of keratinocyte apoptosis by DMBA/PMA. Indeed, we demonstrated that induction by DMBA of apoptotic keratinocytes was reduced 3-fold in skin of K14-survivin compared with non-Tg mice. In this scenario, it is plausible that survivin expression might have a negative impact on clone formation and expansion, and thereby result in delay to tumor formation and decreased tumor yield.

Survivin expression did not appear to affect papilloma growth, as we did not observe significant differences in individual tumor size, percentage of large tumors formed, or mitotic index. These findings are consistent with our previous studies (27) in which Tg expression of survivin did not affect epidermal differentiation. Although previous studies also found that skin of K14-survivin mice demonstrated normal epidermal differentiation, here we observed that in the context of chemical carcinogenesis on a p53+/− background, survivin expression increased the tendency toward cellular atypia, as a larger fraction of papillomas were atypical compared with those in non-Tg mice. Even more striking was our finding that papilloma regression, which occurred in 10–20% of tumors in non-Tg mice, was not observed in K14-survivin mice. In the generation of skin cancers, survivin expression, and by extension apoptosis inhibition, may be an important factor in increasing the likelihood that precursor lesions will persist and accumulate additional mutations to progress toward carcinoma. An alternative mechanism to apoptosis for tumor regression is keratinocyte senescence, but a role for survivin in this process has not been defined. Finally, survivin expression was associated with a higher rate of transformation from papilloma to carcinoma. It may be that SCCs ultimately arise from papillomas (or precursor lesions) that do not regress. We found that survivin was consistently expressed in all of the tumors, including those in non-Tg mice, consistent with our previous findings (20) in both premalignant and malignant human skin tumors. Analysis of survivin expression in tumors was not particularly informative, given the nonquantitative nature of immunohistochemistry and our inability to distinguish between endogenous and Tg survivin.

The multiple effects of survivin expression on chemical carcinogenesis were amplified on a p53+/− genetic background. Our previous studies (27) in the K14-survivin mouse revealed a potential antagonistic relationship between survivin and p53, as resistance to UVB-induced apoptosis in survivin-expressing keratinocytes was dramatically enhanced by loss of a p53 allele. Thus, the combination of survivin expression and loss of p53 (9) may provide a general mechanism for aberrant inhibition of apoptosis that leads to the development and progression of cancer. Recent evidence suggests that both survivin and p53 may be coupled to the apoptotic machinery through Apaf-1/caspase-9, an upstream initiator of the intrinsic (mitochondrial initiated) pathway (39). A physical complex between survivin and caspase-9 has been demonstrated in vivo (17), and apoptosis mediated by p53 requires downstream mitochondrial cytochrome c release and

![Fig. 5. Histological analysis of tumors in K14-survivin p53+/− (□) and non-Tg p53+/− (■) mice. Tumors <2 mm in diameter were assumed to be papillomas. One non-Tg animal developed a s.c. poorly differentiated tumor that was not classified. Shown are percentages of total tumors formed at end point for each group. Actual number of tumors indicated in parentheses above bars.](image)

![Fig. 6. Analysis of proliferation in papillomas from K14-survivin p53+/− (□) and non-Tg p53+/− (■) mice. Shown are mitotic indices determined by PCNA staining of typical and atypical papillomas as indicated for each group. Error bars, ±SE. Actual number of tumors analyzed indicated in parentheses above bars. P values for comparisons between groups of mice are indicated.](image)
CHEMICAL CARCINOGENESIS IN K14-SURVIVIN MICE

caspase-9 activation (40, 41). In addition to converging on the same components of the apoptotic pathway, there may be a direct relationship between survivin and p53, given the recent demonstration that p53 binds to the survivin promoter in vivo and represses survivin gene expression (42).

In summary, we have investigated the effect of survivin expression in the skin on tumor induction using an established regimen of chemical carcinogenesis. Our results indicate that although survivin exerts a paradoxical negative effect on tumor formation, its expression prevented papilloma regression and promoted conversion to SCC, consistent with its expression in most premalignant lesions and skin cancers.

ACKNOWLEDGMENTS

We thank Dr. Jennifer McNiff (Yale University School of Medicine, New Haven, CT) for reviewing the histology of some tumors in the initial phase of this study and Dr. Ken Boucher (Huntsman Cancer Institute) for suggestions on statistical analysis. We also thank Dr. Douglas Brash (Yale University School of Medicine) for helpful discussions.

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