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Tumor suppressor activity of ODC antizyme in MEK-driven skin tumorigenesis

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Abstract

To test the hypothesis that suppression of ornithine decarboxylase (ODC) activity blocks the promotion of target cells in the outer root sheath of the hair follicle initiated by Raf/MEK/ERK activation, we crossed mice overexpressing an activated MEK mutant in the skin (K14-MEK mice) with two transgenic lines overexpressing antizyme (AZ), which binds to ODC and targets it for degradation. K14-MEK mice develop spontaneous skin tumors without initiation or promotion. These mice on the ICR background were crossed with K5-AZ and K6-AZ mice on both the carcinogenesis-resistant C57BL/6 background and the sensitive DBA/2 background. Expression of AZ driven by either the K5 or K6 promoter along with K14-MEK dramatically delayed tumor incidence and reduced tumor multiplicity on both backgrounds compared with littermates expressing the MEK transgene alone. The effect was most remarkable in the MEK/K6-AZ mice from the ICR/D2 F1 cross, where double transgenic mice averaged less than one tumor per mouse for more than 8 weeks, while K14-MEK mice averaged over 13 tumors per mouse at this age. Putrescine was decreased in MEK/AZ tumors, while spermidine and spermine levels were unaffected, suggesting that the primary role played by AZ in this system is to inhibit putrescine accumulation. MEK/AZ tumors did not show evidence of apoptosis, but there was a 15–20% decrease in S-phase cells and a 40–60% decrease in mitotic cells in MEK/AZ tumors. These results indicate that the principal effect of AZ may be to slow cell growth primarily by increasing G_2 /M transit time.

Introduction

The use of transgenic animals provides a unique system for the study of the events and gene interactions involved in the development of tumors. This is particularly true of models of skin tumor development, where the genetic and biochemical alterations that occur in response to the two-stage initiation/promotion protocol have been studied extensively in recent years (reviewed in (1)). In addition, strong promoters are available that can target transgene expression specifically to cells of interest (2). The experiments reported here make use of several transgenic mouse models to gain a greater understanding of the role played by ornithine decarboxylase (ODC) in skin tumorigenesis.

ODC catalyzes the conversion of ornithine to putrescine, the first step in the biosynthesis of intracellular polyamines. High levels of ODC can transform NIH/3T3 cells (3 , 4), and induction of ODC activity and changes in polyamine levels have been demonstrated in a variety of experimental systems designed to study neoplastic growth. Increased ODC activity has been associated with *in vitro* transformation by such oncogenes as H- *ras* and v- *src* (5 – 7). We have shown that ODC is controlled both transcriptionally and translationally by the Ras effector pathways Raf/MEK/ERK and PI 3-kinase (8), and ODC is also a transcriptional target of both c- *myc* and the WT1 tumor suppressor (9 , 10). *In vivo* , transgenic mice have been used to establish that overexpression of ODC in the outer root sheath (ORS) of the hair follicle using both the Keratin 5 (K5) and Keratin 6 (K6) promoters is a sufficient stimulus for skin tumor promotion (11 – 13), and this increased ODC activity can cooperate with the Raf/MEK/ERK pathway to promote invasiveness of keratinocytes from K6/ODC mice (14). Elevated ODC activity was detected in the spontaneous skin tumors that develop on mice expressing an activated MEK protein from the K14 promoter (K14-MEK mice (15)). Our results using K14-MEK mice treated with the ODC inactivator α-difluoromethylornithine (DFMO) provide strong evidence that induction of ODC activity is critical to MEK-induced skin tumorigenesis and link the induction of ODC during skin tumor promotion with alterations in Ras signal transduction pathways (15).

To examine the role of ODC in the molecular mechanism of tumor development in response to Ras effector pathway activation, tumor formation on both carcinogenesis-sensitive and resistant backgrounds was analyzed in K14-MEK mice crossed with transgenic lines designed to block ODC activity. These lines use keratin promoters to overexpress either a dominant negative form of ODC (ODCdn), which we have shown decreases ODC activity when expressed in the skin (16), or antizyme (AZ) (17), an important endogenous regulator of ODC and

polyamine homeostasis. The wild type form of ODC has an extremely short half life (20–30 min), and its degradation is mediated by AZ, which binds to the monomeric form of ODC, preventing formation of the enzymatically active homodimer, and then targets ODC for degradation (18). In addition to its effects on ODC, AZ also controls polyamine content by inhibiting polyamine transport into cells (19). We have shown previously using AZ transgenic mice that AZ suppresses tumor growth in the classic DMBA/TPA skin tumorigenesis model (17) and a UV carcinogenesis model that utilized mice heterozygous for the patched tumor suppressor gene (20).

AZ expression is driven by either the K5 or K6 promoter in these experiments (17), and ODCdn is driven by the K6 promoter (16). The constitutive K5 and K14 promoters are active in the same cell populations (21) and the K6 promoter, while expressed at very low levels in untreated skin, is upregulated by hyperproliferative stimuli (22), which is provided in this model by the activated MEK protein. Therefore, MEK is expressed in the same cell populations as the ODC inhibitor proteins in the double transgenic lines. The use of these transgenic lines targets inhibition of ODC activity to a specific subpopulation of epidermal keratinocytes, rather than the more general effect seen with an inhibitor such as DFMO, thus providing valuable model systems to evaluate the role of ODC and polyamines in skin tumorigenesis.

The results show that overexpression of AZ driven by either the K5 or K6 promoter at the time of the initiating stimulus reduces total tumor numbers and dramatically delays tumor formation in K14-MEK mice. Tumors that form exhibit slower growth rates, characterized by a decrease in S-phase cells and a much larger reduction of mitosis, suggesting a prolonged G_2 transit time. No measurable increase in apoptosis was observed in MEK/AZ tumors, indicating that in this model AZ is acting to slow tumor proliferation without affecting the rate of programmed cell death.

Materials and methods

Transgenic mice

K14-MEK (line 6) mice (15) were maintained on the ICR background by breeding hemizygous K14-MEK males with ICR females (Charles River Laboratories, Wilmington, MA). K5-AZ mice and K6-AZ (line 52) mice (17) were maintained on both the C57BL/6 and DBA/2 backgrounds for at least 5 generations by breeding hemizygous males with nontransgenic females (Jackson Laboratories, Bar Harbor, ME). K6-ODCdn mice (16) were maintained on the DBA/2 background by breeding hemizygous males with nontransgenic females for at least 5 generations. For tumorigenesis experiments, K14-MEK males were bred with K5-AZ, K6-AZ or K6-ODCdn females. The progeny were genotyped by PCR analysis of tail DNA as described (15 – 17) and examined for tumor formation.

Tumor induction experiments

F1 crosses of ICR/B6 and ICR/D2 mice were used for all experiments. Mice expressing either the K14-MEK transgene alone or in combination with K6-ODCdn, K5-AZ or K6-AZ were analyzed for spontaneous tumor formation. Tumors were counted weekly or biweekly after the first tumors became visible on the K14-MEK mice (usually 3–5 weeks of age). Both male and female mice were used in all experiments, and all experiments were performed using littermates. Mice were killed at the times noted in the text.

Biochemical analyses

Tumor samples were harvested and homogenized as described previously (17), and ODC was assayed at 37°C by measuring the release of $^{14}CO_2$ from L-[1-^{14}C] ornithine (23). For polyamine analysis in the skin, mice were killed at 7 weeks while in the resting phase of the hair cycle. Animals were shaved, and dermis and epidermis were isolated as described previously (17). Animals were killed for analysis of polyamines in tumors at 8 weeks

for K14-MEK mice and 13 weeks for MEK/AZ mice. All samples were acid extracted using 10% TCA and analyzed for polyamines using reverse phase HPLC analysis (24).

AZ immunohistochemistry

Tumors were removed immediately after sacrifice, fixed overnight in 10% neutral buffered formalin, embedded in paraffin, and 5 µm sections were obtained. Antiserum to AZ was produced in rabbits immunized with a purified recombinant polyhistidine-tagged AZ fusion protein and then purified using an AZ-affinity column as described previously (17). Following deparaffinization and rehydration in graded alcohols, tumor sections were heated in Vector Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA.) in a microwave oven (88–95°C, 3 × 5 min) before nonspecific binding sites were blocked with goat serum. Sections were incubated overnight at 37°C in a humidified chamber with rabbit anti-AZ antibody at 0.6 ng/µl dilution followed by incubation with biotinylated goat anti-rabbit antibody (1:500 dilution; Vector Laboratories). Slides were then incubated with streptavidin horseradish peroxidase (1:1000 dilution; Dako, Carpinteria, CA). Expression of AZ was localized by a final incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories). Sections were counterstained with hematoxylin and pictures taken on a Nikon TMS microscope.

Cell proliferation analysis

Cell proliferation was analyzed using PCNA immunohistochemistry, BrdU labeling and phospho-histone H3 (ser10) immunohistochemistry in MEK and MEK/AZ tumors. Mouse monoclonal anti-PCNA antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was used along with biotinylated goat anti-mouse antibody (1:500 dilution; Dako). Slides were then incubated with streptavidin horseradish peroxidase (1:1000 dilution; Dako) and PCNA was localized with 3-amino-9-ethylcarbazole chromogen (Vector Laboratories) and hematoxylin counterstain. When BrdU was used, mice were injected i.p. with 100 mg/kg body weight and killed 2 h later. Paraffin-embedded sections were subjected to zinc-buffered formalin postfixation and antigen retrieval as described previously (25), incubated overnight at 4°C with 1 U/ml peroxidase-conjugated mouse monoclonal anti-BrdU Fab fragments (Roche, Indianapolis, IN) and BrdU staining was visualized with DAB and hematoxylin counterstain. For phospho-histone H3 (ser10), sections were deparaffinized and subject to antigen retrieval as described above for AZ staining and then incubated with rabbit anti-phospho-histone H3 (ser10) (1:200, Upstate, Lake Placid, NY). Staining was visualized with the Vectastain Elite ABC kit (Vector Laboratories) with DAB chromogen and hematoxylin counterstain. Labeling index (%) for both BrdU and phospho-histone H3 was calculated by dividing labeled nuclei by the total number of cells counted per tumor section. Between 4 and 6 tumor sections from multiple mice were counted and averaged for each determination, and results are expressed as the mean ± SD.

Apoptosis analysis

The TUNEL assay was used to analyze the number of apoptotic cells in paraffin-embedded tumor sections isolated from MEK and MEK/AZ mice. The 3'-OH end labeling of apoptotic cell DNA was performed using an ApopTag Plus *in situ* peroxidase detection kit (Chemicon, Purchase, NY).

Differentiation analysis

Tumor differentiation was evaluated in MEK and MEK/AZ tumors at approximately 8 weeks for K14-MEK mice and 13–14 weeks for MEK/AZ mice. Paraffin sections were incubated in an anti-keratin 1 antibody (1:500; BAbCo, Richmond, CA) and visualized with a Vectastain Elite ABC kit and DAB (Vector Laboratories).

Statistical analysis

Tumor counts taken over a time course were compared using two-way ANOVA. Tumor incidence data were compared using a log-rank test of the curves. Counts of BrdU-labelled or phospho-histone H3-labelled nuclei were compared using the Student's *t* -test. Polyamine levels were also compared using the Student's *t* -test.

Results

Tumor formation in K14-MEK mice and double transgenic mice

Our results using systemic DFMO treatment suggest that ODC induction is necessary for tumor promotion and maintenance after initiation by MEK activation (15). To extend these experiments, we have crossed K14-MEK mice with transgenic lines that utilize the K6 promoter to overexpress a dominant-negative ODC (K6-ODCdn mice) or the K5 and K6 promoters to express AZ (K5-AZ mice and K6-AZ mice). ODCdn and AZ mice were backcrossed for at least five generations onto the tumor promotion-resistant C57BL/6 strain and the more sensitive DBA/2 strain (26 , 27). The K14-MEK mice were maintained on the ICR strain, and all experiments described use F1 crosses to generate double transgenic mice and littermate controls expressing MEK alone. In both K6-ODCdn mice and K6-AZ mice, expression of the transgene is induced in the ORS and in the interfollicular epidermis in response to hyperplasia. Since K14-MEK mice exhibit spontaneous hyperplasia, the K6 promoter would be expected to be up-regulated in MEK-expressing cells of the double-transgenic mice (22). The K5 and K14 proteins are coexpressed in the same cell lineages (28); therefore K5-AZ mice have constitutive overexpression of the transgene in the same cells that overexpress MEK.

Skin tumors arise spontaneously in K14-MEK mice on the ICR background at multiple sites on the body, without the need for chemical initiation or promotion (15). Introduction of the DBA/2 strain through F1 crosses increased K14-MEK tumorigenicity in the ICR/D2 progeny compared with our previous studies using the ICR strain (15). Expression of AZ from either the K5 or K6 promoter in ICR/D2 crosses reduced both tumor incidence and tumor multiplicity very effectively in the double transgenic mice compared with K14-MEK mice (Figure 1A, B). There was a significant lag in tumor onset in mice expressing AZ on this background, and several double transgenic mice remained tumor-free throughout the experiment (Figure 1A). Tumor multiplicity was reduced at 8 weeks from an average of 13.5 tumors per mouse in the K14-MEK mice to 0.9 tumors per mouse in MEK/K6-AZ double transgenic mice and 5.1 tumors per mouse in MEK/K5-AZ mice. It was usually necessary to sacrifice K14-MEK mice at eight weeks due to large tumor burden. At 12 weeks of age MEK/K6-AZ double transgenic mice still averaged only 4.3 tumors per mouse, while MEK/K5-AZ mice averaged about 11.6 tumors per mouse (Figure 1B). No tumors were observed on non-transgenic littermates or littermates expressing only the AZ transgene.

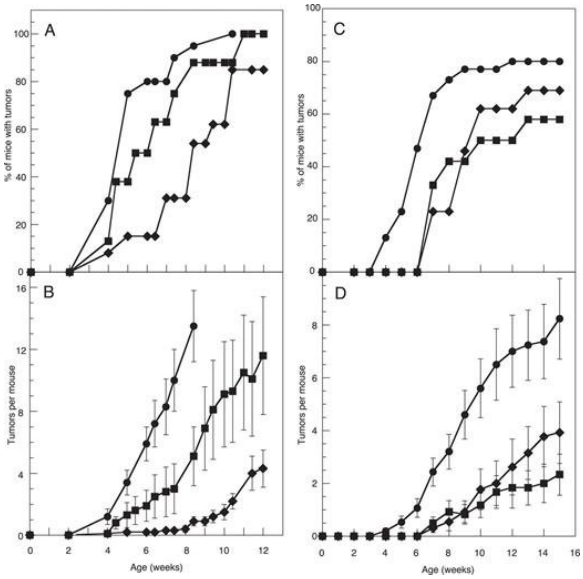

Fig. 1. Effect of AZ expression on spontaneous tumor formation in K14-MEK mice on the ICR/D2 and ICR/B6 backgrounds. On the ICR/D2 background, tumor incidence (**A**) and number of tumors per mouse (**B**) were assessed for K14-MEK mice (filled circle, *N* = 20) and double transgenic MEK/K5-AZ (filled square, *N* = 8) and

MEK/K6-AZ mice (filled diamond, *N* = 13). *P* < 0.001 when tumor incidence of MEK/K6-AZ mice is compared with K14-MEK mice; *P* < 0.0001 when tumor multiplicity of MEK/K6-AZ mice or MEK/K5-AZ mice is compared with K14-MEK mice. *P* < 0.0001 when tumor multiplicity of K5-AZ mice is compared with K6-AZ mice. On the ICR/B6 background, tumor incidence (**C**) and number of tumors per mouse (**D**) were assessed for K14-MEK mice (filled circle, *N* = 30) and double transgenic MEK/K5-AZ (filled square, *N* = 12) and MEK/K6-AZ mice (filled diamond, *N* = 13). *P* < 0.05 when tumor incidence of MEK/K5-AZ mice is compared with K14-MEK mice; *P* < 0.0001 when tumor multiplicity of MEK/K6-AZ mice or MEK/K5-AZ mice is compared with K14-MEK mice. On this background differences between the two AZ lines were not statistically significant. For both ICR/D2 and ICR/B6, tumor multiplicity values are expressed as the mean ± SEM for each time point.

The reduced tumor burden in the double transgenic mice was the result not only of fewer total tumors, but also because tumors in double transgenic mice were much smaller than those in their littermates (data not shown). Expression of MEK on the ICR/D2 background resulted in a high rate of tumor expansion, often leading to the merging of several small tumors into one large mass. K14-MEK mice also frequently had many papilloma-like lesions associated with the vibrissa follicles of the muzzle, making it difficult to count tumor numbers accurately. This pattern of tumor development is similar to that seen previously in K5 *ras* mice (29). MEK/K5-AZ mice exhibited much smaller tumors than MEK mice, while expression of AZ from the K6 promoter blocked tumor formation almost completely (Figure 1A, B and data not shown). Many tumors in MEK/K6-AZ mice were too small to be seen through the coat of the animal, even though double transgenic mice were killed 4 weeks after their MEK littermates.

Crosses were also performed using AZ-overexpressing mice on the C57BL/6 background (Figure 1C, D). As expected, K14-MEK mice from the ICR/B6 cross were more resistant to tumor formation compared with both ICR/D2 mice (Figure 1A, B) and the parental ICR strain (15). In fact, 20% of ICR/B6 MEK mice remained tumor-free for 15 weeks, with an average of 8.2 tumors per mouse visible by this age (Figure 1C, D). As with the DBA/2 crosses, expression of AZ from either the K5 or K6 promoter delayed tumor formation and dramatically reduced tumor multiplicity, with MEK/K5-AZ mice averaging 2.3 tumors per mouse and MEK/K6-AZ mice averaging 3.9 tumors per mouse at 15 weeks (Figure 1D).

Expression of K6-ODCdn in the skin of K14-MEK ICR/D2 mice delayed tumor onset by approximately 3 weeks in double transgenic mice compared with K14-MEK mice, with 100% of K14-MEK mice (*N* = 20) developing tumors by 5 weeks and 100% of MEK/K6-ODCdn mice developing tumors by 8 weeks (*N* = 16). When tumors were counted at sacrifice (8 weeks), expression of ODCdn did not result in a statistically significant difference in tumor number, and ODC activity was not decreased in MEK/K6-ODCdn tumors (data not shown). These results are similar to our previous results using DMBA/TPA treatment of K6-ODCdn mice, where expression of the ODCdn protein did not lead to a significant reduction in ODC activity or polyamine content (16). Therefore, in contrast to our *in vitro* experiments, where the ODC dominant negative mutant effectively reduced ODC activity and reverted oncogenic transformation by H-Ras and RhoA (7), this is not the case in an *in vivo* model.

Analysis of tumors for transgene expression and proliferation

We have shown previously that expression of the MEK transgene results in about a 3-fold increase in MEK protein in transgenic skin, resulting in a high level of phospho-ERK in tumor sections from K14-MEK mice (15). The expression pattern of AZ was analyzed in tumors by immunohistochemistry in K14-MEK mice and MEK/AZ mice on both ICR/D2 and ICR/B6 backgrounds (Figure 2). AZ was undetectable in K14-MEK tumors (Figure 2A, C) and was not consistently detected in MEK/K5-AZ tumors, although a faint expression was observed throughout the basal layer of most samples (Figure 2E, G). Consistent AZ expression was observed from the inducible K6 promoter (Figure 2I, K). Strong (though irregular) expression of AZ in MEK/K6-AZ tumors appeared in the suprabasal layer, with lighter staining in the proliferating basal cell layer. This is consistent with the

pattern of induction of the K6 promoter by hyperplasia in the tumors (22), and has been seen in other models utilizing the K6 promoter for transgene expression (11 , 30). Immunohistochemistry using antibodies directed to the endogenous keratin 6 protein confirmed expression of the K6 promoter in the suprabasal layer of MEK/K6-AZ tumors (data not shown).

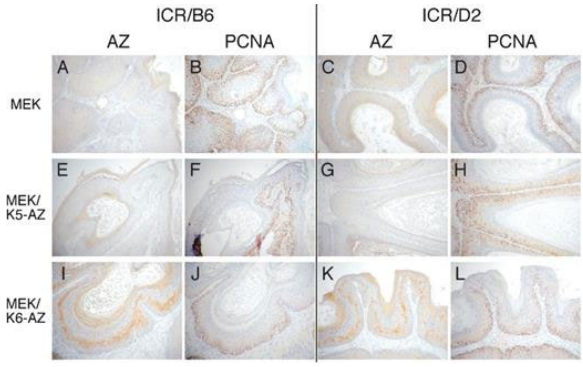

Fig. 2. AZ expression and cell proliferation in K14-MEK and MEK/AZ tumors. Immunohistochemistry for AZ (**A** , **C** , **E** , **G** , **I** , and **K**) and PCNA (**B** , **D** , **F** , **H** , **J,** and **L**) was performed as described in the Materials and Methods on serial sections of tumors from K14-MEK mice and MEK/AZ double transgenic mice on both the ICR/B6 and ICR/D2 backgrounds. Little or no AZ expression is apparent in both K14-MEK tumors (A, C) and MEK/K5-AZ tumors (E, G), while strong AZ staining is detected in the suprabasal cells of MEK/K6-AZ tumors (I, K). Numerous PCNA-positive nuclei are present in the basal layer of all tumors. Note that AZ staining (I, K) and PCNA staining (J, L) are visible in different cell layers in MEK/K6-AZ tumors.

Visual inspection of tumors from MEK and MEK/AZ mice demonstrated a dramatic decrease in tumor volume in double transgenic mice, as discussed above. Serial sections of tumor samples used for AZ immunohistochemistry were analyzed for PCNA-positive nuclei as a measure of tumor proliferation in MEK and MEK/AZ mice. Results are shown for both ICR/D2 mice and ICR/B6 mice (Figure 2). PCNA is an endogenous cell proliferation marker and can be used to identify cell cycle populations. Dark-staining nuclei represent S-phase cells, light-staining nuclei represent G_1 -S and G_2 cells, cells with cytoplasmic staining usually represent mitotic cells and non-staining nuclei represent quiescent cells (31). The results show that, although tumors from K14-MEK mice are visually much larger than tumors from MEK/AZ mice, all tumors are proliferating in the basal cell layer at the time points examined (Figure 2B, D, F, H, J, L). Tumors were generally isolated at sacrifice: 16–18 weeks for all mice on the ICR/B6 background, 8–9 weeks for K14-MEK on the ICR/D2 background and 13–14 weeks for MEK/AZ on the ICR/D2 background.

To quantitate proliferation differences in MEK and MEK/AZ tumors, tumor-bearing mice were injected with BrdU and killed 2 h later, when the labelling index was calculated by counting the number of S-phase cells and dividing it by the number of total cells. Results are shown for ICR/B6 crosses (Figure 3), and results in ICR/D2 mice were similar (data not shown). There was about a 15–20% decrease in labeling index in both MEK/AZ lines when compared with the K14-MEK tumors (Figure 3). Because this decrease alone most likely cannot explain the dramatic difference observed in tumor size with the expression of the AZ transgene, we also examined the phosphorylation of histone H3 in tumors as a measure of mitosis (32). There was a much more substantial decrease in mitotic cells (40–60%) in double transgenic tumors compared with tumors expressing the MEK transgene alone (Figure 3), suggesting that expression of AZ decreases tumor growth rate by increasing the G_2 transit time between S-phase and mitosis.

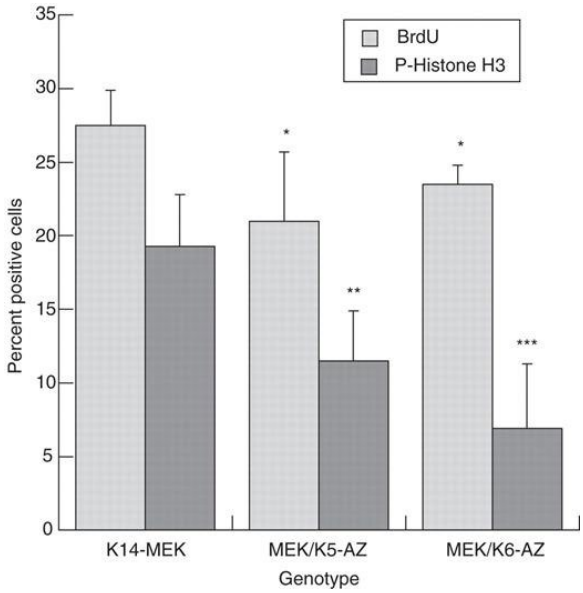

Fig. 3. K14-MEK tumor growth is inhibited by AZ. Tumor proliferation was analyzed by BrdU incorporation and phospho-histone H3 immunohistochemistry as described in the Materials and Methods on tumor sections from K14-MEK mice and MEK/AZ double transgenic mice. Statistical analysis revealed a significant decrease in the percentage of both S-phase cells (* $P < 0.05$) and mitotic cells (** $P < 0.005$; *** $P < 0.0001$) in MEK/AZ tumors.

Analysis of tumors and skin for ODC activity and polyamine levels

ODC activity and protein levels are known to be constitutively upregulated in DMBA/TPA-induced tumors (33 , 34) and in tumors induced by MEK expression from the K14 promoter (15). ODC activity was analyzed in tumor extracts from K14-MEK mice and double transgenic MEK/K5-AZ mice and MEK/K6-AZ mice in F1 ICR/B6 crosses. Although results were variable because of the heterogeneous nature of the tumor tissue and the directed expression of the transgenes, ODC activity was reduced by about 50% in tumors from MEK/K5-AZ mice and 60% in MEK/K6-AZ tumors compared with K14-MEK single transgenic mice (Figure 4). This result is consistent with previous results showing that AZ expression reduced ODC activity in chemically induced tumors (17). ICR/D2 tumors were not analyzed for ODC activity because the very small size of tumors from the double transgenic mice did not allow accurate ODC activity determinations.

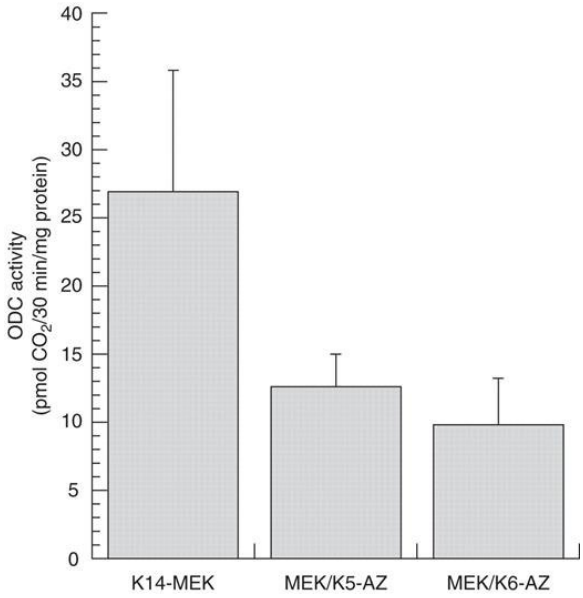

Fig. 4. ODC activity in tumors derived from K14-MEK and MEK/AZ mice on the ICR/B6 background. Mice were killed and tumors were processed for ODC activity as described in the Materials and Methods. Results are shown for tumors from K14-MEK mice (*N* = 6), MEK/K5-AZ mice (*N* = 5) and MEK/K6-AZ mice (*N* = 4). All ODC assays were performed in duplicate. Values are shown as means ± SEM and differences were not statistically significant.

Polyamines were also analyzed in skin and tumors from ICR/B6 mice (Table 1). Polyamine levels are shown for both epidermis and dermis of the following genotypes: wild-type, K14-MEK, K5-AZ, K6-AZ, MEK/K5-AZ and MEK/K6-AZ (Table 1). There is no measurable putrescine in any of the skin samples. This is consistent with the very low levels of ODC in the skin. There was a trend toward increased spermidine and spermine in the dermis of K14-MEK mice, and levels of spermidine and spermine were closer to those of WT mice in dermis of the double transgenics, particularly MEK/K5-AZ mice (Table 1). However, these differences were not statistically significant. This is not unexpected since both the AZ and MEK transgenes are expressed in limited cell populations, while the polyamines measured reflect levels in the entire epidermis or dermis. This makes it very difficult to detect even large changes in a small number of cells. This is especially true of the K6-AZ, which is induced by hyperproliferation but expressed in a very limited number of cells within the hair follicle ORS under unstimulated conditions.

Table 1.

Polyamine levels in skin and tumors from ICR/B6 mice

Genotype	Putrescine	Spermidine (nmol/g wet weight ± SEM)	Spermine
Epidermis			
WT	BD	221 ± 22	196 ± 13
K14-MEK	BD	262 ± 66	221 ± 49
K5-AZ	BD	230 ± 12	216 ± 17
MEK/K5-AZ	BD	283 ± 45	193 ± 15
K6-AZ	BD	278 ± 80	263 ± 80
MEK/K6-AZ	BD	254 ± 64	228 ± 61
Dermis			
WT	BD	169 ± 20	129 ± 10
K14-MEK	BD	295 ± 86	219 ± 68
K5-AZ	BD	181 ± 33	134 ± 26
MEK/K5-AZ	BD	204 ± 39	136 ± 12
K6-AZ	BD	202 ± 21	151 ± 24
MEK/K6-AZ	BD	246 ± 52	168 ± 39
Tumor			
K14-MEK	335 ± 45	1046 ± 128	99 ± 21
MEK/K5-AZ	274 ± 32	949 ± 72	93 ± 9
MEK/K6-AZ	59 ± 11 [a]	903 ± 41	118 ± 22

[a] $P < 0.01$ versus K14-MEK.

BD, below the limit of detection.

For skin measurements, mice were killed at 7 weeks and dermis was separated from the epidermis as described in the Materials and Methods. When tumors were analyzed, mice were killed after 8 weeks for K14-MEK mice

and after 13 weeks for MEK/K5-AZ mice and MEK/K6-AZ mice. Skin and tumors were harvested and processed for polyamine analysis as described in the Materials and Methods. *N* = 3–6 mice for each measurement.

K14-MEK tumors, which have high levels of ODC, contain large amounts of putrescine and spermidine, but have less spermine than skin (Table 1). Consistent with the induced expression of AZ from the K6 promoter, putrescine levels were reduced significantly in MEK/K6-AZ tumors but not in tumors from MEK/K5-AZ mice (Table 1). Again, since polyamines are measured in whole tumor extracts, these numbers do not necessarily reflect localized polyamine changes brought about by the expression of AZ from the K5 promoter, which would be more restricted in tumors than K6-AZ expression. Spermidine and spermine levels were not changed significantly in either double transgenic line. The putrescine reduction seen in MEK/K6-AZ tumors is similar to results previously reported using DFMO to inhibit ODC in several mouse models of skin tumor formation (12 , 13 , 15).

Measurement of apoptosis in tumor samples

Tumor sections from both MEK and MEK/AZ mice were analyzed by TUNEL analysis to determine the extent of apoptosis (Figure 5). Results are shown for ICR/B6 mice and were similar for ICR/D2 mice. The rapidly growing K14-MEK tumors showed very little evidence of apoptosis (Figure 5A). No increase in apoptosis was observed in MEK/AZ tumors, despite their very small size, compared with mice expressing the MEK transgene alone (Figure 5B, C). Many of the TUNEL-positive cells in the MEK/AZ tumors appeared at the edge of the sections, suggesting the possibility of randomly occurring DNA fragmentation due to necrosis or the normal process of differentiation-dependent molecular breakdown (35). Immunohistochemical analysis of cleaved caspase 3 and western blot analysis of PARP cleavage from MEK and MEK/AZ tumors showed no difference among the groups examined (data not shown), also suggesting that MEK/AZ tumors do not undergo apoptosis at an increased rate compared with MEK tumors.

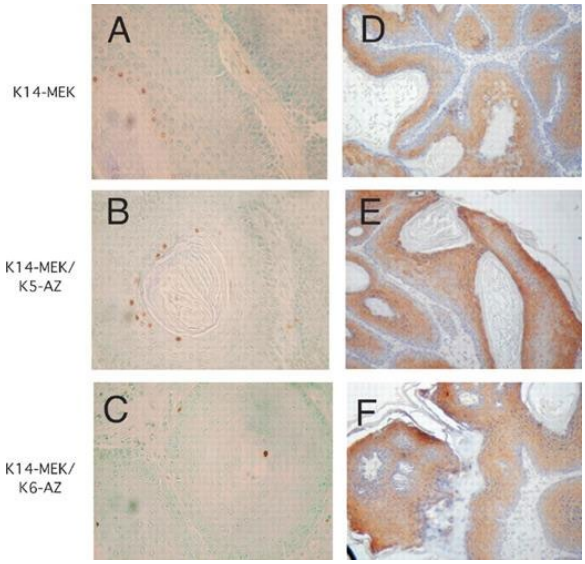

Fig. 5. Apoptosis and differentiation in tumors from K14-MEK, MEK/K5-AZ and MEK/K6-AZ mice on the ICR/B6 background. Apoptotic cells in K14-MEK tumors (**A**), MEK/K5-AZ tumors (**B**) and MEK/K6-AZ tumors (**C**) were visualized by TUNEL assay as described in the Materials and Methods. Results are representative of multiple tumors taken from at least three separate mice in each group. Very little apoptosis was detected in K14-MEK tumors, and increases in apoptosis were not seen with expression of the AZ transgene in combination with MEK. Keratin 1 expression was analyzed in tumors from the same groups (**D** , **E** , **F**) as a measure of differentiation.

Analysis of tumor differentiation

Previous results have shown that spontaneous tumors from K14-MEK mice on the ICR background were well-differentiated papillomas, as measured by keratin 1 protein expression. Similar results were seen here for K14-MEK mice on both the ICR/D2 (data not shown) and ICR/B6 backgrounds when analyzed at 8 weeks (Figure 5D). Mice expressing the MEK transgene alone typically must be killed at this age due to large tumor burdens, especially on the ICR/D2 background. Expression of AZ from either the K5 or K6 promoter did not affect the expression pattern of keratin 1 when analyzed on either background (Figure 5E, F). Western blot analysis confirmed that keratin 1 levels were not different in MEK and MEK/AZ tumors (data not shown). For these measurements double transgenic mice were typically killed at 13–15 weeks.

Discussion

Our previous studies have shown that ODC is highly induced in tumors from K14-MEK mice, and ODC activity and high putrescine are necessary for MEK-induced skin carcinogenesis (15). In the experiments described here, we have inhibited ODC in the K14-MEK mice by crossing them with both K5-AZ and K6-AZ mice. K14-MEK mice on the ICR background were crossed with K5-AZ and K6-AZ mice on both the carcinogenesis-resistant C57BL/6 background and the sensitive DBA/2 background. Combining expression of MEK and AZ results in a striking decrease in both tumor incidence and multiplicity compared with MEK expression alone. MEK/AZ mice not only form fewer tumors than K14-MEK mice, but tumors that form in MEK/AZ mice are also of dramatically reduced size. Although all mice expressing AZ exhibited reduced tumorigenesis, the effect was most remarkable in the MEK/K6-AZ mice from the ICR/D2 F1 cross, where double transgenic mice averaged less than one tumor per mouse for more than 8 weeks, while K14-MEK mice averaged over 13 tumors per mouse at this age.

The tumor response induced by the K14-MEK transgene was much more pronounced in mice resulting from an ICR/D2 F1 cross than from an ICR/B6 cross. Although it is difficult to draw conclusions concerning strain effect on MEK-induced tumor development using mice on mixed backgrounds, the response is similar to the DMBA/TPA sensitivity of the DBA/2 and C57BL/6 strains, and suggests common modulators of MEK-induced tumor development and Ras-induced tumor development in the DMBA/TPA model (36). Multiple genetic loci are known to affect the susceptibility of mice to skin tumor formation (36 , 37), and genes that may modify polyamine-dependent susceptibility to skin tumorigenesis are beginning to be explored (38). The AZ-overexpressing mice may therefore be useful in future experiments designed to answer this question.

AZ expression does not completely prevent tumorigenesis in K14-MEK mice, and tumors do develop over time in MEK/AZ mice, although far fewer and much smaller tumors were observed. ODC activity is 50–60% lower in double transgenic tumors, suggesting that the rate of tumor growth can be correlated with the level of ODC. It has been shown very recently using *Odc*$^{+/-}$ mice that a modest reduction in ODC activity can lead to a marked resistance to tumor development (39). Most of the reduction in ODC activity in MEK/K6-AZ tumors can be accounted for by AZ expression in the suprabasal cell layer. Putrescine is decreased in MEK/K6-AZ tumors, reflecting the higher AZ expression in the MEK/K6-AZ tumors. This result suggests that the primary role played by AZ in this system is to inhibit putrescine accumulation by either inhibiting ODC activity or stimulating ODC protein degradation. The correlation between putrescine content and tumor growth or inhibition is consistent with several other skin tumor models, including Ptch1 $^{+/-}$ /AZ mice (20), our results in K14-MEK mice using DFMO (15), and results in either K5-ODC mice (12) or ODC/Ras double transgenic mice (13) treated with DFMO. Conversely, large increases in putrescine were observed in tumors from mice overexpressing the polyamine catabolic enzyme spermidine/spermine N ^{1} -acetyltransferase (SSAT) driven by the K6 promoter (40). Interestingly, an unusually high number of tumors rapidly progressed to carcinomas in K6-SSAT mice on the C57BL/6 background (40).

AZ staining in the proliferating basal layer of MEK/K5-AZ tumors as seen by immunohistochemistry is of very low intensity. However, ODC activity is reduced by about 50% in all MEK/K5-AZ tumors, suggesting enough AZ is present in these tumors to inhibit ODC. Although induction of K6-AZ expression caused by hyperproliferation of the tumor cells in MEK/K6-AZ tumors is sufficient to reduce overall putrescine levels, the more localized expression of the K5-AZ transgene in tumors results in an absence of measurable changes in putrescine in MEK/K5-AZ whole tumor extracts.

In spite of the obvious difference in tumor size between MEK and MEK/AZ mice, nuclear PCNA expression results suggest similar growth in all tumors at the time of sacrifice. Quantitation of tumor BrdU staining of cells in S-phase revealed a 15–20% difference between MEK and MEK/AZ tumors. This is accompanied by a 40% decrease in mitotic cells in MEK/K5-AZ tumors and more than a 60% decrease in MEK/K6-AZ tumors. These results suggest that AZ inhibits tumor growth primarily by prolonging the transit time between G_2 and M phases of the cell cycle. ODC activity is known to behave in a biphasic manner in the cell cycle, with one peak in late G_1 and a second, higher peak in G_2 (41 – 44). The constitutive AZ expression in these transgenic lines would presumably inhibit both these peaks of activity. The results presented here therefore suggest that inhibition of ODC in G_1 has a moderate effect on the G_1/S transition, while blocking ODC in G_2 has a greater effect on mitosis.

Recent results have implicated AZ in the proteasomal degradation of other proteins, including Smad 1 (45) and cyclin D1 (46). While the results reported here do not rule out the possibility that the delay in cell cycle progression seen in MEK/AZ tumors may be a combination of polyamine depletion and changes in cyclin D1 activity, we did not detect any difference in cyclin D1 expression in MEK and MEK/AZ skin or tumors in this model (results not shown). In addition, the effect of AZ was more pronounced at G_2/M rather than G_1/S, suggesting that it is not related to alterations in cyclin D1 levels or activity, which peak in G_1 (44).

In contrast to our results with DFMO-treated K14-MEK mice (15), MEK/AZ tumors did not show strong evidence of apoptosis, suggesting that the primary effect of AZ is on cell growth in this model. The previous DFMO experiments analyzed apoptosis in established tumors, while the current experiments measure apoptosis rates in developing tumors. DFMO has also been reported to reduce tumor vascularization (13), which may lead to increased rates of apoptosis due to effects that are not tumor-cell autonomous. Overexpression of AZ from the K5 promoter was found to protect zinc-deficient mice from forestomach carcinogenesis brought about by exposure to N-nitrosomethylbenzylamine both by inhibiting uncontrolled cell proliferation and stimulating apoptosis (47). The distinct downstream pathways activated by chemical initiation versus expression of a specific gene such as MEK are likely to contribute to the differing effects of AZ in these models. Tissue specific responses to oncogenic stimuli are also likely to influence these results, and it has been suggested that the primary protective response of keratinocytes may be a program of differentiation rather than apoptosis (48).

The tumors in K14-MEK mice appear as well-differentiated papillomas, and the necessity to sacrifice the K14-MEK mice at relatively young ages due to large tumor burden leaves in question the effect of AZ expression on progression to carcinomas. Backcrosses of K14-MEK mice onto the FVB strain, which are more susceptible to the development of carcinomas (38), will perhaps provide insight into whether AZ can act to inhibit carcinoma formation in a manner similar to that already seen for DFMO (13 , 49 , 50). This will also facilitate additional genetic studies of the pathways that influence MEK-induced carcinogenesis through the combination of the K14-MEK transgene with other transgenic and knockout models.

In summary, the results obtained from these experiments suggest strongly that skin tumorigenesis in response to Ras activation requires an increase in polyamine metabolism, and AZ can act as a tumor suppressor in this model. The results suggest that AZ expression inhibits tumor growth by reducing ODC activity and tumor putrescine accumulation. The inhibition of tumor proliferation appears to be mediated by a mechanism involving a moderate increase in G_1 and a greater increase in G_2 transit times, without inducing apoptosis.

These double transgenic MEK/AZ mice thus provide an excellent model in which to identify growth-related genes and proteins downstream of Ras that are altered in the presence of AZ.

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