Gene expression and cellular sources of inducible nitric oxide synthase during tumor promotion

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Introduction

The cellular alterations that occur during the process of tumor promotion include both an induction of significant epidermal hyperplasia and infiltration of neutrophils into the dermis after a single exposure to a polycyclic aromatic hydrocarbon such as 7,12 dimethylbenz[a]anthracene (DMBA*), followed by multiple topical applications of 12-O-tetradecanoylphorbol-13-acetate (TPA) to the dorsal epidermis of genetically susceptible mice (1-3). Numerous studies have now demonstrated that reactive oxygen intermediates produced during tumor production interact with nucleosides and form oxidative DNA adducts which induce a specific mutational spectrum considered to be a signature of oxidant-mediated DNA damage (3-9). In addition to inducing production of reactive oxygen intermediates, TPA, as well as cytokines and bacterial cell wall products, stimulate production of reactive nitrogen intermediates, including nitric oxide, nitronium ion, nitrogen dioxide radical, peroxynitrite- and peroxynitrous acid (10-14). Nitric oxide is synthesized from L-arginine in an oxygen-dependent reaction that is catalyzed by NADPH-dependent nitric oxide synthase (NOS) (15). Thus far, three distinct types of NOS have been described, including two calcium-dependent constitutive forms of NOS, endothelial NOS (eNOS) and neuronal NOS (16,17). A calcium-independent inducible form of NOS (iNOS) was initially identified as a product of activated monocytes (18), but is now known to be produced by a wide variety of cell types, including epithelial cells from various origins (19-22). Nitric oxide functions both as a cellular messenger and as a diffusible free radical (2). Up-regulation of iNOS and production of nitric oxide occur in many pathological conditions associated with inflammatory processes (24-30) and inhibition of NOS appears to be an effective means of reducing or inhibiting tissue damage induced by the presence of reactive nitrogen intermediates (31-34).

Although it is now clear that nitric oxide and its degradation products can induce oxidative damage during inflammation, the role of nitric oxide and reactive nitrogen intermediates in mutagenesis, tumor initiation, promotion and progression remain to be completely characterized. Nitric oxide alone, or in combination with nitrogen dioxide, has been shown to be mutagenic in the Ames Salmonella reversion assay, in other bacterial systems and in mammalian cells (35-39). Peroxynitrite ion and peroxynitrous acid, formed from the interaction of nitric oxide and superoxide anion, are strong oxidant species. During the degradation of peroxynitrite to nitrite, a hydroxyl-like radical, HOONO·, is formed, however, this radical is not derived from the Fenton reaction and appears to be a more selective oxidant than hydroxyl radicals (40). Peroxynitrite has recently been shown to induce single-strand breaks in supercoiled plasmid DNA (41), to oxidize cellular thiols in rat thymocytes (42) and to induce apoptosis (43,44). The ability of Trolox, a water-soluble form of vitamin E, to inhibit DNA nicks induced by peroxynitrite and the ability of radical scavengers to enhance DNA damage further implicates an 'hydroxyl-like' reactive nitrogen intermediate which can induce oxidative damage but which is not inhibited by radical scavengers. Other studies have demonstrated that peroxynitrite...
induces formation of 8-hydroxydeoxyguanosine (45), which is a DNA adduct that has been used as a signature marker for oxidative DNA damage that occurs following production of reactive oxygen intermediates during tumor promotion (3,4). Peroxynitrite also forms a newly described DNA adduct, 8-nitroguanine, which may be useful as a marker of peroxynitrite-induced DNA damage (46). Peroxynitrite decomposes to form reactive intermediates, including nitrogen dioxide and tyrosyl radicals, which induce nitration of aromatic amino acids such as tyrosine (47–49). The detection of nitrotyrosine using either chromatographic methods or 3-nitrotyrosine-specific antibodies have been used to detect the presence of nitric oxide-derived reactive nitrogen intermediates, including peroxynitrite, within tissue and fluids (50,51).

Initial evidence for the potential importance of nitrogen intermediates in tumor promotion is based on studies using the C3H/10T1/2 in vitro transformation system. These studies demonstrated that iNOS gene expression as well as endogenous production of nitric oxide and nitrogen oxides increased following exposure of these cells to either 3-methylcholanthrene or a combination of γ-interferon and bacterial lipo-polysaccharide (52). Furthermore, arginine analogs that block production of NOS and nitric oxide, including aminoguanidine, Nω-methyl-L-arginine and Nω-nitro-L-arginine, inhibited transformation. These results suggest that the promotional phase of carcinogenesis may be better defined as a process that involves multiple genetic mutations due to endogenous mutagens, such as reactive nitrogen intermediates, being produced in the presence of proliferating cell populations. Other support for the role of nitric oxide and reactive nitrogen intermediates in tumor promotion has come from studies demonstrating that γ-interferon acted as a co-promoter with TPA in induction of papilla growth, which was believed to be based on increased infiltration of activated leukocytes that may be a source of reactive intermediates (53).

Tumor promotion in murine skin has a significant inflammatory component (1–4) that is characterized by the activation of infiltrating leukocytes to produce significant amounts of reactive oxygen species. The role of these reactive oxygen intermediates in formation of oxidative DNA adducts that are mutagenic and result in formation of base pair mutations has been extensively examined (3–9). In contrast, there have been no studies that have characterized the temporal sequence of NOS gene expression over the time course of multi-stage carcinogenesis, nor has the formation of protein nitrotyrosination as a marker for tissue-based formation of peroxynitrite during papilloma growth been examined. The goal of the present investigation was to examine iNOS gene expression in cutaneous tissue isolated at short times (4–96 h) following a single topical treatment of mice with 10 μg TPA and over the 22 weeks of treatment of Sencar mice with a single 25 nmol dose of DMBA followed by twice weekly applications of 2 μg TPA. Immunohistochemical studies were performed to characterize the cellular location of iNOS protein production within the dermis and epidermis and to determine the extent to which 3-nitrotyrosine was present as a result of interaction of dermal tissue with reactive nitrogen intermediates, such as peroxynitrite, over the time of tumor promotion. The present studies demonstrate that there is a compartmentalization of iNOS production during tumor promotion. Infiltrating neutrophils within the dermis are the primary cellular source of iNOS and 3-nitrotyrosine, which serves as a signature for the production of reactive nitrogen intermediates derived from iNOS, including peroxynitrite, during multi-stage carcinogenesis.

Materials and methods

Treatment of Sencar mice with DMBA and TPA

For all studies described, female Sencar mice (6–8 weeks old, 25–32 g; DCT Biological Testing, Frederick, MD) were used. Mice were housed in vivarium facilities at the Ohio State University that meet American Association for Accreditation of Laboratory Animal Care requirements. All protocols were approved prior to beginning the study by the Ohio State University Institutional Animal Care Utilization Committee. Animals were fed a basal diet ad libitum and kept in a room maintained on a 12 h light/dark cycle. At 24 h prior to initial treatment with acetone, DMBA or TPA, the dorsal skin of mice was shaved and mice in the hair re-growth cycle were not used in these studies. Mice treated topically with a 0.2 ml acetone served as the solvent control. DMBA was dissolved in acetone and mice were treated topically with a final concentration of 25 nmol DMBA in 0.2 ml acetone. TPA, at a final concentration of either 2 or 10 μg, was dissolved in reagent grade acetone and applied in a volume of 0.2 ml to the dorsal epidermis of Sencar mice. Cutaneous tissue was collected at various time points following treatment, including 4, 24, 48, 72 or 96 h following a single topical treatment with 2 or 10 μg TPA or acetone as the solvent control. For long-term studies, mice were treated with a single topical application of 25 nmol DMBA and at 7 days later were treated twice weekly with either 2 μg TPA or acetone for time periods including 1, 3, 6, 10, 16 and 22 weeks of tumor promotion. Tissue samples were isolated at 4 h after the final topical application of 2 μg TPA.

Quantitation of iNOS gene expression during tumor promotion

Methods used for quantitative analysis of gene expression were as previously described (54–57). Briefly, RNA was isolated from skin or papillomas using guanidinium thiocyanate and phenol. RNA was quantitated spectrophotometrically and analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining of agarose–formaldehyde gels. Reverse transcription of total RNA was performed and the reverse transcribed cDNA obtained from total RNA was then added to a reaction mixture that included 5′ primers that were homologous to the 5′-end and 3′ primers that were specific to a murine homologue of a rat cDNA. iNOS cDNA from the TBR label reacts with tripropylamine in an electrochemiluminescent reaction. Tissue samples were isolated at 4 h after the final topical application of 2 μg TPA. The goal of the present investigation was to examine iNOS gene expression in cutaneous tissue isolated at short times (4–96 h) following a single topical treatment of mice with 10 μg TPA and over the 22 weeks of treatment of Sencar mice with a single 25 nmol dose of DMBA followed by twice weekly applications of 2 μg TPA. Immunohistochemical studies were performed to characterize the cellular location of iNOS protein production within the dermis and epidermis and to determine the extent to which 3-nitrotyrosine was present as a result of interaction of dermal tissue with reactive nitrogen intermediates, such as peroxynitrite, over the time of tumor promotion. The present studies demonstrate that there is a compartmentalization of iNOS production during tumor promotion. Infiltrating neutrophils within the dermis are the primary cellular source of iNOS and 3-nitrotyrosine, which serves as a signature for the production of reactive nitrogen intermediates derived from iNOS, including peroxynitrite, during multi-stage carcinogenesis.
The present results demonstrate that there were relatively high levels of iNOS gene expression in cutaneous tissue isolated from the dorsal epidermis of Sencar mice treated with single or multiple applications of acetone, with an average level of iNOS in acetone treated skin of 0.23 ± 0.02 LU (mean ± SE) (Figure 1A, lane a, and B). At 4 h following a single topical application of 10 μg TPA, the levels of iNOS mRNA were comparable with those detected in acetone-treated tissue (0.22 ± 0.02 LU) (Figure 1A, lane b, and B). There was a significant decrease in iNOS gene expression beginning at 24 h after topical treatment of the dorsal epidermis of Sencar mice with a single dose of 10 μg TPA (0.05 ± 0.0 LU) (Figure 1A, lane c, and B). The diminution of iNOS gene expression detected at 24 h after a single exposure to TPA was a consistent finding in cutaneous tissue isolated from Sencar mice treated over the time of 22 weeks with a single application of 25 nmol DMBA followed a week later by multiple applications of 2 μg TPA (Figure 1A, g–j, and B). Levels of iNOS gene expression in papillomas isolated from Sencar mice at 22 weeks following a single dose of 25 nmol DMBA and twice weekly applications of 2 μg TPA were similar to the level of iNOS detected in skin isolated over the time of tumor promotion. In comparison, the level of iNOS in cutaneous tissue isolated at 24 h after a single application of 25 nmol DMBA was 0.139 ± 0.01 LU, which was below that detected in acetone-treated mice but was not diminished to the level detected in cutaneous tissue isolated from DMBA–TPA-treated mice.

Cellular localization of iNOS during tumor promotion

Further studies were performed to identify the location of cells producing iNOS using immunohistochemical detection methods. iNOS immunoreactive protein was detectable in the stratum corneum of murine epidermis, in suprabasal keratinocytes and in interfollicular cells of cutaneous tissue isolated at 3 weeks from Sencar mice following multiple applications with acetone (Figure 2A), which is representative of the levels of iNOS protein within acetone-treated cutaneous tissue at all time points examined. Cutaneous tissue isolated at 1 week following a single topical application of 25 nmol DMBA to the dorsal epidermis of Sencar mice did not appear to significantly alter iNOS protein levels within the epidermal layers compared with iNOS present in acetone-treated skin. iNOS immunoreactive protein was low but detectable in the outer-most suprabasal keratinocytes of hyperplastic epidermis isolated at 3 weeks from Sencar mice treated with a single dose of 25 nmol DMBA followed a week later by twice weekly applications of 2 μg TPA (Figure 2B). In contrast to the low level of iNOS immunoreactive protein present within the epidermal cells during tumor promotion, in tissue isolated at 3 weeks following a single application of 25 nmol DMBA followed by repetitive applications of 2 μg TPA, neutrophils within the dermis stained intensely with anti-iNOS antibodies (Figure 2C). Beginning at 10 weeks of the tumor promotion protocol, neutrophils intensely stained with anti-iNOS antibodies were located within the dermal–epidermal junction, in close proximity to basal keratinocytes. The extent of iNOS staining by infiltrating neutrophils within tissue isolated at all times from mice treated topically with a single application of acetone followed by multiple applications of 2 μg TPA was similar to that observed in tissue isolated from mice treated with a DMBA–TPA tumor promotion protocol. Isotypic control IgG antibody was used to confirm the specificity of iNOS immunoreactive protein localization (Figure 2D).
Fig. 2. Immunochemical localization of iNOS in cutaneous tissue during tumor promotion. Immunoreactive iNOS protein was present in high amounts in the stratum corneum, suprabasal keratinocytes and interfollicular cells of dorsal epidermis isolated from Sencar mice at 3 weeks following twice weekly treatment with 0.2 ml acetone (A). At 24 h following a topical application of 10 μg TPA, there was a decrease in immunoreactive iNOS protein within the suprabasal keratinocytes and interfollicular cells (B). In contrast, infiltrating neutrophils within the dermis were the primary source of iNOS protein (C). The low level of isotypic antibody demonstrates the specificity of the immunoreactive iNOS localization (D).

lack of activation of neutrophils within skin treated topically with acetone. 3-Nitrotyrosine immunoreactive protein was present within dermal neutrophils at 10 weeks following twice weekly topical application to Sencar mice of 2 μg TPA (Figure 3A and B), which was representative of the levels of nitrotyrosinated protein within the dermis at all times examined over the 22 weeks of the tumor promotion process. The specificity of the 3-nitrotyrosine antibody staining was deter-
Fig. 3. Immunochemical localization of 3-nitrotyrosine in cutaneous tissue during tumor promotion. Nitrotyrosine immunoreactive protein was localized to dermal tissue (A) and infiltrating neutrophils (A and B) of the dermis of Sencar mice at 10 weeks following treatment with a single dose of DMBA and twice weekly application of 2 μg TPA. Note the lack of 3-nitrotyrosine antibody staining within the epidermal layers.

Discussion

Although the biology of NOS isoforms and the chemistry of formation and reactivity of nitric oxide-derived intermediates is the subject of intensive investigation, there is less known about the regulation and role of NOS and reactive nitrogen intermediates in tumor growth. Previous studies demonstrated that NOS, as measured by immunolocalization of NAD(P)H diaphorase, was decreased in human colorectal carcinomas and human preneoplastic polyps, compared with detectable NOS activity in adjacent normal mucosa (58). Recent studies have demonstrated that DLD-1 colon carcinoma cells engineered to overexpress NOS grew more slowly in vitro and that the inhibition of proliferation was reversed by the addition of N-ethyl-L-ornithine, an inhibitor of NOS (59). In contrast, DLD-1 cells that overexpress NOS grew more rapidly when implanted in vivo in nude mice and formed highly vascularized, invasive tumors. Other studies that examined iNOS protein within human breast tumors found that NOS was present in infiltrating macrophages, vascular endothelial cells and breast tumor cells and that the levels of NOS positively correlate with grade of tumor (60). Recent evidence suggests that the level of iNOS enzyme activity was highest in poorly differentiated ovarian tumors (61). The present results are in agreement with previous observations that suggest that iNOS appears to play a paradoxical role in tumor growth (61), since it can act as a cytostatic agent that inhibits proliferation (62–65), while simultaneously generating leukocyte-derived reactive nitrogen intermediates that damage DNA (41,45) and stimulate neovascularization (59).

The present studies are one of the first to characterize the timing and cellular sources of iNOS and nitric oxide-derived intermediates during multi-stage carcinogenesis. iNOS was up-regulated in the epidermis under homeostatic conditions and there was an inverse correlation between iNOS gene expression and TPA-induced epidermal hyperplasia over the 22 weeks of the tumor promotion protocol during which preneoplastic papillomas develop. These observations are consistent with previous studies that have shown that nitric oxide is down-regulated during processes involving epidermal proliferation, such as in the initial phases of the wound healing process (57), and in the presence of growth factors, such as epidermal growth factor (66). Recent studies suggest that epithelial cells isolated from lining epithelium contain both constitutive and inducible NOS isoforms that differ in their molecular and functional characteristics (22). Epithelial cells from the epidermis, like those of other lining epithelium, reside at the interface of the external environment and the isoforms of NOS within these cells may have differential roles in protecting the host from physical and chemical trauma to the integument. The present immunohistochemical studies localized iNOS protein within the stratum corneum and suprabasal epidermal cells under homeostatic conditions. Although levels of iNOS protein were lower in the suprabasal cells following topical application of TPA than in acetone-treated epidermis, iNOS remained detectable in the upper-most epidermal cells. These results suggest that iNOS is produced locally by more differentiated cells within the epidermis during the tumor
The extent to which reactive nitrogen intermediates produced by neutrophils induce formation of specific oxidative DNA adducts and the differential role that reactive nitrogen intermediates may have in the tumor promotion process is currently not known. In contrast to the low levels of iNOS in suprabasal keratinocytes and in papillomas, infiltrating neutrophils within the dermis appear to be the primary cellular sources of both iNOS and 3-nitrotyrosine during tumor promotion. Recent reports demonstrate that direct exposure of guanine to peroxynitrite induces formation of a newly described DNA adduct, 8-nitroguanine, which may serve as a signature for the presence of nitrogen intermediates (46). A very recent study demonstrated that peroxynitrite induced predominantly base pair mutations, primarily involving G:C→T:A transversions and secondarily induced G:C→C:G transversions (67). Taken together with previous studies which documented the timing of production of reactive oxygen intermediates during tumor promotion (3,4), the present studies suggest that both reactive nitrogen and oxygen intermediates are produced by infiltrating neutrophils within the dermis and are therefore present in the microenvironment within the same time frame following single and multiple topical applications of TPA. Nitric oxide and superoxide anion interact to form peroxynitrite, which can induce DNA strand breaks and subsequently form oxidative DNA adducts that can result in base pair mutations (41,42,45). It is possible that reactive nitrogen intermediates contribute to the misreplication and misrepair once attributed solely to reactive oxygen intermediates produced during tumor promotion. In contrast to previous studies that have demonstrated that both infiltrating leukocytes as well as epidermal keratinocytes produce reactive oxygen intermediates during tumor promotion (68–73), it appears that neutrophils within the dermis serve as the source of iNOS and nitric oxide-derived intermediates during the tumor promotion process and that iNOS is significantly down-regulated within the epidermis during the development of papillomas.

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References


