Inhibition of Ornithine Decarboxylase (ODC) Decreases Tumor Vascularization and Reverses Spontaneous Tumors in ODC/Ras Transgenic Mice

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ABSTRACT

We have shown that ornithine decarboxylase (ODC) overexpression in the skin of TG.AC v-Ha-ras transgenic mice induces the formation of spontaneous skin carcinomas. Treatment of ODC/Ras double transgenic mice with α-difluoromethylornithine (DFMO), a specific inhibitor of ODC enzyme activity, causes a rapid regression of these spontaneous tumors. DFMO treatment led to dramatic decreases in ODC activity and putrescine levels, but v-Ha-ras expression was not affected in the regressed tumors. Moreover, cyclin D1 continued to be strongly expressed in the basal epithelial cells of regressed tumors, and there was no decrease in the proliferative index of these same tumor cells. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling analyses revealed increased DNA fragmentation in DFMO regressed tumors compared with similarly sized spontaneous tumors from ODC/Ras transgenic mice not treated with DFMO. Moreover, the blood vessel count was significantly decreased in regressed tumors within the first four days of DFMO treatment. The decreased vasculature in DFMO regressed tumors was not attributable to altered expression of murine vascular endothelial growth factor (VEGF) isoforms. Elevated levels of ODC activity in the skin of K6/ODC transgenic mice increased the dermal vascularization compared with that in nontransgenic normal littermates. Our results suggest that ODC stimulates an angiogenic factor(s) other than VEGF and/or may play a key role in a cell survival effector pathway of Ras that is independent of a Ras-induced proliferation pathway.

INTRODUCTION

The polyamines have long been known to be associated with rapid cell proliferation in both normal and neoplastic cells and tissue (1, 2). ODC is the initial rate-limiting enzyme involved in the biosynthesis of polyamines and is responsible for converting L-ornithine to putrescine. The polyamines putrescine, spermidine, and spermine are some of the major cations present in cells, and they are essential for normal cell growth and differentiation. ODC is aberrantly regulated in tumor cells and results in high basal levels of polyamines in many epithelial tumors (3–7). In large part, this results from the up-regulation of ODC expression by oncogenes such as c-myc (8–10), v-src (11), v-raf (12), or an activated Ras or RhoA protein (12, 13). Indeed, the mutation of c-Ha-ras is an early genetic change in mouse skin initiated with the carcinogen DMBA (14). Although mutations in the ras gene occur in 30% of all human tumors (15), aberrant Ras signaling probably contributes even more to the development of human tumors because mutations in other genes can also lead to chronic up-regulation of the Ras pathway without any mutations in ras (16–18).

To study the effect of elevated levels of ODC and polyamines and a mutant ras gene in mouse skin tumorigenesis, we generated double transgenic mice by breeding K6/ODC transgenic mice with TG.AC v-Ha-ras transgenic mice (19). A keratin 6 promoter drives the ODC transgene in K6/ODC transgenic mice, which results in elevated ODC/polyamine levels directed to the outer root sheath of hair follicles (20). TG.AC transgenic mice carry a v-Ha-ras transgene while still retaining two normal c-Ha-ras alleles (21, 22). Transgenic mice that possess only the K6/ODC or the v-Ha-ras transgene do not develop skin tumors unless treated with either a carcinogen or tumor promoter, respectively (23–25). However, we found that ODC overexpression, targeted to hair follicles in conjunction with an activated Ras protein, are sufficient to produce spontaneous skin carcinomas in ODC/Ras double transgenic mice (19).

The ODC/Ras transgenic mouse offers several advantages as a model for studying epithelial tumorigenesis, including the ability to focus on the effects of aberrant expression of only two genes (Ras and ODC) without any complicating effects of chemical carcinogens or tumor promoters on other genes. Another advantage is the that the K6/ODC transgene expression can be manipulated with the highly specific ODC inhibitor, DFMO (26). DFMO has been shown to inhibit the development of skin tumors in carcinogen-treated mice when it is given during the promotion phase (27, 28), and it is effective in treating glioblastomas when combined with 1,3-bis(2-chloroethyl)-1-nitrosourea (29). Currently, DFMO is being used with moderate success in cancer chemopreventive trials with patient populations at high risk for the development of colon, esophageal, breast, skin, and prostate cancer (30).

We report here that treatment of ODC/Ras transgenic mice with 1% DFMO in the drinking water not only prevents the formation of spontaneous skin tumors but also causes the rapid regression of tumors that are allowed to spontaneously develop. Surprisingly, we found that the proliferation index of the tumor epithelial cells in regressed ODC/Ras tumors was not affected by DFMO. Our results suggest that polyamines play an important role in the survival of epidermal tumors, perhaps by controlling essential angiogenic factors.

MATERIALS AND METHODS

Animals. ODC/Ras double transgenic mice were generated by crossing K6/ODC transgenic mice on a C57BL/6 background with TG.AC transgenic mice homozygous for the v-Ha-ras transgene and on a FVB/N background, as described previously (19). Use of animals was approved by the Institutional Animal Care and Use Committee of the Lankenau Institute for Medical Research. Skin tumors spontaneously developed in ODC/Ras transgenic mice hemizygous for both the ODC and the v-Ha-ras transgenes 6–10 weeks after birth. Tumors >1 mm in size were counted and measured with calipers at least twice a week. To delay the formation of spontaneous tumors, 4-week-old mice were administered DFMO (Ilex Oncology, Inc., San Antonio, TX) at 1% (w/v) in the drinking water for 5 weeks. For tumor regression studies, mice with tumors were administered 1% DFMO in the drinking water for up to 6 weeks. Two h before sacrifice, all mice were injected i.p. with bromodeoxyuridine (Sigma, St. Louis, MO) at a dose of 100 μg/kg body weight.

Processing of Tissues. A portion of each tumor was processed for histology, and the remainder was snap frozen in liquid nitrogen. To circumvent problems arising from tumor heterogeneity, frozen tumor tissue was ground to a fine powder and used for subsequent RNA, protein, and polyamine analyses. For ODC enzyme activity, a portion of the ground tissue was lysed in 25 mM

Received 3/23/00; accepted 8/25/00.

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2 Supported in part by Grant CA70739 from the National Cancer Institute.

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The abbreviations used are: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; TdT, terminal deoxynucleotidyl transferase; DMBA, 7,12-dimethylbenz[a]anthracene; RT-PCR, reverse transcription-PCR; HPRT, hypoxanthine phosphoribosyltransferase; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor.

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Tris-HCl (pH 7.5), 2.5 mM DTT, 0.1 mM EDTA, and protease inhibitors. Homogenates were assayed for ODC enzyme activity by quantifying the production of $^{14}$CO$_2$ from l-[$^{14}$C]ornithine (31). A portion of ground tissue was also lysed in 0.2 N perchloric acid for measurement of polyamine levels. Putrescine, spermidine, and spermine levels were determined by dansylation and separation on a reversed phase C$_18$ high-performance liquid chromatography column (32).

**Protein Analyses.** For immunoblots, tissues were homogenized in RIPA buffer [50 mM Tris-HCl (pH 7.5), 1% NP40, 0.25% sodium deoxycholate, 0.25% SDS, 150 mM NaCl, and 1 mM EGTA] containing 1 $\mu$g/ml each of aprotinin, leupeptin, and pepstatin, 1 mM NaF, 1 mM sodium orthovanadate, and 1 mM Pefabloc by passing through a syringe needle after a 30-min incubation on ice. Tissue lysates were clarified by centrifugation, and protein content was determined by Lowry assay. Equal amounts of protein were separated by 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). To assess for equal protein loading and transfer to the membranes, the protein on the membranes was stained with Ponceau S solution (Sigma Chemical Co., St. Louis, MO). Immunoblots were incubated for 1 h at room temperature in blocking solution (PBS with 10% milk and 0.05% Tween 20), followed by the primary antibody diluted in blocking solution containing 0.1% milk for 1–2 h. Blots were probed with a polyclonal anti-keratin 1 or loricrin antibody (Covance, Richmond, CA) and a monoclonal anti-β-actin antibody (Sigma). The immunoblots were developed with a horseradish peroxidase-conjugated secondary antibody, followed by detection using enhanced chemiluminescence according to the manufacturer’s directions (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

**Immunohistochemistry/in Situ Hybridization.** Tissues were fixed in either Fekete’s solution (60% ethanol, 3.2% formaldehyde, and 0.75 M acetic acid) or in 4% p-formaldehyde in PBS overnight and then embedded in paraffin. Sections were deparaffinized, hydrated, and incubated with primary antibody. Slides were then incubated with the appropriate biotinylated secondary antibody, followed by an incubation with an avidin and biotinylated peroxidase complex (Vectorstain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA), all at room temperature. Immunoreactive cells were localized by incubating the sections with a chromagen solution containing diaminobenzidine and H$_2$O$_2$ and then counterstaining with hematoxylin. Cyclin D1 expression was detected using a rabbit polyclonal anti-cyclin D1 antibody (Upstate Biotechnology, Lake Placid, NY). Keratin 1 and loricrin expression was detected using rabbit polyclonal antibodies obtained from Covance (Richmond, CA). BrdUrd incorporated in cells undergoing DNA synthesis was detected in tumor sections using a rat monoclonal anti-BrdUrd antibody (Zymed Laboratories, San Francisco, CA). The proliferative index was determined by multiplying the number of BrdUrd-positive cells/500 tumor epithelial cells in the basal layer by 100. To visualize the vasculature of tumors, endothelial cells were stained with a monoclonal antimouse CD31 (NeoMar) antibody. Slides were then incubated with the appropriate biotinylated second antibody. Immunoreactive cells were localized by incubating the sections with a chromagen solution containing diaminobenzidine and H$_2$O$_2$, and a monoclonal anti-keratin 1 or loricrin antibody (Covance, Richmond, CA). Shown by incubating the sections with a chromagen solution containing diaminobenzidine and H$_2$O$_2$, and a monoclonal anti-keratin 1 or loricrin antibody (Covance, Richmond, CA). Shown by incubating the sections with a chromagen solution containing diaminobenzidine and H$_2$O$_2$, and a monoclonal anti-keratin 1 or loricrin antibody (Covance, Richmond, CA). The proliferative index was determined by multiplying the number of BrdUrd-positive cells/500 tumor epithelial cells in the basal layer by 100.

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**Apoptosis Analysis.** Freshly sectioned tissue was deparaffinized with xylene, and apoptotic cells were detected using the ApoTACS TdT-based TUNEL assay ( Trevigen, Gaithersburg, MD), following the manufacturer’s instructions. Briefly, tumor sections were deparaffinized with xylene and then rehydrated and permeabilized with proteinase K in PBS for 30 min. After washing the sections, DNA fragments in apoptotic cells were end-labeled with biotinylated nucleotides using a TdT/horseradish peroxydase reaction in conjuction with an antibody detection system targeting nucleotides incorporated onto the 3’-OH ends of the DNA fragments. DNA fragmentation was detected by staining with diaminobenzidine, and cells were counterstained with methyl green. Positive control tissue sections were prepared by nicking DNA with DNase I. In negative control tissue sections, deionized water was substituted for TdT in the working solution. Apoptotic cells were identified on the basis of a combination of positive staining and morphological criteria as described by Kerr et al. (35). The apoptotic index was determined by dividing the number of apoptotic cells by the total number of cells in the tumor and multiplying by 100.

**RESULTS**

We have shown previously that transgenic mice expressing both the K6/ODC and v-Ha-ras transgenes (ODC/Ras transgenic mice) develop spontaneous skin tumors early in life. The development of these tumors is dependent upon elevated levels of ODC enzyme activity because tumor formation can be blocked if mice are administered 1% DFMO in their drinking water (19, 25, 37). No skin tumors developed in littersmates that were hemizygous for the v-Ha-ras transgene but lacked the ODC transgene. Removal of the DFMO from the drinking water resulted in formation of spontaneous tumors in ODC/Ras mice approximately 4–7 weeks later (Fig. 1). The rate of tumor incidence is similar, regardless of whether tumor formation was delayed with prior DFMO treatment, thus reflecting that spontaneous tumors are initially observed in ODC/Ras transgenic mice within a very narrow window of time. On average, fewer skin tumors developed in mice that had been treated previously with DFMO for 5 weeks (mean, 5.5 tumors/mouse) compared with the number of tumors that developed in mice never treated with DFMO (mean, 12 tumors/mouse).

DFMO treatment of ODC/Ras transgenic mice caused a rapid regression of the tumors that had developed spontaneously (Fig. 2). Tumors >1 cm$^2$ almost completely regressed within 2–4 weeks of DFMO treatment, with no apparent toxicity to the animals. Table 1 shows that most tumors regressed quite dramatically in the first week or two after administration of DFMO. Interestingly, histological examination revealed that the tumor epithelial cells that remained in the regressing tumors never completely disappeared with DFMO treatment. Despite almost complete visible regression of the tumors after 6 weeks of DFMO treatment, small clusters of hyperplastic growths of epithelial cells were still present upon histological evaluation of
tumor cells of regressed tumors that expressed v-Ha-
was still detected in the remaining basal epithelial cells and some suprabasal cells of all DFMO-regressing tumors after 1 month of DFMO treatment. These data imply that the major contribution of elevated levels of ODC/polyamines in cooperating with a mutated ras to produce tumors is some process other than increased tumor cell proliferation or sustained cyclin D1 expression. Because there was no effect on the proliferative index of basal epithelial cells in the DFMO regressed tumors, increased cell death, through either...
terminal differentiation or apoptosis, must account for the decrease in tumor mass after inhibition of ODC enzyme activity.

Immunohistochemical staining of tumors detected no increased expression of differentiation markers such as keratin 1 or loricrin in regressing tumors as compared with tumors from mice not treated with DFMO (Fig. 4a). Immunoblot analyses revealed that keratin 1 expression is elevated in the epidermis of K6/ODC transgenic mice (Fig. 4b). Expression of keratin 1 appears to be dependent upon polyamine levels because 4 days of DFMO treatment lowered the level of keratin 1 protein in the epidermis of both K6/ODC transgenic mice and their normal littermates (Fig. 4b). Although an activated Ha-ras has been shown to block the expression of keratin 1 in epidermal cells and tumors (46, 47), some keratin 1 protein was still expressed in the spontaneous ODC/Ras tumors (Fig. 4). Interestingly, inhibiting ODC enzyme activity via treatment with DFMO resulted in less keratin 1 expression in the regressed tumors (Fig. 4). In addition,

<table>
<thead>
<tr>
<th>Days of DFMO</th>
<th>% BrdUrd-positive tumor cells</th>
<th>Apoptotic index (%)</th>
<th>Tumor size (&lt;80 mm²)</th>
<th>Tumor size (&lt;80 mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>49 ± 2.1</td>
<td>2.53 ± 1.5</td>
<td>0.96 ± 0.73</td>
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<tr>
<td>1</td>
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<td>6.60</td>
<td>2.85 ± 1.23</td>
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<tr>
<td>4</td>
<td>47 ± 7.5</td>
<td>3.75</td>
<td>2.73 ± 0.73</td>
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<tr>
<td>12</td>
<td>45 ± 3.8</td>
<td>6.47</td>
<td>3.72 ± 0.70</td>
<td></td>
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<tr>
<td>42</td>
<td>54 ± 1.5</td>
<td>4.72</td>
<td>3.54</td>
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* Tumor-bearing ODC/Ras double transgenic mice were administered 1% DFMO in their drinking water and sacrificed after 0, 1, 4, 12, and 42 days of DFMO treatment. Two h before sacrifice, mice received injections of BrdUrd at a dose of 100 μg/g body weight. After detection using an anti-BrdUrd antibody, tumor cells with BrdUrd-positive nuclei were identified as BrdUrd-positive cells. BrdUrd-positive cells/500 cells in the basal layer were counted in multiple sections of each tumor. The percentage of BrdUrd-positive tumor cells as the proliferative index for each group was expressed as the mean ± SD.

* Apoptotic cells were detected using the ApoTACS terminal deoxynucleotidyl transferase-based TUNEL assay. For each sample, a total of 1500–2500 cells in three to five areas were counted at ×10 (eyepiece) and ×40 (objective). The apoptotic index for each sample was determined as the average value from five areas. The apoptotic index is expressed as the mean ± SD of determinations on multiple specimens.
observed increased vasculature in the dermis of K6/ODC skin compared with dermis from nontransgenic littermates (Table 4). These results suggest that elevated ODC enzyme activity contributes to the survival of these epidermal tumors by up-regulating essential angiogenic factors.

Because VEGF is known to be a potent mediator of vasculogenic and angiogenic events associated with tumor growth (48–52), we looked at the effect of DFMO inhibition on VEGF expression in the regressed tumors. Similar to the human VEGF gene, the mouse gene for VEGF has been shown to encode three alternatively spliced forms of VEGF (VEGF_{188}, VEGF_{164}, and VEGF_{120}) but no isoform corresponding to the human VEGF_{206} (53). RT-PCR analysis of mRNA isolated from ODC/Ras tumors from transgenic mice not treated with DFMO, or from regressed tumors from mice treated 1, 4, or 12 days with DFMO, revealed no change in the expression level of VEGF_{120}-VEGF_{164} or VEGF_{188} (Fig. 6). Moreover, Western analysis showed no increased expression of VEGF protein in K6/ODC transgenic skin compared with normal littermate skin, despite the increased vasculature in K6/ODC skin (data not shown). However, VEGF protein remained elevated in spontaneous tumors from ODC/Ras transgenic mice. VEGF mRNA up-regulation has been shown to correlate with activated Ha-ras levels (54), and our results showing no change in the level of VEGF isoforms in regressed ODC/Ras tumors reflects that v-Ha-ras expression is not affected in the DFMO regressed tumors. Thus, our findings suggest that the decreased vasculature in the regressed tumors is attributable to altered activity of an essential angiogenic factor(s) other than VEGF.

### Table 4 Effect of ODC overexpression on the vasculature in transgenic mouse skin and tumors

<table>
<thead>
<tr>
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<th>Vessel count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td></td>
</tr>
<tr>
<td>Normal littermates</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>K6/ODC transgenic mice</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>ODC/Ras tumors</td>
<td></td>
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<tr>
<td>Days DFMO treatment</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>1</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>3.7 ± 1.3</td>
</tr>
</tbody>
</table>

Tissue sections from K6/ODC transgenic mouse skin and normal, nontransgenic littermates and also ODC/Ras tumors after 0, 1, or 4 days of DFMO treatment were stained with anti-CD31 (PECAM-1) to visualize blood vessels. Vessels were counted in five fields at ×10 (eyepiece) and ×10 (objective). The vessel count for each sample was determined as the average number of vessels measured in five areas. Values are expressed as the mean ± SD of determinations on multiple specimens. For each group, the vessel count of ODC/Ras tumors after 12 or 42 days of DFMO treatment was not quantitated because of lack of sufficient tumor tissue in the regressed tumors.
DISCUSSION

More than 90% of skin tumors induced by the standard DMBA and TPA tumorigenesis protocol possess a mutation at codon 61 of the Ha-ras gene (14, 55), resulting in the production of a constitutively activated Ras protein. Although a mutated Ha-ras oncogene plays an important role in the initiation step of mouse skin carcinogenesis (14, 55, 56), our data indicate that tumor development and progression to a malignant phenotype are also dependent upon elevated levels of ODC and polyamines. In the ODC/Ras transgenic mouse model, an activated ras is still expressed in regressed tumors after inhibition of tumor ODC enzyme activity by DFMO. Although DFMO treatment results in a dramatic reduction in tumor size, the continued expression of v-Ha-ras presumably permits the observed continued proliferation and up-regulation of cyclin D1 in epithelial tumor cells (42–45).

These seemingly paradoxical results of tumor regression despite continued proliferation were initially surprising in light of the strong association of ODC activity induction with proliferating cells and tissue (1, 2, 57, 58). Moreover, we have demonstrated previously that sustained elevated levels of ODC and polyamines stimulate proliferation in the skin of K6/ODC transgenic mice compared with that in normal littermate skin (59). Indeed, our results show no inhibitory effect of DFMO on DNA synthesis in regressing ODC/Ras tumors differ from those reported by O’Brien et al. (37), who examined the effect of DFMO on DMBA-initiated tumors in K6/ODC transgenic mice and found a dramatic decrease in tumor cell proliferation in regressed tumors. Because these were carcinogen-induced tumors, it is important to note that carcinogens, including DMBA, target many genes other than Ha-ras, which may exert different effects on cell cycle progression (60). Moreover, there may be a ras dosage effect because ODC/Ras transgenic mice carry a v-Ha-ras transgene, having point mutations in codons 12 and 59 in addition to two normal c-Ha-ras alleles. Other factors contributing to differences in DFMO-induced regression of ODC/Ras tumors and DMBA-initiated K6/ODC tumors may lie in the different etiology of the tumors or in the genetic background of the mice. In fact, all of the spontaneous tumors that develop in ODC/Ras double transgenic mice are keratoacanthomas, unlike the squamous papillomas that form in the DMBA-initiated K6/ODC transgenic mice, which may reflect the FVB/N genetic background in the ODC/Ras double transgenic mice. In contrast to its human counterpart, keratoacanthomas in mice often convert to squamous carcinomas and usually do not regress (61). Thus, it is significant that these aggressive keratoacanthomas in ODC/Ras mice regress so quickly with DFMO treatment. However, our results illustrate that elevated levels of polyamines not only affect the proliferation of tumor cells but also promote tumorigenesis and the survival of tumors through downstream effectors that control pathways other than proliferation.

Because DFMO-induced regression of spontaneous tumors in the ODC/Ras transgenic mice does not involve altered expression of v-Ha-ras or changes in the proliferative index of the remaining epithelial tumor cells, then ODC and polyamines must regulate other pathways leading to either terminal differentiation or apoptosis that would yield a net decrease in a proliferating population of tumor cells. One possible explanation for the regression of ODC/Ras tumors promoted by DFMO treatment is that DFMO may block one of the effector pathways of activated Ha-Ras (62) that determines cell survival. For instance, it has been reported that the phosphatidylinositol 3-kinase pathway, acting through PKB/Akt, mediates the aberrant survival of Ras-transformed epithelial cells in the absence of attachment (63). If polyamines play a key role in this survival pathway, then it would be expected that DFMO treatment would act to abrogate this protection against apoptosis, thus resulting in tumor regression.

Our data suggest that differentiation of tumor epithelial cells is not accelerated in the regressing tumors because there is no increase in the expression of genes normally associated with the terminal differentiation program of keratinocytes. However, there is a positive correlation of DFMO-induced regression of ODC/Ras tumors with decreased vascularization and increased apoptosis of both tumor epithelial and stromal cells. It remains to be determined what essential survival factors and/or angiogenic factors are regulated by polyamines that play a key role in the maintenance of these tumors. This pattern of increased apoptosis and decreased tumor vascularization in regressing tumors is similar to that observed upon treatment of primary tumors with angiogenesis inhibitors such as angiostatin and endostatin (64, 65). Indeed, angiogenesis is essential for the growth and persistence of solid tumors (66–68), and it is possible that DFMO inhibition of ODC activity and the resultant decreased vascularization of tumors leads to a critical reduction of paracrine factors essential for the survival of tumor cells. Accumulating reports indicate that polyamines play an important role in tumor neovascularization, DFMO has been found to inhibit B16 melanoma-induced angiogenesis in chick embryo chorioallantoic membranes (69) and to affect the first step in the metastatic cascade, intravasation (70). In addition, ODC-transformed NIH3T3 cells produce well-vascularized, rapidly growing fibrosarcomas (71, 72).

Neovascularization is the result of the net balance between positive and negative regulators of this process. Not only do tumors up-regulate the production of a variety of angiogenic factors such as VEGF, acidic FGF, and basic FGF (54, 73), they also generate inhibitors of angiogenesis including angiostatin (64), thrombospondin (74), and endostatin (65). Expression of well-known angiogenic factors such as VEGF, basic and acidic FGFs, or matrix metalloproteinases has not been found to be stimulated by elevated levels of ODC (71). VEGF is a potent angiogenic factor that is unique in that it acts specifically on vascular endothelial cells (75, 76), and different VEGF isoforms have been reported to be selectively expressed in tumors and various tissues of TG.AC transgenic mice, thus suggesting different functions for these various VEGF isoforms (36). However, we found that DFMO had no effect on the expression of murine VEGF isoforms in regressing ODC/Ras tumors. These findings are expected because they agree with published reports that describe the proliferative effects and the up-regulation of VEGF expression as important downstream effectors of an activated ras oncogene in contributing to tumor growth (54, 77). However, we have demonstrated that the proliferative effects of an activated Ha-ras and continued expression of VEGF are not sufficient to support the continued development and survival of these epidermal tumors.

The effect of ODC on the vascularization of tumors is attributable, at least in part, to the production of a yet unidentified angiogenic factor(s). Indeed, our preliminary studies have shown that primary cultures of ODC-overexpressing keratinocytes from K6/ODC transgenic skin attract significantly more endothelial cells to migrate across a Matrigel-coated filter than do normal primary keratinocytes when normal or K6/ODC keratinocytes are cultured in serum-free medium. Because polyamines added to the culture medium of normal keratinocytes are not sufficient to increase the migration of endothelial cells, it is likely that ODC-overexpressing keratinocytes produce a factor that stimulates the migration of endothelial cells. These findings agree with previous reports that conditioned medium from ODC-overexpressing fibroblasts promotes the migration of bovine capillary endothelial cells in collagen gels and increases the growth rate of these endothelial cells in vitro (71). Although the identity of ODC-induced angiogenic factor(s) that are responsible for tumor vascularization remains elusive, both the angiogenesis-inhibiting factors, thrombospondin-1 and -2, were found to be down-regulated in ODC-
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