The mRNA expression of inducible nitric oxide synthase in DMBA-induced hamster buccal-pouch carcinomas using reverse transcription-polymerase chain reaction

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Abstract

Background: Three isoforms of NO synthase (NOS) have been identified: endothelial NOS, neuronal NOS, and inducible NOS (iNOS). The enhanced expression of iNOS, at the protein level, has been reported previously in certain chemically induced oral carcinomas in hamster buccal-pouch mucosa, however, the corresponding expression of iNOS, at the mRNA level, has not yet been demonstrated. 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 a reverse transcription-polymerase chain reaction (RT-PCR).

Methods: Thirty-three outbred, young (six-weeks old), male, Syrian golden hamsters (Mesocricatus auratus) were randomly divided into one experimental group (13 animals) and two control groups (10 animals each). The pouches of a group of 13 animals of the experimental group were bilaterally painted with a 0.5% DMBA solution three times a week for 12 weeks. Each animal of one control group was similarly treated with mineral oil only, while the other control group of 10 animals remained untreated throughout the experiment.

Results: Areas of dysplasia and squamous-cell carcinomas, with a 100% tumor incidence, developed for all of the DMBA-treated buccal pouches. The mineral oil-treated and untreated pouches had no obvious changes. A band of 499-bp, corresponding to iNOS mRNA, was present in all the DMBA-treated hamster buccal-pouch mucosa animals, but not in the untreated animals or the animals treated with mineral oil. Upon direct sequencing, this 499-bp band was confirmed to be part of the iNOS gene.

Conclusions: This study demonstrated that increased iNOS mRNA expression could contribute to the mechanism for experimentally induced oral carcinogenesis.

Key words: DMBA-carcinogenesis; hamster; inducible nitric oxide synthase; mRNA; RT-PCR

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Nitric oxide (NO), a gaseous free radical, is a short-lived molecule that possesses a multitude of biological functions including the potential to induce inflammation and cancer (1). Nitric oxide is synthesized

from a family of enzymes named NO synthase (NOS; E.C.1.14.13.39) that convert L-arginine to L-citrulline and thus generate NO (2). Three isoforms of NO have been identified and characterized to date: two of them (endothelial NOS or eNO, a 132-kDa protein and neuronal NOS or nNOS, a 162-kDa protein) are present constitutively and are calcium dependent. The other isoform (inducible NOS or iNOS, a 130-kDa protein), which is induced in the body during the inflammatory processes, is calcium independent (3).

Nitric oxide reacts rapidly and nonenzymatically with the superoxide anion to form a peroxynitrite anion, a highly toxic molecule which is responsible for eliciting a wide range of DNA damage and protein modifications (4). The peroxynitrite ion 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 of NO could be essential to its participation in carcinogenesis.

The hamster buccal-pouch mucosa provides one of the most widely accepted experimental models for oral carcinogenesis (6). Despite anatomical and histological differences 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 (7). To date, little is known of the role of NO in experimentally induced oral carcinogenesis. The purpose of this study was to investigate the mRNA expression of iNOS for 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch carcinomas.

Materials and methods

Hamsters and treatments

Thirty-three outbred, young (six-weeks old), male, Syrian golden hamsters (Mesocricatus auratus) (purchased from National Science Council Animal Breeding Centre, Taipei, ROC), weighing about 100 g each at the commencement of the experiment, were randomly allocated to the experimental group (13 animals) or one of the two control groups (10 animals each). The animals were housed under constant conditions (22°C, 12h light/dark cycle) and fed with tap water and standard Purina laboratory chow ad libitum. The animal-handling protocol ensured that the test animals were humanely treated throughout the experimental process. Subsequent to allowing the animals to acclimatize to their new surroundings for one week, both pouches of the 13 animals in the experimental group were painted with a 0.5% DMBA solution at 9a.m. on the 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 were similarly treated by topical application of approximately 0.2ml mineral oil to the medial walls of both pouches at each painting. Another control group of 10 animals remained untreated throughout the experiment.

After 12weeks of such treatment (three days after the last treatment), to avoid the potential influence of any diurnal variation (8), all the animals from each group were simultaneously killed at 9a.m., using a lethal dose of diethyl ether. The animals' pouches were exposed by dissection, and cut from the oral opening to the caudal ends along the middle of the lateral walls and examined grossly. Both pouches were then excised. A portion of the pouch tissue was immediately frozen in liquid nitrogen for subsequent RNA extraction, and another portion was fixed in 10% neutral-buffered formalin solution, dehydrated in ascending alcohols, cleared in xylene, and embedded in paraffin for light microscopy.

Extraction of RNA

Total RNA was extracted by homogenizing the hamster buccal-pouch tissue specimens in guanidium isothiocyanate followed by ultracentrifugation in caesium chloride, as described previously (9). The RNA concentration was determined from the optical density at a wavelength of 260nm (using an OD_{260} unit equivalent to 40μg/ml of RNA). In brief, the pouch tissue (~250mg) was added to 500μl of denaturing solution (4M guanidium thiocyanate, 25mM sodium citrate [pH = 7.0], 0.5% sodium N-sarosine) with 50μl of 2M NaOAc (pH = 4.0), 500μl of water-saturated phenol, and 100μl of chloroform:isoamyl alcohol (1:1, v/v). The homogenized pouch tissue, plus solution, was then vortexed thoroughly. The RNA was subsequently precipitated with an equal volume of isopropanol. The pellet was rinsed twice with 1ml of 75% ethanol and dried in air. The RNA was resuspended in 100μl of diethlpyrocarbonate (DEPC)-treated water at a final concentration of 1μg/μl and stored at −80°C until further use.

Reverse transcription reaction (cDNA synthesis)

Isolated total RNA (1μg) was reverse-transcribed to cDNA in a reaction mixture (with a final volume of 20μl) containing 4μl of $MgCl_2$ (5mM), 2μl of 10 x reverse transcription buffer (10mM Tris-HCl [pH = 9.0], 50mM KCl, 0.1% Triton® X-100), 2μl of dNTP mixture (1mM each), 0.5μl of recombinant RNasin® ribnonuclease inhibitor (1μ/μl), 15 units of avian-myeloblastosis-virus (AMV) reverse transcriptase (High Conc.) (15μ/μg), 0.5μg of oligo$(dT)_{15}$ primer (Promega, catalogue no. A3500, WI, USA). The reaction mixture was incu-

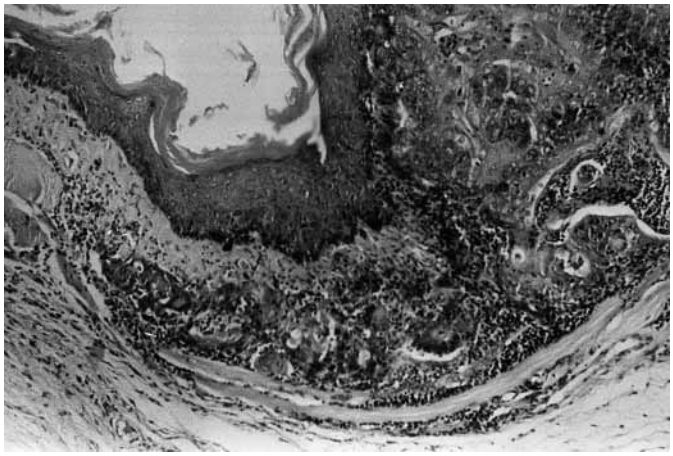

Fig. 1. Areas of epithelial dysplasia and squamous-cell carcinoma were noted in a representative sample of a 12-week DMBA-treated hamster buccal-pouch carcinoma (H&E ×40).

bated for 15 min at 42 °C. The AMV reverse transcriptase was inactivated by heating for five minutes at 99 °C and then incubated at 0–5 °C for a further five minutes.

Polymerase chain reaction amplification

All oligonucleotide primers were purchased from Genset corp. [La Jolla (CA), USA]. The primer pairs were chosen from the published cDNA sequences of iNOS (10, GenBank accession no. D14051), and β-actin (11, GenBank accession no. X-00351). Oligonucleotide primers for PCR reactions were as shown in Table 1.

The 20 μl first-strand cDNA-synthesis reaction product obtained from the reverse transcriptase (RT) reaction was diluted to 100 μl with nuclease-free water. The PCR amplification reaction mixture (with a final volume of 100 μl) contained 20 μl of diluted, first-strand cDNA reaction product (< 10 ng/μl), 2 μl of cDNA reaction dNTPs (200 μM each), 4 μl of $MgCl_2$ (2 mM), 8 μl of 10 x reverse transcription buffer (10 mM Tris-HCl, [pH = 9.0], 50 mM KCl, 0.1% Triton® X-100), 50 pmol of upstream primer, 50 pmol of downstream primer, and 2.5 units of *Taq* DNA polymerase (Promega, catalogue no. M7660).

The PCRs were carried out on a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Thermocycling conditions included denaturing at 94 °C for one minute (one cycle), then denaturing at 94 °C (60 s), annealing at 55 °C (60 s) for iNOS or at 60 °C (60 s) for β-actin, and extending at 72 °C (60 s) for 30 cycles and a final extension at 72 °C for seven minutes. The β-actin primers were utilized as positive controls. Negative controls without RNA and without reverse transcriptase were also performed. Amplification products were analyzed by electrophoresis in a 2% agarose gel along with the DNA molecular weight marker (Boehringer Mannheim, Mannheim, Germany) containing ethidium bromide. The PCR products were visualized as bands with a UV transilluminator. Photographs were taken with a Polaroid DS-300 camera. The PCR products were then sequenced to confirm their identities using a T7 Sequenase kit (version 2.0, Amersham International, Little Chalfont, UK).

Results

Gross observations and histopathology

Gross and histopathological changes in the 12-week DMBA-treated pouches were similar to those described in our previous study (12). Areas of dysplasia and squamous-cell carcinomas (Fig. 1) with a 100% tumor incidence were apparent for all of the 12-week DMBA-treated pouches. The mineral oil-treated and untreated pouches revealed no obvious changes.

Reverse transcription-polymerase chain reaction

A band corresponding to 499-bp was observed for all the hamster buccal-pouch tissue specimens treated with DMBA for 12 weeks (Fig. 2). Upon direct sequencing, this 499-bp band was confirmed to be part of the iNOS gene. No such bands were noticed for the untreated animals, the mineral oil-treated tissues and the negative-control samples (Fig. 2). All samples, except the negative-control samples, revealed bands of β-actin (350-bp) (Fig. 2).

Table 1. Oligonucleotide primers used to amplify iNOS and β-actin 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′	
β-actin sense	628–657	5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′	350-bp
β-actin antisense	1048–1077	5′-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3′	

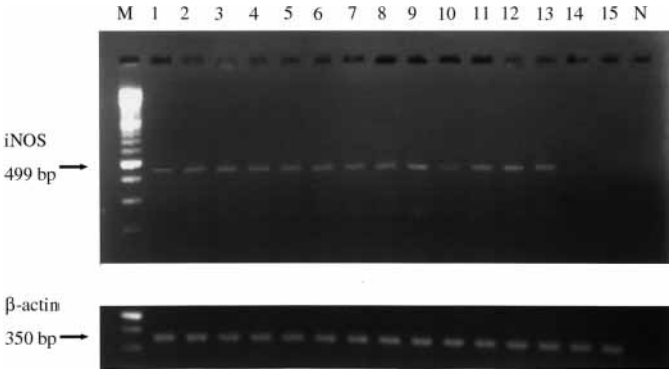

Fig. 2. A band corresponding to approximately 499-bp was observed for all the hamster buccal-pouch tissue specimens treated with DMBA for 12 weeks (lanes 1–13). No bands were noted for untreated (lane 14), mineral oil-treated (lane 15), and the negative control (lane N) samples. All samples (lanes 1–15) except the negative control sample (lane N) revealed bands of β-actin (350-bp). Lane M is the DNA molecular-weight marker.

Discussion

The expression of iNOS mRNA in chemically induced oral carcinomas has not, to the best of our knowledge, been reported previously for hamster buccal-pouch mucosa. Using RT-PCR, iNOS mRNA was predominantly found in the DMBA-treated pouch carcinomas in the current study, however, the expression of mRNA of iNOS has already been noted for patients demonstrating gastric carcinomas (13). This suggests that the high activity of iNOS mRNA may be closely related to chemically induced oral carcinogenesis.

The current study merely 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 the role (initiation or promotion) of iNOS in oral carcinogenesis. Furthermore, subsequent study using *in situ* RT-PCR may help demonstrate the distribution of iNOS mRNA in hamster buccal-pouch carcinomas.

A large number of both experimental and clinical reports suggested the expression of iNOS in the tissues of chronic inflammatory diseases including cancer (14). A considerable portion of the carcinogenic process involves chronic inflammation, particularly in the post-initiation stage (15). The hypothesis explaining the role of inflammation in carcinogenesis chiefly focuses on the cytotoxicity and mutagenesis elicited by oxygen-radicals, generated by phagocytic cells. This hypothesis is further supported by the discovery that NO and its derivatives are produced in copious quantities in inflamed tissues (16). Such a high level of NO following inflammation, without homeostatic regulation, may then lead to DNA damage and mutations (16). Despite it being a subject of some conjecture, it is worth considering that due to episodes of chronic inflammation following DMBA treatment, pouch keratinocytes typically suffered from the high concentration of NO. This prolonged exposure of tissue to NO may induce an accumulation of mutations in the DMBA-treated pouch keratinocytes either as a direct result of the presence of NO itself or through the potentiation of DMBA.

The high concentration of NO produced in the DMBA-treated pouch tissue could contribute to carcinogenesis by impairing the tumor suppressor function of p53 gene (17). A study of early adenocarcinoma in the lung found that an excess of NO may have induced a p53 gene mutation containing, mainly, a G:C-to-T:A transversion (18).

The high level of NO in some types of cancer cells may induce apoptosis (19), however, cells with a mutated or non-functional p53 gene have demonstrated resistance to NO-mediated cell killing (20). An aberrantly high expression of iNOS mRNA was found in the DMBA-treated pouch keratinocytes in the present study. Hence, clonal selection may favor those pouch keratinocytes containing either a mutated or non-functional p53 gene able to resist NO-mediated killing. These altered keratinocytes subsequently utilize NO in their microenvironment, permitting cancer development via a plethora of possible pathways such as development of an enhanced vascular permeability and increased blood flow (21).

Recently, iNOS expression was correlated with p53 for human oral epithelial dysplasia (22) and oral squamous-cell carcinoma (23). It therefore appears worthwhile to attempt to verify whether a similar correlation exists between NOS and p53 in cases of DMBA-induced hamster buccal-pouch carcinogenesis. Subsequent work focusing upon the interaction between iNOS and p53 in DMBA-induced hamster buccal-pouch carcinogenesis may provide a new insight into the mechanism of chemically induced carcinogenesis in the oral cavity.

Inducible NOS protein has been induced in premalignant and malignant, but not normal, clinical specimens obtained from stomach (24), colon (25), lung (26), esophagus (27), prostate (28), and the oral cavity (22, 23, 29, 30), suggesting that iNOS is an immunohistochemical marker for these malignant neoplasia. Being assessed at an mRNA level in the present study, we have also demonstrated that iNOS is a valuable marker at an mRNA level for DMBA-induced hamster buccal-pouch carcinomas.

The 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 using immunohistochemical techniques at the protein level (12). This suggests that iNOS gene expression may be regulated at the level of mRNA, however, more studies needs to be conducted done to unravel the molecular mechanism(s) that regulate the increased iNOS mRNA expression in experimentally induced oral carcinogenesis.

References

1. Nathan C, Xie Q. Nitric oxide synthases: roles, tolls, and controls *Cell* 1994; **78**: 915–8.
2. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994; **298**: 249–58.
3. Morris SM, Billiar TR. New insights into the regulation of inducible nitric oxide synthesis. *Am J Physiol* 1994; **266**: E829–39.
4. Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR. DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc Nat Acad Sci, USA* 1992; **89**: 3030–4.
5. Henry Y, Lepoivre M, Drapier JC, Ducrocq C, Boucher JL, Guissani A. EPR characterization of molecular targets for NO in mammalian cells and organelles. *FASEB J* 1993; **7**: 1124–34.
6. Gimenez-Conti IB, Slaga TJ. The hamster cheek pouch carcinogenesis model. *J Cell Biochem* 1993; **17F**: 83–90.
7. Morris AL. Factors influencing experimental carcinogenesis in the hamster cheek pouch. *J Dent Res* 1961; **40**: 3–15.
8. Lin LM, Chen YK. Diurnal variation of γ-glutamyl transpeptidase activity during DMBA-induced hamster buccal pouch carcinogenesis. *Oral Dis* 1997; **3**: 153–6.
9. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–9.
10. Gradini R, Realacci M, Ginepri A, et al. Nitric oxide synthases in normal and benign hyperplastic human prostate: immunohistochemistry and molecular biology. *J Pathol*; **189**: 224–9.
11. Briggs JP, Todd-Turla K, Schnermann JB, Killen PD. Approach to molecular basis of nephron heterogenicity: application of reverse transcription-polymerase chain reaction to dissected tubule segments. *Semin Nephrol* 1993; **13**: 2–12.
12. Chen YK, Lin LM. Immunohistochemical expression of inducible nitric oxide synthase in DMBA-induced hamster buccal pouch carcinogenesis. *Oral Oncol* 2000; **36**: 221–4.
13. Doi C, Noguchi Y, Marat D, et al. Expression of nitric oxide synthase in gastric cancer. *Cancer Lett* 1999; **144**: 161–7.
14. Ohshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res* 1994; **305**: 253–64.
15. Murakami A, Kawabata K, Koshiba T, et al. Nitric oxide synthase is induced in tumor promoter-sensitive, but not tumor promoter-resistant, JB6 mouse epidermal cells cocultured with interferon-γ-stimulated RAW 264.7 cells: The role of tumor necrosis factor-α. *Cancer Res* 2000; **60**: 6326–31.
16. Tamir S, Tannenbaum SR. The role of nitric oxide (NO) in the carcinogenesis process. *Biochim Biophys Acta* 1996; **1288**: F31–6.
17. Calmels S, Hainaut P, Ohshima H. Nitric oxide induces conformational and functional modifications of wild-type p53 tumor suppressor protein. *Cancer Res* 1997; **57**: 3365–9.
18. Fujimoto H, Sasaki JI, Matsumoto M, et al. Significant correlation of nitric oxide synthase activity and p53 gene mutation in stage I lung adenocarcinoma. *Jpn J Cancer Res* 1998; **89**: 696–702.
19. Xie K, Huang S, Dong Z, et al. Transfection with inducible nitric oxide synthase gene suppresses tumorigenicity and abrogates metastasis in K-1735 murine melanoma cells. *J Exp Med* 1995; **181**: 1333–43.
20. Ambs S, Hussain SP, Harris CC. Interactive effects of nitric oxide and the p53 tumor suppressor gene in carcinogenesis and tumor progression. *FASEB J* 1997; **11**: 433–8.
21. Fukumura D, Jain RK. Role of nitric oxide in angiogenesis and microcirculation in tumors. *Cancer Meta Rev* 1998; **17**: 77–89.
22. Brennan PA, Conroy B, Sinclair D, Spedding AV. Expression of inducible nitric oxide synthase and p53 in oral epithelial dysplasia. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000; **90**: 624–9.
23. Brennan PA, Palacios-Callender M, Sinclair D, Spedding AV, Zaki GA. Correlation between type II nitric oxide synthase and p53 expression in oral squamous cell carcinoma. *Br J Oral Maxillofac Surg* 2000; **38**: 627–32.
24. Fu S, Ramanujam KS, Wong A, Fantry GT. Increased expression and cellular localization of iNOS and CSO-2 in Helicobacter pylori gastritis. *Gastroenterology* 1999; **116**: 1319–29.
25. Dijksta G, Moshage H, van Dullemen HM, et al. Expression nitric oxide synthase formation nitrotryosine reactive oxygen species inflammatory bowel disease. *J Pathol* 1998; **186**: 416–21.
26. Liu CY, Wang CH, Chen TC, Lin HC, Yu CT, Kuo HP. Increased level of exhaled nitric oxide and up-regulation of nitric oxide synthase in patients with primary lung cancer. *Br J Cancer* 1998; **78**: 534–41.
27. Wilson KT, Fu S, Ramanujam KS, Meltzer SJ. Increased expression of iNOS and COX-2 in the Barrett's esophagus and associated adenocarcinomas. *Cancer Res* 1998; **58**: 2929–34.
28. Klotz T, Bloch W, Volberg C, Engelmann U, Addicks K. Selective expression of inducible nitric oxide synthase in human prostate carcinoma. *Cancer* 1998; **82**: 1897–903.
29. Brennan PA, Palacios-Callender M, Sinclair D, Spedding AV, Zaki GA. Does expression of inducible nitric oxide synthase correlate with severity of oral epithelial dysplasia? *J Cranio-Maxillofac Surg* 2000; **28**: 44–8.
30. Brennan PA, Palacios-Callender M, Zaki GA, Spedding AV, Langdon JD. Type II nitric oxide synthase (NOS2) expression correlates with lymph node status in squamous cell carcinoma. *J Oral Pathol Med* 2001; **30**: 129–34.

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