Inducible Nitric Oxide Synthase mRNA Is Upregulated in Skin Tumors of v-Ha-ras Transgenic TG-AC Mice Treated with 12-o-Tetradecanoylphorbol-13-acetate

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The correlation between the steady-state level of inducible nitric oxide synthase (iNOS) mRNA and skin tumors induced following treatment with 12-o-tetradecanoylphorbol-13-acetate (TPA) was investigated in transgenic TG-AC mice, which carry the v-Ha-ras oncogene fused to the promoter of the mouse embryonic β-like, ζ-globin gene. In animals treated with TPA (2.5 μg x 2/week, for 2 weeks), the increase of iNOS mRNA was locally confined only to the regions of papillomas, but not to the skin tissues surrounding the papillomas. However, the tissues surrounding the papillomas showed only a minor increase in the steady-state level of iNOS mRNA. These data suggest that iNOS gene expressions may underlie tumorgenesis during TPA promotion in TG-AC mice.

Key words TPA; inducible nitric oxide synthase; transgenic mice

Nitric oxide (NO) is a paramagnetic free radical, which is involved in the mediation of various physiological functions, such as neurotransmission, vasodilation, platelet aggregation, inflammation, cytotoxicity, and antitumor activities. Among three isoforms of NO Synthase (NOS), inducible NOS (iNOS) produces high concentrations of NO, in order to counter pathogens and to coordinate T cell responses. NO and NOS have been studied in mouse skin during carcinogenesis. Macrophage-derived NO synthesis delays the progression of UV-induced murine skin cancer, suggesting that it has antitumor activity.

High levels of NO have been shown to suppress the activation of T lymphocytes in response to antigens or mitogens, and implicated in tumor-induced immunosuppression in rats. Evidence exists to show that excessive NO production by iNOS in rat solid tumors might also be involved in increases of vascular permeability and blood flow, as well as tumor growth.

It has been recently hypothesized that NO synthesis needs to be elevated during the vascular stage of a malignant melanoma, which leads to a decrease in endothelial cell proliferation and the maintenance of vasodilator tone in and around the tumor. A tumor promoter 12-o-tetradecanoylphorbol-13-acetate (TPA) produced a transient increase in the transcription of iNOS gene in primary neonatal rat hepatocytes. On the other hand, the protein kinase C (PKC, a receptor of TPA) inhibitor Ro31-8220 abolished the induction of iNOS activity in murine macrophages. However, the phorbol type of tumor promoters (TPA, mezerein) downregulated the constitutive form of NOS in Sencar mouse epidermis. In murine melanoma cells, an inverse correlation between the expression of iNOS activity and the production of metastases has been observed.

It has recently been reported that iNOS is downregulated in hyperplastic epidermis and in papillomas during tumor promotion in Sencar mice treated with 7,12-dimethylbenz(a)anthracene (DMBA) followed by repetitive application of TPA. A transgenic TG-AC mouse line has been developed that contains an activated Harvey-ras gene, thus mimicking initiation by ras activation following treatment. The TG-AC mice developed from FVB/N mice are homozygous for a v-Ha-ras transgene that contains point mutations at codons 12 and 59, fused to a fetal-ζ-globin promoter. These mice rapidly produce skin papillomas or other tumors and have thus been proposed for identifying chemical carcinogens in short-term bioassays. However, not much mechanistic information on chemical carcinogenesis in TG-AC mice has been available either to understand better the mouse system or to compare with other normal rodent system. Since iNOS has been reported to play a role in tumorgenesis, it is worth while to investigate iNOS in TG-AC mice. In this study, the iNOS gene expression was examined in skin tumors of TG-AC mice following treatment with TPA or benzo[a]pyrene (BP) and BP was compared with TPA in terms of tumorigenicity.

MATERIALS AND METHODS

Chemicals TPA was purchased from Sigma (St. Louis, MO, U.S.A.). Acetone and formalin were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Animal Treatments TG-AC mice were treated and maintained in accordance with the NIH guidelines for human care and used under an institutional, peer-reviewed animal study protocol. Groups of 5—10 female homozygous TG-AC mice (obtained from Taconic Laboratories, Germantown, NY), housed 4 mice per cage, were first treated when 7—8 weeks old (20±2 g b.w.). Mice were fed Purina Pico Chow, no. 5058 and water ad libitum. Mice were shaved on the dorsal surface at the application site 1 d prior to the administration of the first dose, and then as needed basis during the dosing period. Chemicals, in 200 μl of acetone, were administered topically to the shaved area 2 times per week. The mice were maintained on corncob bedding and on a 12-h light/dark cycle. In a promotion study, TPA (2.5 μg x 2/week) was administered to mice for 2 weeks. Mice with tumors were killed by CO2 asphyxiatation. Immediately afterwards each tumor and surrounding skin were swabbed with 70% ethanol, and each skin tissue was aseptically removed and

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frozen at −80°C in a sterile tube. Skin tissues were prepared 14 weeks after final treatment with TPA.

**RT-PCR Analysis of Transgene and iNOS** Following animal treatment, total RNA was isolated from each tissue treated and used for reverse transcription-polymerase chain reaction (RT-PCR) analyses. Each pool of total RNA was prepared using TRI-reagent according to the method of Chomczynski and Sacchi. The level of iNOS mRNA transcription was measured according to the competitive RT-PCR analysis described by Jeon et al. Briefly, one microgram of total RNA (isolated from skin tissues or tumors in TG-AC mice) from each treatment was used for the reverse transcription reaction in 20 μl using the Superscript™ Preamplification System (Gibco-BRL, Gaithersburg, MD, U.S.A.), and 1.5 μl of the reaction was used for PCR, using the Gene Amp PCR kit with AmpliTaq DNA polymerase (Perkin Elmer Corp., Norwalk, CT, U.S.A.) in 10 μl and amplified with each fixed cycle number in a Perkin Elmer PCR cycler Model 9600. The temperatures for denaturing, annealing and the extension reaction for the PCR were as follows: 1st cycle, denaturing for 3 min at 95°C, annealing for 1 min at 62°C, and the extension reaction for 1.5 min at 72°C; 2nd cycle, denaturing for 1 min at 95°C, annealing for 1 min at 60°C, and the extension reaction for 1.5 min at 72°C; 3rd to the last cycle, denaturing for 30 s at 94°C, annealing for 30 s at 58°C, and the extension reaction for 45 s at 72°C; the extra-extension reaction for 7 min at 72°C. The optimum number of cycles for detecting each mRNA using RT-PCR was determined by pilot studies: the optimum numbers were as follows; iNOS, 32 cycles and β-2-microglobin, 29 cycles. The primers and the competitive DNA fragment (MIMIC) for the quantitative and competitive RT-PCR analysis of iNOS mRNA transcripts were purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). The sequence of the primer set and size of target amplified band were as follows;

**Table 1. Tumor Induction in Homozygous TG-AC Mice Treated with TPA**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Average tumor incidence/mouse</th>
<th>Survival(b)</th>
</tr>
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<tbody>
<tr>
<td>TPA 2.5 μg</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Acetone 200 μl</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(a) Number of weeks. (b) Number of mice alive at the end of experiment (16th week). (c) Mice treated 2X/week for 2 weeks. All tumors are papillomas in the TPA treated mice. After 16 weeks, mice were sacrificed.

**Table 2. The Level of iNOS mRNA Transcripts in Skin Tumors or Surrounding Tissues of Tumors in TG-AC Mice Treated with TPA**

<table>
<thead>
<tr>
<th>iNOS mRNA/MIMIC</th>
<th>Control&lt;0.02(&lt;1.1)(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1=0.459 (24.6)</td>
<td>15&lt;0.02(&lt;1.1)</td>
</tr>
<tr>
<td>2=0.478 (26.2)</td>
<td>25&lt;0.02(&lt;1.1)</td>
</tr>
<tr>
<td>3=0.218 (11.9)</td>
<td>35&lt;0.02(&lt;1.1)</td>
</tr>
<tr>
<td>0.382±0.143*</td>
<td>0.02 (1.1)</td>
</tr>
<tr>
<td>(20.9±7.8)*</td>
<td></td>
</tr>
</tbody>
</table>

(a) iNOS mRNA transcripts (<1.1×10⁻³ atomol/μg of total RNA). 1, 2, and 3 represent sample No. 1—3 (lane 1—3). RNA isolated from papilloma of mouse skin treated with TPA. 1S, 2S, and 3S represent each surrounding tissue of the sample No. 1—3 (lane 1—3). The data represent mean±S.D and significantly different from those of surrounding tissues assuming that their values are all 0.02 (1.1) (p<0.05).

**RESULTS**

The steady-state level of iNOS mRNA in samples of normal, papilloma, and surrounding tissues of papillomas or carcinomas from TG-AC mice skin treated with TPA was investigated using competitive RT-PCR analysis. Tumor inductions following treatment with TPA are summarized in Table 1. Tumors were induced within 6 weeks after the TPA treatment. In the case of animals treated with TPA, all tumors were shown to be papillomas, as determined by histopathological analysis. For the RT-PCR analysis of the steady-state level of iNOS mRNA transcripts, competitive RT-PCR was performed; the competitive DNA fragment (MIMIC) was used to exclude the PCR amplification of pseudogene; and the level of iNOS mRNA was expressed as the ratio, iNOS mRNA/MIMIC (Table 2). Figure 1C shows an example of RT-PCR analysis of iNOS mRNA in the selected sample (lanes 1—5, Fig. 1C). In the quantitative RT-PCR analysis, the cDNA sample was two-fold serially-diluted (from right to left; upper band) and 0.031 attomole of the competitive DNA fragment was two-fold serially-diluted (from left to right; lower band). In all the TPA-induced papillomas (lane 1—3), the increase in the steady-state level of iNOS mRNA was observed while that of each corresponding surrounding tissue (lane 1S, 2S, or 3S) of papillomas (lane 1—3) remained at background level (iNOS mRNA/MIMIC, <0.02) (Fig. 1A). The level of iNOS mRNA for the selected sample was about 0.035 attomol/μl of total RNA, as determined by identifying the point at which the serial dilutions yielded 1:1 molar ratio of PCR products. The numbers of papillomas (lane 1, papilloma RNA isolated from mouse skin bearing 3 papillomas; lane 2, RNA from mouse skin with 9 papillomas; lane 3, RNA from mouse skin with 30 or more papillomas) had no effect on the steady-state level of iNOS mRNA in both papillomas and surrounding tissues of papillomas.
mRNA/MIMIC, <0.02) (Table 2). H & E staining of control mouse skin, and papilloma from TPA treatment was also summarized in Fig. 2.

DISCUSSION

Transgenic TG-AC mice have been considered a useful animal model to screen carcinogens and to investigate the mechanism of chemical carcinogenesis in a short period of time. About 20 known carcinogens were examined in TG-AC mice and the assay results were highly concordant with those from 2-year tumor bioassays.20) BP, a prototype of polycyclic aromatic hydrocarbons (PAH), has been studied to compare with TPA in terms of tumorigenicity in the skin and to investigate the association of iNOS with tumorigenesis. In tumor formation, TPA (a promoter) produced only papillomas whereas BP (a complete carcinogen) produced mostly carcinomas (4 out of 5 mice). It is likely that to produce a carcinoma in TG-AC mice both genetic and epigenetic alteration would be required by a complete carcinogen such as BP. To produce a papilloma, only epigenotoxic promoter (incomplete carcinogen) such as TPA would be needed in genetically altered sensitive TG-AC mice (data not shown).

NOS has been shown to be involved in the promotion and progression of tumorigenesis. In previous studies in humans, iNOS levels were positively correlated with the progression of breast tumors21) and highly expressed in poorly differentiated ovarian tumors.22) During the tumor promotion in Sencar mice treated with 7,12-dimethylbenz(a)anthracene and TPA, the temporal sequence of iNOS gene expression was examined and shown to be downregulated in hyperplastic epidermis and papillomas.23) In this study with TG-AC mice, however, iNOS mRNA was upregulated in all the papillomas during tumor promotion. These two different observations might be related to species difference. Our data, however, is concordant with the results from recent reports of various types of human or animal tumors.24–29) On the other hand, in the control group or in the surrounding epithelial tissues of tumors (papillomas or carcinomas) induced by treatment with TPA, an increase in iNOS mRNA levels was not detectable, suggesting that iNOS expression was locally involved only to the regions of tumors. It is highly possible that upregulation of

![Fig. 1. Competitive RT-PCR Analysis of iNOS mRNA in the Papillomas Induced after Treatment with TPA](image)

(Control): iNOS mRNA of skin from acetone treatment, (1, 2, and 3): papillomas from TPA treatment, (1S, 2S, 3S): surrounding (S) tissue of corresponding papilloma (1, 2, 3), iNOS mRNA (A), housekeeping gene (Mβ) (B), and the competitive DNA fragment (C) were analyzed. (C) shows a standard competitive RT-PCR of iNOS with MIMIC and the total sum of band density from iNOS and MIMIC is equal on each lane (1 – 5). Expression levels are also numerically summarized in Table 2.

![Fig. 2. H & E Staining of Control Mouse Skin (a), and Papilloma from TPA Treatment (b), in TG-AC Mouse Skin](image)

Arrows indicate a normal epidermis (·), hair follicle (↑), and encapsulated papilloma (→). Magnification, ×100.
iNOS elevate the level of nitric oxide, which both inhibits proliferation during promotion and stimulates neovascularization during tumor progression.\textsuperscript{29,30} These results suggest that the upregulation of the iNOS gene may underlie the induction of tumors during TPA promotion in TG-AC mice. In the future, chemopreventive effects of iNOS inhibitors need to be investigated on tumorigenesis in TG-AC mice to support this data.

REFERENCES