Haploinsufficiency for Odc Modifies Mouse Skin Tumor Susceptibility

Yongjun Guo, John L. Cleveland, and Thomas G. O’Brien

Abstract
Numerous studies have linked overexpression of ornithine decarboxylase (Odc) gene with enhanced susceptibility to mouse skin tumorigenesis. However, there is little experimental evidence suggesting that modest reductions in Odc expression might reduce tumor susceptibility. To address this issue, here we report the use of the Odc<sup>-/-</sup> haploinsufficiency model, in which one copy of the murine Odc gene has been inactivated by a homologous recombination. Compared with model, in which one copy of the murine Odc substantially fewer skin papillomas developed in mice subjected to a two-stage initiation-promotion protocol, Odc<sup>-/-</sup> epidermis was diminished in treatment, the characteristic hyperplastic response of the enzyme activity and polyamine accumulation following treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Furthermore, following chronic TPA treatment, the characteristic hyperplastic response of the epidermis was diminished in Odc<sup>-/-</sup> mice. Finally, when subjected to a two-stage initiation-promotion protocol, substantially fewer skin papillomas developed in Odc<sup>-/-</sup> mice compared with wild-type littersmates. These results support the concept that differences in tissue polyamine levels, resulting from either overexpression or reductions in ODC, are important modifiers of tumor susceptibility. (Cancer Res 2005; 65(4): 1146-9)

Introduction
Expression of the ornithine decarboxylase (Odc) gene, which encodes the rate-limiting enzyme of polyamine biosynthesis, has a powerful modifying influence on the susceptibility of murine skin to tumor development. First, most, if not all, tumor-promoting agents induce transient increases in Odc expression in epidermis (1) and the magnitude of Odc induction correlates well with the potency of tumor promoters (2). Second, the highly specific inhibitor of ODC enzymatic activity, 2-difluoromethylornithine, is a very effective chemopreventative agent when given during skin tumorigenesis protocols (3, 4). Finally, targeted overexpression of an Odc transgene in skin greatly enhances the susceptibility of this tissue to carcinogenesis (5). The metabolic consequences of increased Odc expression are increases in intracellular polyamine pools, especially putrescine, the immediate product of the decarboxylation of ornithine. Evidence to date suggests that sustained elevations in cellular polyamine pools likely account for the increased susceptibility to tumorgenesis conferred by Odc overexpression. Indeed, increased catabolism of spermidine and spermine in skin, which leads to elevated putrescine levels, also enhances tumor susceptibility (6).

Based on previous studies, it is clear that large increases in Odc expression can increase susceptibility of mouse skin to tumor development. Conversely, it is also evident that complete inhibition of the very high levels of ODC enzymatic activity in Odc transgenic mice after treatment with high concentrations of the ODC-specific inhibitor difluoromethylornithine completely prevents tumor development following carcinogen treatment (5). However, between these two extremes there is little evidence that more subtle changes in Odc expression influence tumor development. With the recent development of the Odc<sup>-/-</sup> mouse model (7), one approach to this question would be to investigate the effect of Odc gene dosage on skin carcinogenesis. Odc<sup>-/-</sup> mice appear identical to Odc<sup>+/+</sup> littersmates, have no apparent abnormalities, and are fertile. Whereas targeted inactivation of one Odc allele has no obvious phenotypic effect, loss of function of both alleles results in very early lethality during embryonic development (7); therefore, there are no redundant pathways for putrescine biosynthesis in mice as there are in lower organisms (8). To determine if Odc haploinsufficiency has an impact on tumor development, we compared induced levels of ODC activity and polyamine levels as well as tumor yield in standard initiation-promotion experiments in wild-type Odc<sup>-/+</sup> versus Odc<sup>−/−</sup> mice. Our results establish surprisingly substantial effects of Odc haploinsufficiency on skin tumor susceptibility.

Materials and Methods

Animals and Tumorigenesis Experiments. Odc<sup>-/-</sup> mice on a mixed C57BL/6;129 strain background were back crossed repeatedly to C57BL/6 mice to produce a congenic strain. Odc genotypes were determined from tail DNAs by a PCR-based protocol (7). Mice used in experiments were between the fifth and eighth back cross generations. Skin tumorigenesis experiments were done by initiating 7-week-old mice of both sexes by topical application of either 400 or 800 nmol (two independent experiments) of 7,12-dimethylbenz(a)anthracene (DMBA) dissolved in 200 μl acetone followed, beginning 1 week later, by twice weekly application of 12-O-tetradecanoylphorbol-13-acetate (TPA). Tumors <2 mm were counted biweekly beginning 7 weeks after DMBA treatment. Differences in tumor multiplicity between Odc<sup>-/-</sup> and Odc<sup>−/−</sup> mice were analyzed statistically using the Wilcoxon rank sum test.

Biochemical Assays. To assess the effects of Odc gene dosage on ODC activity and polyamine levels, groups of two to three mice were treated once with 17 nmol TPA and sacrificed at several intervals up to 24 hours. The epidermis was separated from the dermis and epidermal extracts, prepared by homogenization in 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM sodium orthovanadate, and 1 × protease inhibitor cocktail, were centrifuged to remove nuclei and cell debris. ODC activity is expressed as units per milligram protein, where 1 unit of ODC activity corresponds to 1 nmol of C02 liberated per hour. Polyamine levels are expressed as nanomoles per milligram DNA.

Histology. Cohorts of wild-type and Odc<sup>-/-</sup> mice were treated with 17 nmol TPA for up to 4 weeks at 3- to 4-day intervals, whereas control mice were treated with acetone. After shaving the treated area, mice were euthanized and areas of skin from the mid dorsum fixed in Fekete's fixative, sectioned, and stained with hematoxylin-eosin or with a monoclonal antibody against keratin 14. The numbers of hair follicles in the epidermis were counted.
Results

The basal levels of ODC activity in mouse epidermis are extremely low, making comparisons of uninduced ODC activity in Odc+/+ versus Odc−/− mice technically challenging. We therefore addressed the effects of Odc gene dosage on ODC expression levels by examining the induced levels of ODC activity following TPA treatment. TPA is among the best inducers of ODC and was chosen as an inducing agent because it would be used in subsequent initiation-promotion experiments. Figure 1 illustrates the time course of induction of ODC enzyme activity in the epidermis of Odc+/+ and Odc−/− mice following a single treatment with 17 nmol TPA. The magnitude of the ODC induction was ~2- to 3-fold higher in Odc+/+ mice compared with Odc−/− mice at all time points studied. Therefore, targeted inactivation of the Odc gene produced by homologous recombination results in a loss-of-function Odc gene product (7), and Odc haploinsufficient mice express approximately half the levels of active enzyme.

To address the consequences of Odc haploinsufficiency on polyamine levels we determined TPA-induced changes in epidermal polyamine levels (Table 1). The basal levels of individual and total polyamines were not markedly different in Odc+/+ versus Odc−/− mice; however, at 24 hours after TPA treatment [when the maximal effect of TPA would be expected (10)] epidermal putrescine levels were almost 3-fold higher and spermidine levels 2-fold higher in Odc+/+ versus Odc−/− mice. Therefore, differences in ODC activity observed after TPA treatment of Odc+/+ versus Odc−/− mice is also reflected in differences in epidermal polyamine pools, which are the proximal effector molecules for downstream Odc-mediated phenotypic changes, including effects on tumor susceptibility (1-4).

The reduction in Odc gene dosage did not alter the histologic appearance of the skin or adnexa such as hair follicles or sebaceous glands in untreated mice. To assess whether Odc haploinsufficiency altered the tissue response to chronic TPA treatment, mice of both Odc genotypes were treated for various lengths of time (1–28 days) with twice weekly doses of 17 nmol TPA, a regimen typically used in a tumorigenesis protocol (11). No differences in histology of the skin of Odc+/+ versus Odc−/− mice were noted following acute exposure to TPA (1-7 days), but the typically pronounced hyperplastic response of the epidermis to chronic TPA exposure (2 weeks) was reduced in Odc−/− mice (Fig. 2). After 2 weeks of TPA treatment, the epidermis of wild-type mice was generally three to five nucleated cell layers thick, whereas that of Odc−/− mice was only two to three nucleated cell layers thick. The total thickness of the epidermis of wild-type mice was thus approximately double that observed in chronically treated Odc−/− mice. Another notable histologic difference following chronic exposure to TPA was the appearance of hair follicles: these were characteristically hyperplastic in wild-type mice but this response was diminished in Odc−/− mice. This difference in hair follicle proliferative response to chronic TPA treatment seems to be more important than the response of interfollicular epidermis because hair follicle keratinocytes are the likely progenitors for most of the papillomas that develop in the initiation-promotion model (12). Furthermore, after 4 weeks of TPA treatment, Odc+/+ mice maintained a hyperplastic response in both the interfollicular epidermis and in hair follicles, whereas these responses were essentially lacking in Odc−/− mice (data not shown).

To determine the effects of reduced Odc gene dosage on tumor susceptibility, we performed standard initiation-promotion experiments in skin. In one experiment, groups of mice were initiated with 800 nmol DMBA and promoted twice weekly with TPA for 23 weeks. Strikingly, Odc haploinsufficiency affected both the time of onset and the multiplicity of tumors in treated mice. Tumor multiplicity was 3-fold higher in Odc+/+ mice compared with Odc−/− mice (Fig. 3) and significantly more Odc−/− mice developed tumors, with a tumor incidence (percent tumor-bearing mice) of 77.8% for Odc+/+ mice versus 29.6% for Odc−/− mice. To confirm these findings, we repeated this experiment using a lower initiating dose of DMBA (400 nmol) and, again, a very similar result was obtained: tumor multiplicity in Odc+/+ mice was 1.72 tumors per mouse (with a tumor incidence of 83.3%), whereas in Odc−/− mice there were, on average, only 0.32 tumors per mouse (with a tumor incidence of 25.6%). All tumors that developed in both experiments were typical exophytic, well-differentiated, squamous papillomas. However, an obvious effect of Odc gene dosage was also noted in terms of their size, where, in general, tumors were larger in Odc+/+ mice than in Odc−/− mice.

Discussion

Numerous studies using a variety of pharmacologic (2, 3), transgenic (4, 13), and nutritional (14) approaches have showed that up-regulation of polyamine biosynthesis is associated with enhanced susceptibility to skin tumor development. We show here using a targeted gene inactivation approach that the converse is also true: limiting Odc expression reduces susceptibility to skin tumor development. Similar conclusions have been reached using high concentrations of the specific inhibitor 2-difluoromethylornithine given systemically throughout the promotion period (4), but these studies could be criticized on the grounds that ODC function in the skin is completely absent (see, e.g., data in ref. 9) Rather than completely suppress ODC activity, the haploinsufficiency model described herein results in ~50%
reduction of activity following induction by TPA. Despite this rather modest effect on Odc expression, the effect on tumor yield is substantial: 3.5- to 5.4-fold fewer tumors are produced in Odc+/- mice compared with wild-type mice following a standard initiation-promotion protocol, a finding that is genetic proof of principle that even relatively modest reductions of ODC indeed affect tumor development in a rather profound manner.

Rather than considering the impact of Odc gene dosage on levels of enzyme activity, it is physiologically more important to examine the changes in tissue polyamine pools because these molecules are the actual effectors acting on downstream targets relevant to tumorigenesis. From the data in Table 1, it seems clear that the effect of Odc gene dosage on polyamine pools following TPA treatment is more substantial than might be predicted based on enzyme activity measurements (Fig. 1). For example, in Odc+/- mice at 24 hours after TPA treatment, the putrescine level was elevated 3-fold, whereas spermidine levels were unchanged and total polyamine levels were slightly reduced versus control values. In contrast, in wild-type mice, the putrescine level is 18-fold higher, spermidine is 3-fold higher, and total polyamine levels are double the control values. Thus, relatively modest differences in Odc expression can have rather large effects on individual and total tissue polyamine pools.

As might be expected from the ODC activity and polyamine data described above, the histologic response of the skin after TPA treatment is shown in Figure 2. Seven-week-old wild-type (A and C) or Odc+/- (B and D) mice were treated topically with either acetone (A and B) or 17 nmol TPA (C and D) at 3- to 4-day intervals for 2 weeks (four treatments). One day after the last treatment, mice were sacrificed and their skins processed histologically as described in Materials and Methods.

### Table 1. TPA-induced polyamine levels in Odc+/- versus Odc+/- mice

<table>
<thead>
<tr>
<th>Odc genotype</th>
<th>Hours after TPA</th>
<th>Polyamine nmol/mg DNA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Putrescine</td>
<td>Spermidine</td>
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<tr>
<td>+/-</td>
<td>0</td>
<td>7.0</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>14.3</td>
<td>114</td>
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<tr>
<td></td>
<td>6</td>
<td>47.6</td>
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<td></td>
<td>24</td>
<td>20.4</td>
<td>146</td>
</tr>
<tr>
<td>+/+</td>
<td>0</td>
<td>3.3</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>38</td>
<td>121</td>
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<td>114</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>58</td>
<td>306</td>
</tr>
</tbody>
</table>

NOTE: Groups of two to three mice of each Odc genotype were treated topically with 17 nmol TPA and sacrificed 4, 6, or 24 hours later. Control mice (0-hour point) were treated with acetone and sacrificed 6 hours later. Polyamine levels in epidermal extracts were measured as described previously (8).
treatment varied by Odc gene dosage. An epidermal hyperplastic response to TPA treatment, which is considered a necessary prerequisite for tumor promotion (15), was observed in Odc+/- mice but was less robust and of shorter duration than the response of wild-type mice. This was true for interfollicular epidermis, but especially so for hair follicles. The presumptive origin of most tumors in the initiation-promotion model (12). Although it is clear that one active Odc gene is capable of supplying sufficient polyamines to support the rapid proliferation that occurs during embryonic and early postnatal development (7), this conclusion may not apply to pathophysiologic conditions such as chronic tumor promotion. Our results in this animal model lend support to chemopreventative approaches using low dose 2-difluoromethylornithine in humans (16), in which the rationale is to limit "excessive" polyamine synthesis in preneoplastic cells that drives their progression toward a more malignant phenotype.

An important issue is whether our results in this mouse model are relevant to human cancer, especially given the findings that rather small differences in Odc expression can have a significant impact on tumor susceptibility. Early studies of the human ODC gene reported a Pst I RFLP (17) that ultimately was shown to be due to an A/G single nucleotide polymorphism in a transcriptional regulatory region in intron 1 (18). The A/G polymorphism lies between two closely spaced Myc-binding E-boxes, 5 bases from the more distal one. Importantly, the A allelic variant defined by this single nucleotide polymorphism is functionally more active in luciferase-based reporter assays (18, 19). Luciferase expression was 3-fold higher in fibroblasts transfected with the A allele–containing construct with the G allele construct and 10-fold higher in colonic epithelial cells. Thus, it seems quite likely that there may be interindividual differences in ODC expression based on genotype at the ODC locus. Indeed, in a test of the hypothesis that genetic variation at the ODC locus might influence cancer risk, a recent epidemiologic study has showed a positive association between the AA ODC genotype and increased prostate cancer risk in men who smoked or who had high-risk allelic of the androgen receptor gene (20). This result supports our conclusion from the Odc+/- mouse model that relatively modest differences in ODC gene expression can affect cancer susceptibility. Additional studies in both Odc+/- mice and humans are needed to determine if this conclusion is broadly applicable to a wide range of tissues at risk for tumor development.

Acknowledgments

Grant support: NIH grants ES 01664 (T.G. O'Brien) and CA-100603, the Cancer Center Support (Core) grant CA21765, and the American Lebanese Syrian Associated Charities (J.L. Cleveland).

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We thank Dr. Janet Sawicki for comments on the manuscript.

References


www.aacrjournals.org 1149 Cancer Res 2005; 65: (4). February 15, 2005

Figure 3. Tumor response of Odc+/- and Odc-/- mice to an initiation-promotion protocol. Groups of Odc+/- (●) and littermate Odc-/- (●) mice were initiated with 800 nmol DMBA and promoted with TPA as described in Materials and Methods. There were 43 Odc+ and 36 Odc-/- mice of both sexes in the experiment. There was no sex difference in tumor multiplicity in either group of mice. The difference in tumor multiplicities was statistically significant (P < 0.0001).
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