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The mRNA expression of inducible nitric oxide synthase in DMBA-induced hamster buccal-pouch carcinomas: an *in situ* RT-PCR study

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Summary. Three isoforms of nitric oxide synthase (NOS) have been identified: endothelial NOS, neuronal NOS, and inducible NOS (iNOS). The enhanced expression of iNOS at the protein level using immunohistochemical technique has been reported previously in chemically induced oral carcinomas in hamster buccal-pouch mucosa. However, the corresponding expression of iNOS at the mRNA level has not yet been demonstrated using *in situ* reverse transcription-polymerase chain reaction (IS RT-PCR). The purpose of the present study is to assess the iNOS mRNA expression level in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch carcinomas using IS RT-PCR. Thirty outbred, young (6-weeks old), male, Syrian golden hamsters (*Mesocricatus auratus*) were randomly divided into one experimental group (10 animals), and two control groups (10 animals each). The pouches of a group of 10 animals of the experimental group were painted bilaterally with a 0.5% DMBA solution three times a week for 15 weeks. Each animal of one of the control groups was similarly treated with only mineral oil. Another control group of 10 animals remained untreated throughout the experiment. Invasive squamous-cell carcinomas with a 100% tumour incidence developed in all of the DMBA-treated buccal pouches. The mineral oil-treated and untreated pouches revealed no obvious changes. Inducible NOS mRNA was demonstrated amongst all the 15-week DMBA-treated hamster buccal-pouch mucosa animals, but not in the untreated animals and not in the animals for which the buccal-pouch was treated with mineral oil. Further study is necessary to evaluate the mechanism(s) which contribute to the increased iNOS mRNA expression for experimentally induced oral carcinogenesis.

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Introduction

Nitric oxide (NO), an important mediator in various physiological and pathological activities including inflammatory process and cancer formation, is produced when L-arginine is metabolized to L-citrulline by nitric oxide synthase (NOS; E.C.1.14.13.39). At least three isoenzymes have been identified, i.e. calcium-dependent neuronal and endothelial isoforms that are constitutively expressed, and a calcium-independent (iNOS) isoform that is inducible (Ambs *et al*. 1998; Thomsen & Miles 1998; Reveneau *et al*. 1999). Neuronal and endothelial NOS usually produce small amounts of NO that last a short time (minutes), whereas iNOS generates large amounts of NO that last up to several days (Ambs *et al*. 1998; Thomsen & Miles 1998; Reveneau *et al*. 1999).

Nitric oxide has been postulated to have play a multifaceted role in cancer (Wink *et al*. 1998). It has been implicated in a positive role, in permitting tumour growth, including mutagenicity, angiogenesis and metastasis, but has also been involved in the cytotoxicity of macrophages toward tumour cells (Hevel *et al*. 1991). Additionally, NO reacts with the superoxide anion to form a per oxynitrite anion, a highly toxic molecule causing both DNA damage and protein modifications (Nguyen *et al*. 1992).

The hamster buccal-pouch mucosa provides one of the most widely accepted experimental models for oral carcinogenesis (Gimenez-Conti & Slaga 1993). Despite anatomical and histological variations between (hamster) pouch mucosa and human buccal tissue, experimental carcinogenesis protocols for the former induce premalignant changes and carcinomas that are similar to the development of premalignancy and malignancy in human oral mucosa (Morris 1961). To date the role of NO in experimental-induced oral carcinogenesis remains to be fully elucidated. The aim of this study was to investigate the mRNA expression of iNOS in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch carcinomas.

Materials and methods

Animals

Thirty outbred, young (6-weeks old), male, Syrian golden hamsters (*Mesocricatus auratus*) (purchased from National Science Council Animal Breeding Centre, ROC), weighing about 100 g each at the commencement of the experiment, were randomly divided into one experimental group (10 animals), and two control groups (10 animals each). 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 animal-handling protocol ensured humane practices throughout the experimental process. Subsequent to allowing the animals 1 week of acclimatization to their new surroundings, both pouches of each animal in the experimental group of 10 animals were painted with a 0.5% DMBA solution (Sigma, purity: approx. 95%) was dissolved in mineral oil (Sigma, purity: 100%) at 9 a.m. on Monday, Wednesday and Friday of each week, using a no. 4 sable-hair brush. Both pouches of each animal of one of the control groups of 10 animals were similarly treated with mineral oil (Sigma, purity: 100%). Approximately 0.2 mL of the respective solution was applied topically to the medial walls of both pouches at each painting. Another control group of 10 animals remained untreated throughout the experiment.

At the end of 15 weeks of such treatment (three days following the last treatment), in order to avoid the potential influence of any diurnal variation (Lin & Chen 1997), all of the animals from each group were simultaneously killed at 9 a.m. by inhaling a lethal dose of diethyl ether. The animals' pouches were exposed by dissection, and 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 fixed in 10% neutral-buffered formalin solution for about 24 h, dehydrated in ascending alcohols, cleared in xylene, and embedded in paraffin for light microscopy. Two serial sections of each specimen were cut at 4-μm-thickness. One of the sections was prepared for IS RT-PCR study while another was used for haematoxylin–eosin staining. In order to test the specificity of the iNOS primers, a portion of selective samples of DMBA-treated pouch tissues was immediately frozen in liquid nitrogen for subsequent RNA extraction and routine RT-PCR reaction.

Preparation of tissue sections

RNase-free conditions were used throughout the slide preparation and the IS RT-PCR procedure. After deparaffinization and dehydration, the section was pretreated with 10 μg/mL proteinase K (Sigma, St. Louis, MO) for 1 min at room temperature at which time enzyme activity was prevented with 0.1 M glycine in PBS.

Table 1. Oligonucleotide primers used to amplify iNOS cDNAs

Oligonucleotide primers	cDNA positions	Sequences	PCR products
iNOS sense	1425–1441	5′-GCC TCG CTC TGG AAA GA-3′	499 bp
iNOS antisense	1908–1924	5′-TCC ATG CAG ACA ACC TT-3′	

Reverse transcription and amplification

The Titan one-tube RT-PCR system (Boehringer Mannheim, Indianapolis, IN) was used to perform this IS RT-PCR reaction. The final concentration for the RT-PCR reaction mixture was as follows: 200 μM each of deoxyribonucleotide (dATP, dCTP, dGTP), 180 μM dTTP, 40 μM Dig-11-DTP, 0.4 μM downstream primer, 0.4 μM upstream primer, 5 mM dithiothreitol solution (DTT), 1.5 mM $MgCl_2$ and 1 μL Expanol high-fidelity enzyme mix (all of the reagents were obtained from Boehringer Mannheim, with the exception of primers). The oligonucleotide primers specific for iNOS were purchased from Genset Corp. (La Jolla, CA) (Table 1). The primer pairs were chosen from the published cDNA sequences of iNOS (GenBank accession no. D14051). The specificity of the primers has been tested with selective fresh tissues of the DMBA-induced hamster buccal-pouch carcinomas using the standard procedures for routine RT-PCR. Hybaid Sure-Seal (Hybaid Instruments, Holbrook, NY) was placed around the sample on the slides. The reaction mixture was then carefully pipetted onto each tissue section. After coverslips were applied, slides were placed on the block of a thermal cycler (TaKaRa MP, Tokyo, Japan). Reverse transcription was carried out at 50 °C for 30 min. PCR amplification was carried out with an initial denaturing step at 94 °C for 2 min and then 20 cycles of amplification with denaturing at 94 °C for 30 s; annealing at 55 °C for 30 s and elongation at 68 °C for 2 min; and a final extension of 68 °C for 7 min.

Immunohistochemical detection of digoxigenin

We used the DIG nucleic acid detection kit (Boehringer Mannheim) to detect the digoxigenin tagged IS RT-PCR amplified products. The slides, after rinsing with PBS, were incubated in anti-digoxigenin antibody conjugated with alkaline phosphatase diluted at 1 : 500 for 2 h. A subsequent enzyme-catalysed colour reaction solution with 5-bromo-4-chloryl-3-indolyl phosphate and nitro-bluetetrazolium salt was applied on the slides to produce an insoluble purple coloration. Colour development, checked under the microscope, was terminated by washing the slides with Tris buffer containing EDTA within approximately 10 min or as soon as the purple colour was observed in any of the slides. Slides were then counterstained with nuclear Fast Red, and cover-slipped with a water-soluble mounting medium. The positivity of iNOS mRNA of each section was observed using a light microscope.

Results

Gross observations and histopathology

Gross and histopathological changes in the DMBA-treated pouches were similar to those described in our previous study (Chen & Lin 1996). Invasive squamous cell carcinomas with a 100% tumour incidence developed in all of the DMBA-treated pouches. The mineral oil-treated and untreated pouches revealed no obvious changes.

In situ *reverse transcription-polymerase chain reaction*

The iNOS mRNA was detected in all of the samples of hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks (Figure 1). The iNOS mRNA staining was also present in cells of the tumour stroma, presumed to be macrophages (Figure 1). Consistent with our previous study using immunohistochemistry (Chen & Lin 2000),

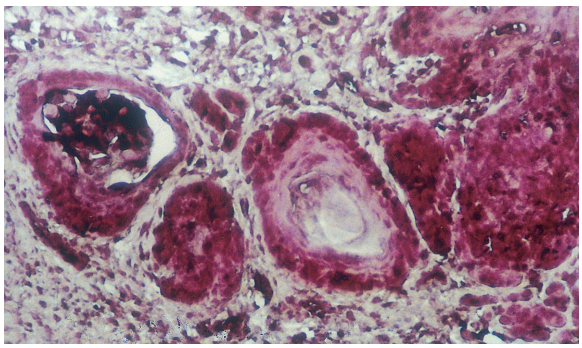

Figure 1. Inducible NOS mRNA as shown by the purple coloration was identified in the invasive tumour islands of a representative sample of hamster buccal-pouch tissue specimen treated with DMBA for 15 weeks. Note that iNOS mRNA staining was also present in cells of the tumour stroma, presumed to be macrophages (×100).

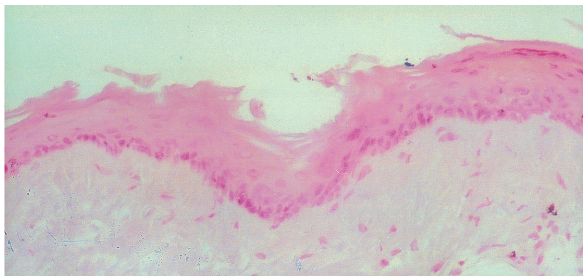

Figure 2. Inducible NOS mRNA activity could not be found in a representative sample of non-treated pouch tissue specimen (×100).

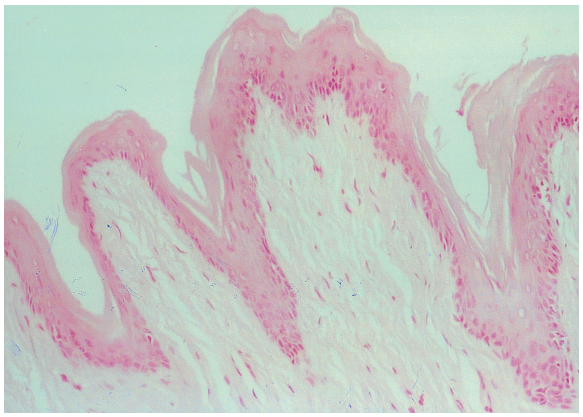

Figure 3. Inducible NOS mRNA activity could not be detected in a representative sample of mineral oil-treated pouch tissue specimen (×100).

a purple staining for the detection of iNOS mRNA using IS RT-PCR was noted in comparable sites (cytoplasm and nuclei of the tumour islands). Inducible NOS mRNA activity could not be found in the untreated (Figure 2) and the mineral oil-treated (Figure 3) pouches. Omission of the primers in control sections showed negative findings for iNOS mRNA activity in all specimens (Figure 4).

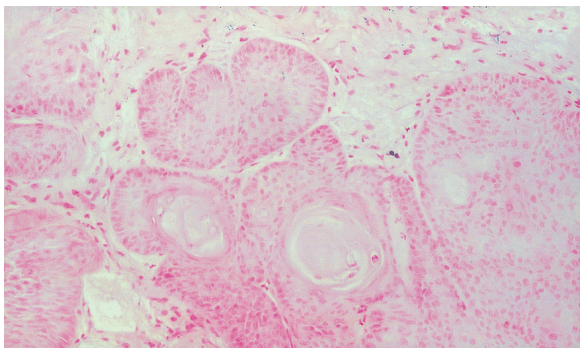

Figure 4. Omission of the primers in control sections showed negative finding for iNOS mRNA activity (×100).

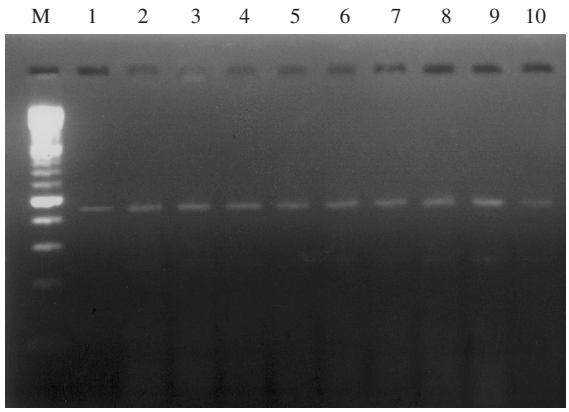

Figure 5. A band corresponding to 499 bp was observed for all the selected samples of hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks (lanes 1–10). Lane M is the DNA molecular-weight marker.

A band corresponding to 499-bp was observed for all the selected samples of hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks (Figure 5).

Discussion

Using immunohistochemical techniques, we have previously demonstrated an enhanced expression of iNOS at the protein level in chemically induced oral carcinomas in hamster buccal-pouch mucosa (Chen & Lin 2000). However, the corresponding expression of iNOS at the mRNA level using IS RT-PCR has not, to the best of our knowledge, been reported previously for hamster buccal-pouch carcinomas. Using the same experimental model system, we now report the presence of iNOS mRNA in DMBA-induced hamster buccal-pouch carcinomas with IS RT-PCR. Therefore, it appears reasonable to suggest that the escalation in activity of iNOS mRNA may be closely related to chemically induced oral carcinogenesis.

Compared with the demonstration of iNOS mRNA by RT-PCR, this method (IS RT-PCR) provides important additional information about the cellular localization of mRNA. Furthermore, the IS RT-PCR technique can use archival tissues and is not constrained by methods of tissue fixation. Compared to immunohistochemical approaches, the preservation of tissue morphology was less than ideal, but the general outlines of tissue architecture, especially the epithelial vs. stromal boundaries, were clearly distinguishable.

This study shows that iNOS mRNA is over-expressed in the hamster buccal-pouch carcinomas that develop from DMBA treatment. It does not demonstrate whether or not over-expression of iNOS mRNA in the early stages of

DMBA treatment is a risk factor for the development of carcinomas. Further study on the sequential expression of iNOS mRNA during DMBA-induced hamster buccal-pouch carcinogenesis is required to understand a role (e.g. in initiation or promotion) of iNOS on oral carcinogenesis.

The exposure of normal human hepatocytes to a high level of NO could initiate a series of events that rapidly leads to damage of both nuclear and mitochondrial DNA, such damage subsequently leading to cell-cycle arrest and mitochondrial dysfunction, and ultimately resulting in the pathological events associated with the development of hepatocellular carcinoma (D'Ambrosio *et al*. 2001).

Recently, iNOS expression has been correlated with p53 for human oral epithelial dysplasia (Brennan *et al*. 2000a) and oral squamous-cell carcinoma (Brennan *et al*. 2000b). Thus it appears worthwhile to attempt to verify whether or not a similar correlation between NOS and p53 exist in cases of DMBA-induced hamster buccal-pouch carcinogenesis. Subsequent experiments focusing upon the interaction between iNOS and p53 in DMBA-induced hamster buccal-pouch carcinogenesis may provide a new insight to the mechanism of chemically induced carcinogenesis in the oral cavity.

Inducible NOS protein has been suggested as an immunohistochemical marker for malignant neoplasia in prostate tissue (Klotz *et al*. 1998). Assessed at the mRNA level in the present study, we have demonstrated that iNOS may also be a potential marker at the mRNA level for DMBA-induced hamster buccal-pouch carcinomas. In addition, high expression of iNOS mRNA in DMBA-induced hamster buccal-pouch carcinomas as shown in this study may suggest a possible therapeutic approach through the inhibition of tumour growth by a novel inhibitor of iNOS such as N-(3-(aminomethyl)-benzyl)acetmidine (1400 W) (Thomsen *et al*. 1997).

In conclusion, enhanced expression of iNOS mRNA in DMBA-induced hamster buccal-pouch carcinomas compared with the untreated and mineral oil-treated counterparts in the current study, is consistent with our previous work at the protein level using immunohistochemical techniques (Chen & Lin 2000), suggesting that iNOS gene expression may be regulated at the level of mRNA. However, further work remains to be done to unravel the molecular mechanism(s) that modulate the increased iNOS mRNA expression in experimentally induced oral carcinogenesis.

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