



Immunohistochemical expression of inducible nitric oxide synthase in DMBA-induced hamster buccal pouch carcinogenesis

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Abstract

Nitric oxide (NO) plays a key role in the processes of inflammation and carcinogenesis. Three isoforms of NO synthase have been identified: endothelial nitric oxide synthase (NOS), neuronal NOS, and inducible NOS (iNOS). The purpose of this study was to investigate the characteristics of iNOS in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis. Thirty outbred young (6-week-old) male Syrian golden hamsters were randomly divided into three groups: DMBA (0.5%) painted group ($n=10$); mineral oil-treated group ($n=10$); and non-treated group ($n=10$). The average number of iNOS positive foci per section in the DMBA-treated group was approximately 12.2 ± 4.7 . Both cytoplasmic and nuclear stainings were observed in the DMBA-treated pouch keratinocytes. No iNOS activity could be detected in the untreated or mineral oil-treated pouches. In conclusion, this study has demonstrated that iNOS is expressed in DMBA-induced hamster pouch carcinomas. This finding suggests that iNOS expression may be associated with the development of chemically induced oral carcinomas. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Following its identification in 1987 as endothelium-derived relaxing factor, nitric oxide (NO) has been recognized as having a key role in many and diverse biological processes including inflammation and cancer [1]. NO is synthesized from a family of enzymes named NO synthase (NOS; EC 1.14.13.39) that convert L-arginine to L-citrulline and generate NO [2]. Three isoforms of NO synthase have been identified and characterized to date. Two of them [endothelial NOS (eNOS), a 132-kDa protein; neuronal NOS (nNOS), a 162-kDa protein] are present constitutively and are calcium dependent. The other isoform [inducible NOS (iNOS), a 130-kDa protein], which is induced in the body during inflammatory processes, is calcium independent [3].

NO, a highly reactive radical, may react with other radicals to form cytotoxic compounds, such as peroxy-nitrite, which may cause DNA damage [4]. It can also react directly with a variety of enzymes and other proteins to either activate or inhibit their functions by

oxidizing SH groups, complexing with metal ions, or reacting with tyrosine [5]. All of these effects could be essential for the participation of NO in carcinogenesis.

The hamster buccal pouch mucosa model is one of the most widely accepted experimental models of oral carcinogenesis [6]. In spite of anatomical and histological differences between pouch mucosa and human buccal tissue, carcinogenesis protocols induce premalignant changes and carcinomas that recapitulate many of the features observed during human oral carcinogenesis [7]. Despite the rapid growth in the field of NO research, the role of NO in oral carcinogenesis has not yet been fully elucidated. The purpose of this study was to investigate the presence, characteristics, and cellular localization of iNOS in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis.

2. Materials and methods

2.1. Hamsters and treatments

Outbred young (6-week-old; 30 animals) male Syrian golden hamsters (*Mesocricetus auratus*) (purchased

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from National Science Council Animal Breeding Centre, Republic of China), weighing about 100 g at the beginning of the experiment, were randomly divided into three groups, each containing 10 animals. The animals were housed under constant conditions (22°C, 12-h light/dark cycle) and fed with tap water and standard Purina laboratory chow ad libitum. The protocol ensured humane practices. After allowing the animals 1 week of acclimatization to their new surroundings, both pouches of a group of 10 animals were painted with a 0.5% DMBA solution at 09:00 on Monday, Wednesday and Friday of each week, using a No. 4 sable-hair brush. Bilateral pouches of each animal of another group were similarly treated with mineral oil. Approximately 0.2 ml of each solution was applied topically to the medial walls of both pouches at each painting. The last group of 10 animals were untreated throughout the experiment.

At the end of 15 weeks (3 days following the last treatment), all of the animals from each group were simultaneously killed at 09:00 by a lethal dose of diethyl ether to avoid the influence of diurnal variation [8]. The dead hamsters were then fixed in a supine position with pins. Their pouches were exposed by dissection, cut from their oral opening to their caudal ends along the middle of their lateral walls, and examined grossly. Both pouches were then excised and placed on cardboard to prevent distortion of the pouch tissues.

Six specimens of approximately equal length were taken from each pouch [9–11]. They were routinely processed for light microscopy by fixation in 10% neutral buffered formalin solution, dehydration in ascending alcohols, clearing in xylene and then embedding in paraffin. Two serial sections of each specimen were cut at 4- μ m thickness. One of the sections was prepared for iNOS immunohistochemistry study while another was used for hematoxylin–eosin staining. Thus, a total of six slides from each pouch and 12 from each animal were used for iNOS immunostaining.

2.2. Immunohistochemical staining of iNOS

Stainings were performed by a standard avidin–biotin peroxidase complex (ABC) method [12]. Polyclonal antibodies of mouse iNOS were raised on rabbits and obtained from Calbiochem-Novabiochem Corporation (Catalog. No. 482728). This antibody recognizes iNOS protein in human, rat and mouse and exhibits no cross-reactivities with eNOS and nNOS (information from manufacturer). After deparafinization in xylene and ethanol, the tissue sections were treated in 0.3% H₂O₂-methanol, and 10% normal goat serum. All sections were subsequently incubated with the primary antibody (1:500) at room temperature for 30 min. These sections were then incubated for 30 min at room temperature with biotin-conjugated goat anti-rabbit IgG (Vector,

Burlingame, USA; 1:100) and then for 30 min with ABC (Dako, Santa Barbara, USA). The sites of peroxidase binding were visualized as brown reaction products with benzidine reaction. The sections were counterstained with hematoxylin. The total number of iNOS-positive foci for each animal was calculated as the sum of the numbers of stained foci from the eight representative slides from both treated pouches. Negative controls for the specificity of anti-iNOS antisera were included by omitting the primary antisera.

3. Results

3.1. Gross observations and histopathology

Gross and histopathological changes in the DMBA-treated pouches were similar to those described in our previous study [9]. Areas of dysplasia and invasive squamous cell carcinomas with a 100% tumor incidence were developed in all of the DMBA-treated pouches. The mineral oil-treated and untreated pouches revealed no obvious changes.

3.2. iNOS immunohistochemistry

The possible patterns of iNOS positivity, randomly distributed in a pouch mucosa, may vary from a single cell to a cluster of two or more cells without the interruption of unstained cells. The average number of iNOS positive foci per section in the DMBA-treated group was approximately 12.2 ± 4.7 . Both cytoplasmic and nuclear stainings were observed in hyperplastic (Fig. 1), and dysplastic (Fig. 2) segments of the epithelium, as well as in invasive squamous cell carcinoma (Fig. 3). No iNOS activity could be detected in the untreated or mineral oil-treated pouches. Omission of the primary antisera in control sections disclosed negative findings for iNOS activity in all specimens.

4. Discussion

In the current study, the immunoreactivity was predominantly found in the DMBA-treated pouch keratinocytes. This suggests that high iNOS activity may be closely linked to carcinogenesis. To our knowledge, this may be the first study to report the presence of iNOS activity in oral chemical carcinogenesis. However, iNOS activity has previously been reported in patients with carcinomas of head and neck, lung, colon and prostate [13–16].

Chronic inflammation is well recognized as a risk factor for a variety of human cancers [17]. The central hypothesis used to explain the role of inflammation in carcinogenesis focuses on the role of oxygen radicals

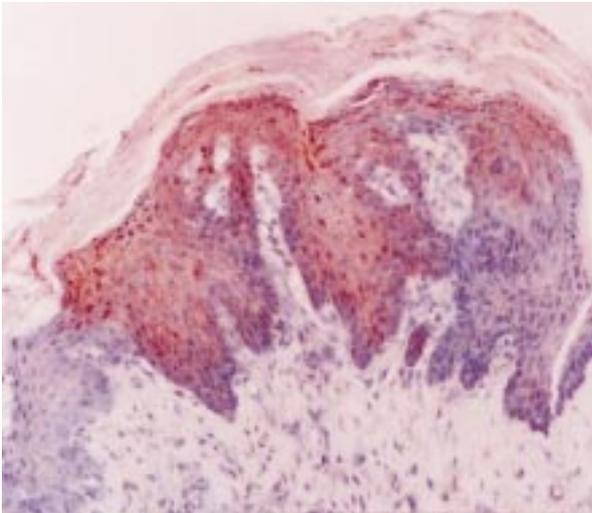


Fig. 1. An inducible nitric oxide synthase (iNOS)-positive focus in the hyperplastic segment of the hamster buccal pouch epithelium following 15 weeks of topical DMBA treatment. A large portion of the focus involved the whole layer but spared the keratin layer, which contained a few positive cells (ABC stain, $\times 100$).

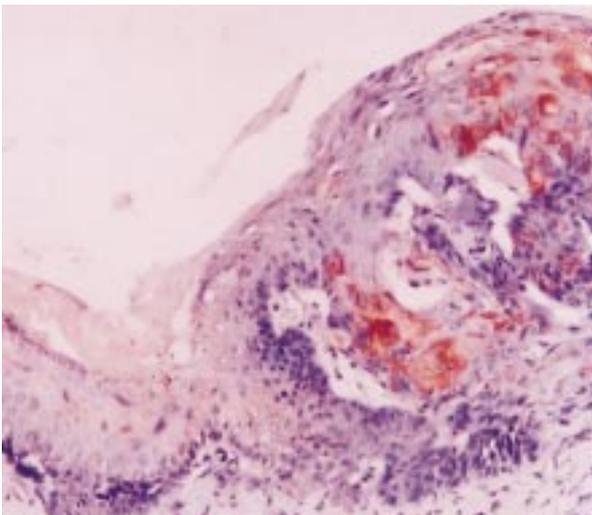


Fig. 2. Multiple inducible nitric oxide synthase (iNOS)-positive foci distributed randomly within the dysplastic segment of the hamster buccal pouch epithelium following 15 weeks of topical DMBA treatment (ABC stain, $\times 100$).

generated by phagocytic cells, which cause cytotoxicity and mutagenesis. This hypothesis has been fortified and expanded by the finding that NO and its derivatives are produced in copious quantities in inflamed tissues. Such a high level of production of NO following inflammation, despite its short-term immune benefit, may generate toxic intermediates capable of causing direct and indirect tissue damage, as well as genotoxicity and its resultant potential for carcinogenic effects [18].

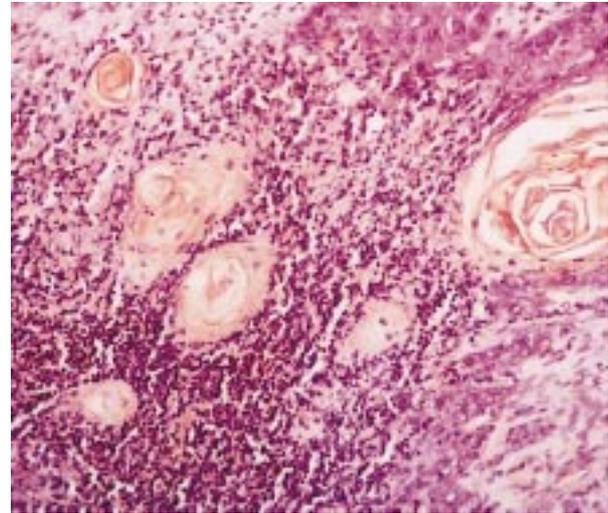


Fig. 3. Multiple invasive squamous cell carcinoma epithelial islands stained positive with inducible nitric oxide synthase (iNOS) after 15 weeks of topical DMBA treatment (ABC stain, $\times 100$).

Furthermore, excess NO produced in inflamed tissues could play a role in carcinogenesis by impairing the tumor-suppressor function of p53 [19]. A study of early adenocarcinoma in the lung found that an excess of NO may induce a p53 gene mutation containing mainly G:C-to-T:A transversion [20]. It is, therefore, reasonable to suggest that during episodes of chronic inflammation following DMBA treatment, pouch keratinocytes may have been exposed to a high concentration of NO over long periods of time. This long-term exposure may have resulted in an accumulation of mutations in the altered pouch keratinocytes either because of NO itself or through the potentiation of the genotoxic agent, DMBA. Future study of the co-expression of iNOS and p53 in inflamed tissues in DMBA-induced hamster buccal pouch carcinogenesis may substantiate this implication.

The induction of a high concentration of NO in some types of cancer cells may cause apoptosis [21]; however, cells with missing, non-functional or mutated p53 gene are found to exhibit resistance to NO-mediated killing [22]. In the present study, we demonstrated the presence of aberrant high expression of iNOS in DMBA-treated pouch keratinocytes. Given the fact that mutated p53 has been found in chemically induced hamster pouch carcinomas [23], it is reasonable to suggest that in a heterogeneous population of DMBA-treated keratinocytes, clonal selection, and hence growth advantage, favor those pouch keratinocytes with either a mutated or non-functional p53 gene which is capable of resisting NO-mediated killing. These altered keratinocytes may then further utilize NO in their microenvironment, facilitating cancer promotion and progression through a variety of possible mechanisms such as enhanced vascular permeability and increased blood flow [24]. A similar finding of excessive NO production permitting a

rapid tumor growth in rat solid tumor has been previously reported [25]. Therefore, it is conceivable that the mutated *p53* gene, or other as yet unknown genetic determinants, may function in determining the role of NO in oral experimental carcinogenesis. Further research on the nature of the interaction between iNOS and *p53* in DMBA-induced hamster buccal pouch carcinomas may shed new light on the molecular mechanism of the oral chemical carcinogenesis.

iNOS has been suggested to be an immunohistochemical marker for malignant neoplasia of the prostate [16]. Whether this relationship is also present in oral carcinomas cannot be determined from the results of the present study. Further detailed study to establish the sequential expression and subsequent fate of iNOS-stained keratinocytes in precancerous lesions during DMBA-induced hamster buccal pouch carcinogenesis may help to determine whether iNOS is a marker for oral carcinoma.

In conclusion, the present study used immunohistochemistry to demonstrate the expression of iNOS in DMBA-induced hamster pouch carcinomas. This finding indicates a possible association of iNOS with the development of chemically induced oral carcinomas. Much work remains to be done to unravel the precise role(s) played by iNOS in oral chemical carcinogenesis.

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