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Experimental Oral Pathology

Correlation between inducible nitric oxide synthase and p53 expression for DMBA-induced hamster buccal-pouch carcinomas

YK Chen, SS Hsue, LM Lin

Oral Pathology Department, School of Dentistry, Kaohsiung Medical University, Kaohsiung, Taiwan

OBJECTIVES: Three isoforms of nitric oxide synthase (NOS) have been identified previously – endothelial NOS, neuronal NOS, and inducible NOS (iNOS). It has been reported previously that there may be a negative feedback loop existing between nitric oxide (NO) production and wild-type p53 tumor-suppressor gene, but the relationship has not previously been studied for oral experimental carcinogenesis. The purpose of the present study is to assess whether iNOS expression correlates with p53 expression at both protein and mRNA levels for 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch carcinomas.

MATERIALS AND METHODS: Thirty-five out-bred, young (6 week old), male, Syrian golden hamsters (*Mesocricatus auratus*) were randomly divided into one experimental group (15 animals), and two control groups (10 animals each). Bilaterally, the pouches of a group of 15 animals from the experimental group were painted with a 0.5% DMBA solution three times a week for 12 weeks whilst each animal from one of the control groups was similarly treated with only mineral oil. Another control group of 10 animals remained untreated throughout the experimental procedure. Specimens obtained from the hamster buccal-pouch mucosa were evaluated using immunohistochemical assessment of iNOS and p53 protein and *in situ* reverse transcription–polymerase chain reaction (IS RT–PCR), as well as reverse transcription–polymerase chain reaction (RT–PCR) for iNOS and p53 mRNA.

RESULTS: Two of the 15 animals of the DMBA-treated group died during the experiment. Squamous-cell carcinomas with a 100% tumor incidence were apparent for all of the 15-week DMBA pouch-treated animals. Animals from the mineral oil-treated and untreated pouch groups revealed no obvious changes. Inducible NOS mRNA was identified as a band corresponding to a 499-bp PCR product and was observed for all 13 of the hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks. The p53 mRNA was found as a band corresponding to a 370-bp polymerase chain reaction (PCR) product and was noted for nine (9/13, 69%) of the 15-week DMBA-treated pouches. No such bands (iNOS and p53) were noted for the untreated animals, the mineral oil-treated tissues and the negative-control samples. Using IS RT–PCR, the proportional (percentage) expression of iNOS (13/13, 100%) and p53 (8/13, 62%) mRNA observed for the hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks was noted to be consistent with the findings using RT–PCR. Furthermore, the proportional expression of iNOS (13/13, 100%) and p53 (8/13, 62%) proteins for the 15-week DMBA-treated hamster buccal-pouch tissue specimens was noted to be consistent with the findings using RT–PCR and IS RT–PCR. A significant association between iNOS and p53 expression (at both protein and mRNA levels) was noted (Fisher's exact probability test, $P < 0.05$). Neither iNOS nor p53 activity (at both protein and mRNA levels) was found for any of the untreated and mineral oil-treated pouches.

CONCLUSIONS: Enhanced expression of iNOS and p53 at both protein and mRNA levels in DMBA-induced hamster buccal-pouch carcinomas compared with the untreated and mineral oil-treated counterparts, has been demonstrated in the current study. Furthermore, we report what is, to the best of our knowledge, the first identification of a significant association between iNOS and p53 expression (at both protein and mRNA levels) in this experimental model system for oral carcinogenesis, although their precise interactions remain to be clarified.
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Keywords: inducible nitric oxide synthase; p53; DMBA-induced carcinogenesis; hamster

Correspondence: Dr Li-Min Lin, Oral Pathology Department, School of Dentistry, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung, Taiwan. E-mail: k0285@ms22.hinet.net
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Introduction

Nitric oxide (NO), a reactive gaseous free radical, is produced from its precursor amino acid L-arginine by the enzyme NO synthase (NOS; E.C.1.14.13.39) (Palmer, Ashton and Moncada, 1988; Bredt and Snyder, 1990). Based on calcium dependence, three different NOS isoforms have been identified to date. Neuronal and endothelial NOS are constitutively expressed, and are calcium-dependent, whilst inducible NOS (iNOS) is calcium-independent (Ambs *et al*, 1998b; Thomsen and Miles, 1998; Reveneau *et al*, 1999). Under inductive conditions, iNOS results in the production of high levels of NO, which can mediate antibacterial and anti-tumor activities, although the prolonged induction of NOS may contribute to a variety of pathological phenomena associated with inflammatory processes and cancer formation (Nathan and Xie, 1994).

The hamster buccal-pouch mucosa provides one of the most widely accepted experimental models for oral carcinogenesis (Gimenez-Conti and Slaga, 1993). Despite the existence of anatomic and histologic variations between hamster pouch mucosa and human buccal tissue, experimental carcinogenesis protocols for the former are able to induce premalignant changes and carcinomas that are similar to the development of premalignancy and malignancy in human oral mucosa (Morris, 1961). Over-expression of iNOS at both protein and mRNA levels has been reported previously for chemically induced oral carcinomas located in hamster buccal-pouch mucosa (Chen and Lin, 2000; Chen, Hsue and Lin, 2002a,b). Given that p53 tumor-suppressor gene expression has been previously reported for hamster buccal-pouch cancerous tissue (Gimenez-Conti *et al*, 1996; Chang *et al*, 2000), the relationship between iNOS and p53 remains an interesting area for study. To the best of our knowledge, the correlation between iNOS and p53 expression has not yet been demonstrated for the hamster buccal-pouch carcinogenesis model. The purpose of the present study is to assess whether iNOS expression is associated with p53 expression at both protein and mRNA levels for 7,12-dimethylbenz[a] anthracene (DMBA)-induced hamster buccal-pouch carcinomas applying immunohistochemistry, *in situ* reverse transcription–polymerase chain reaction (IS RT–PCR), and reverse transcription–polymerase chain reaction (RT–PCR) studies.

Materials and methods

Animals

Thirty-five out-bred, young (6 weeks old), male, Syrian golden hamsters (*Mesocricatus auratus*; purchased from the National Science Council Animal Breeding Center, Taipei, Taiwan), weighing about 100 grams each at the commencement of the experiment, were randomly divided into one experimental group (15 animals), and two control groups (10 animals each). The animals were housed under constant conditions (22°C, a 12-h light/dark cycle) and fed tap water and standard Purina laboratory chow *ad libitum*. The animal-handling protocol ensured humane practices were continued throughout the experimental process. Subsequent to 1 week of acclimatization to their new surroundings, both pouches of each animal from the experimental group of 15 animals were painted with a 0.5% DMBA solution (Sigma Chemical Co., St. Louis, MO, USA, purity: approx. 95%) which was dissolved in mineral oil (Sigma, purity: 100%), using a No. 4 sable-hair brush. Both pouches of each animal of one of the control groups 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 session. Another control group of 10 animals remained untreated throughout the experiment.

At the end of 15 weeks of such treatment (3 days following the final treatment), in order to avoid the potential influence of any diurnal variation (Lin and Chen, 1997), all animals from each group were simultaneously killed by inhalation of a lethal dose of diethyl ether. The pouches were dissected and then excised. A portion of the tissue was immediately frozen in liquid nitrogen for subsequent RNA extraction and RT–PCR reaction investigation, whilst another portion was fixed in 10% neutral-buffered formalin solution for about 24 h, dehydrated in a series of ascending-concentration alcohol solutions, cleared in xylene, and embedded in paraffin for IS RT–PCR and immunohistochemistry studies.

Reverse transcription–polymerase chain reaction

Total RNA was extracted by homogenizing the hamster buccal-pouch tissue specimens in guanidium isothiocyanate followed by ultracentrifugation in caesium chloride, as described previously (Chomczynski and Sacchi, 1987). The RNA concentration was determined from the optical density at a wavelength of 260 nm (by using an OD_{260} unit equivalent to 40 μg ml^{-1} of RNA). In brief, the pouch tissue ($\sim$250 mg) was added to 500 μl of denaturing solution [4 M guanidium thiocyanate, 25 mM sodium citrate (pH = 7.0) and 0.5% sodium N-sarosine] with 50 μl of 2 M NaOAc (pH = 4.0), 500 μl of water-saturated phenol, and 100 μl of chloroform:isoamyl alcohol (1:1, v/v). The homogenized pouch tissue plus solution was then vortexed thoroughly. RNA was subsequently precipitated with an equal volume of isopropanol, following which the pellet was rinsed twice with 1 ml of 75% ethanol and dried in air. RNA was resuspended in 100 μl of diethlpyrocarbonate (DEPC)-treated water at a final concentration of 1 μg μl^{-1} and stored at −80°C until further use.

Isolated total RNA (1 μg) was reverse-transcribed to cDNA in a reaction mixture (with a final volume of 20 μl) containing 4 μl of $MgCl_2$ (5 mM), 2 μl of 10 × reverse transcription buffer [10 mM Tris–HCl (pH = 9.0), 50 mM KCl, 0.1% Triton® X-100], 2 μl of dNTP mixture (1 mM each), 0.5 μl of recombinant RNasin® ribnonuclease inhibitor (1 μ μl^{-1}), 15 units of avian-myeloblastosis-virus (AMV) reverse transcriptase (High Conc.) (15 μ μg^{-1}), 0.5 μg of oligo(dT)$_{15}$ primer (Promega, Madison, WI, USA) catalogue no. A3500. The reaction mixture was incubated for 15 min at 42°C.

Table 1 Oligonucleotide primers used to amplify iNOS, p53 and β-actin cDNAs

Oligonucleotide primers	*Sequences*	*PCR products*
iNOS sense	5′-GCCTCGCTCTGGAAAGA-3′	499-bp
iNOS antisense	5′-TCCATGCAGACAACCTT-3′	
P53 sense	5′-CTGAGGTTGGCTCTGACTGTACCACCATCC-3′	370-bp
P53 antisense	5′-CTCATTCAGCTCTCGGAACATCTCGAAGCG-3′	
β-actin sense	5′-AACCGCGAGAAGATGACCCAGATCATGTTT-3′	350-bp
β-actin antisense	5′-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3′	

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

All oligonucleotide primers were purchased from Genset corp. (La Jolla, CA, USA). The primer pairs were chosen from the published cDNA sequences of iNOS (Chen *et al*, 2002a), p53 (Cai *et al*, 2000) and β-actin (Chen *et al*, 2002c). Oligonucleotide primers used for polymerase chain reaction (PCR) reactions are listed 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^{-1}), 2 μl of cDNA reaction dNTPs (200 μM each), 4 μl of $MgCl_2$ (2 mM), 8 μl of $10 \times$ 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 1 min (one cycle), then denaturing at 94°C (1 min), annealing at 55°C (1 min) for iNOS/p53 or at 60°C (1 min) for β-actin, and extending at 72°C (1 min) for 30 cycles and a final extension at 72°C for 7 min. The β-actin primers were utilized as positive controls. Negative controls without the presence of any RNA or 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, Germany) containing ethidium bromide. The PCR products were visualized as bands with an UV transilluminator. Photographs were taken with a Polaroid DS-300 camera (Applied Biosystems Taiwan, Taipei, Taiwan).

In situ *reverse transcription–polymerase chain reaction*

RNase-free conditions were used throughout slide preparation and the IS RT–PCR procedure. Following deparaffinization and dehydration, the section was pretreated with 10 $\mu g\ ml^{-1}$ proteinase K (Sigma, St Louis, MO, USA) for 1 min at room temperature at which time further enzyme activity was precluded with 0.1 M glycine in phosphate-buffered saline (PBS).

The Titan one-tube RT–PCR system (Boehringer Mannheim, Indianapolis, IN, USA) was used to perform this IS RT–PCR reaction. The oligonucleotide primers specific for iNOS and p53 were purchased from Genset corp. (Table 1). Reverse transcription was carried out at 50°C for 30 min on the block of a thermal cycler (TaKaRa MP), following which 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.

The digoxigenin nucleic acid-detection kit (Boehringer Mannheim, Germany) was used to detect the digoxigenin-tagged IS RT–PCR-amplified products. The sampled slides, following rinsing with PBS, were incubated, for 2 h, in anti-digoxigenin antibody conjugated with horseradish peroxidase diluted at 1:50. A subsequent enzyme-catalyzed color reaction solution with 3,3-diaminobenzidine tetrahydrochloride ($500\ \mu g\ ml^{-1}$) and hydrogen peroxide (0.015%) was applied to the slides in order to produce an insoluble brown coloration. Color development was checked under a microscope, and slides were washed with tris buffer containing ethylenediaminetetraacetic acid (EDTA) to terminate the reaction, and then counterstained with hematoxylin. The relative 'positivity' of iNOS/p53 mRNA for each section was observed utilizing a light microscope. Negative control experiments were performed for each specimen and on the same slide by omitting the primers in order to exclude endogenous priming of degraded DNA.

Immunohistochemical staining

Paraffin-embedded, 4-μm-thick tissue sections were stained for the iNOS and p53 proteins. Staining was performed by using a standard avidin–biotin peroxidase complex (ABC) method (Hsu, Raine and Fanger, 1981). Polyclonal antibodies of mouse iNOS were generated in rabbits and obtained from Calbiochem-Novabiochem Corporation, San Diego, CA, USA [Cat. no. 482728]. This antibody recognizes iNOS protein in tissue deriving from humans, rats and mice and exhibits no cross-reactivity with eNOS and nNOS (information from manufacturer). The specificity of this antibody has been established in a previous study (Chen and Lin, 2000). By contrast, monoclonal antibody NCL-p53-D07 (mAb D07; Novocastra, Newcastle, UK) was used for the identification of p53 protein. The mAb D07 antibodies used herein detect both wild-type and mutant forms of p53 (Vojtesek *et al*, 1992). Subsequent to deparafinization in xylene and ethanol, the tissue sections were treated in 0.3% H_2O_2-methanol in order to block endogenous peroxidase activity. The antigenicity was subsequently

unmasked by microwave heating for a period of 3 min in a 10 mM solution of sodium citrate. Following this, a 10% solution of normal goat serum was applied in order to reduce non-specific staining for all tissue sections stained for iNOS protein while a blocking solution of 2% dry milk in PBS (with 0.02% sodium azide) was applied to those sections stained for p53 protein. All sections prepared for iNOS staining were subsequently incubated with the primary antibody (1:500) at room temperature for 30 min whereas those sections prepared for p53 staining were treated with mAB D07 at a dilution of 1:200 for 2 h at room temperature. Following subsequent rinsing with PBS, those sections intended for iNOS staining were then incubated for 30 min at room temperature in the presence of biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA, USA; 1:100). By contrast, the sections intended for p53 staining were treated with biotinylated anti-mouse IgG antibody (Vector; 1:100) for 30 min, following which all the sections (both those intended for iNOS and p53 staining) were again washed with PBS and then incubated with avidin–biotin complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA, USA) for a further 30 min. Subsequent to a PBS rinse, the sites of peroxidase binding were visualized as brown reaction products via a benzidine reaction. The sections were then counterstained with hematoxylin. Each set of experiments included a human buccal squamous-cell carcinoma specimen known to express iNOS and p53, which served as a positive control and ensured the reproducibility of the staining process. Immunohistochemical staining was classified as negative, if no staining occurred or if positive staining was present for $<25\%$ of the cells, or classified as positive, if $>25\%$ of the cells stained positively. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments.

Results

Gross observation and histopathology

Unfortunately, two of the 15 animals from the DMBA-treated group died during the experimental procedure. Gross and histopathological changes amongst the 15-week DMBA-treated pouches were similar to those described in a previous study (Chen *et al*, 2002c). Squamous-cell carcinomas with a 100% tumor incidence were apparent for all of the 15-week DMBA-treated pouches. Mineral oil-treated and untreated pouches revealed no obvious changes associated with such treatment.

Reverse transcription–polymerase chain reaction

Inducible NOS mRNA was identified as a band corresponding to a 499-bp PCR product and was observed for all 13 of the hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks (Table 2). p53 mRNA was found as a band corresponding to a 370-bp PCR product and was noted for nine (9/13, 69%) 15-week DMBA-treated pouches (Figure 1). In addition, a significant association was detected between iNOS and p53

Table 2 Correlation between iNOS and p53 expression (both protein and mRNA) for DMBA-induced hamster buccal-pouch carcinomas (Fisher's exact probability test, $P < 0.05$)

iNOS mRNA/protein	*p53 mRNA/protein*	
	Positive	*Negative*
Positive	[a]9 (8)/[8]	4 (5)/[5]
Negative	0 (0)/[0]	0 (0)/[0]

[a]Results obtained from RT–PCR; data in rounded parenthesis are obtained from IS RT–PCR whereas data in square parenthesis are obtained from immunohistochemical staining.

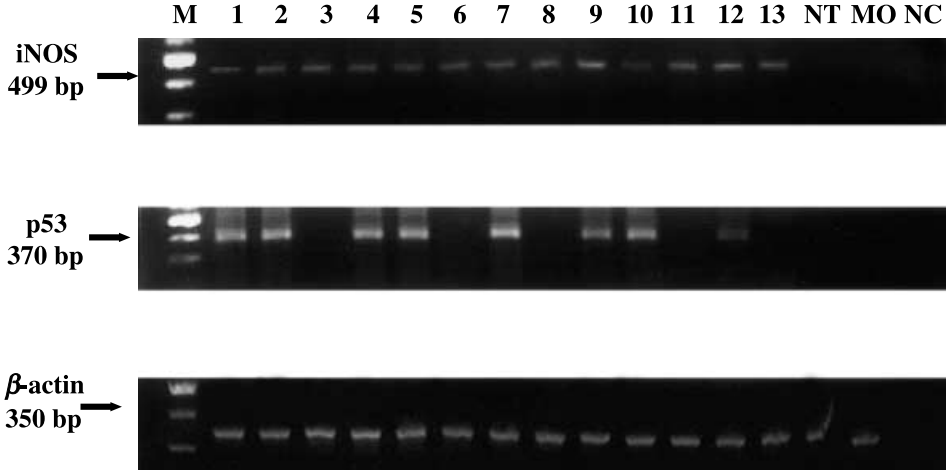

Figure 1 Expression of iNOS and p53 mRNA in hamster buccal-pouch carcinomas using RT–PCR. A band of a 499-bp PCR product corresponding to iNOS mRNA is observed for all the hamster buccal-pouch tissue specimens treated with DMBA over a period of 15 weeks (lanes 1–13) whereas nine specimens of buccal-pouch carcinoma reveal a 370-bp PCR product corresponding to p53 mRNA (lanes 1,2,4,5,7,9,10, and 12). No bands are noted for untreated (lane NT), mineral oil-treated (lane MO), and the negative control (lane NC) samples. All samples (lanes 1–13, NT, MO), apart from the negative control sample (lane NC), reveal bands of β-actin (350-bp). Lane M is the DNA molecular-weight marker

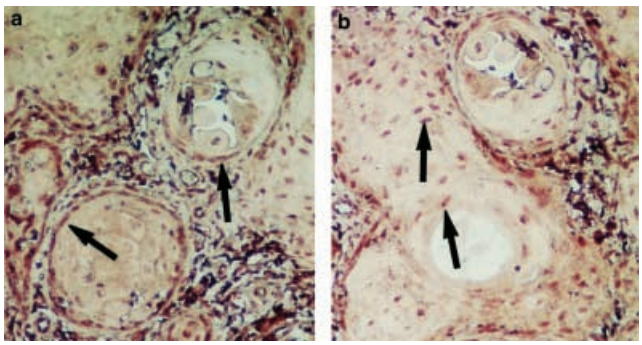

Figure 2 Some of the iNOS mRNA staining, as demonstrated by brown coloration, was located around the keratin pearls (**a**) whereas p53 mRNA staining distributed randomly within the tumor islands (**b**) (×100) (typical examples of positivity shown by arrows)

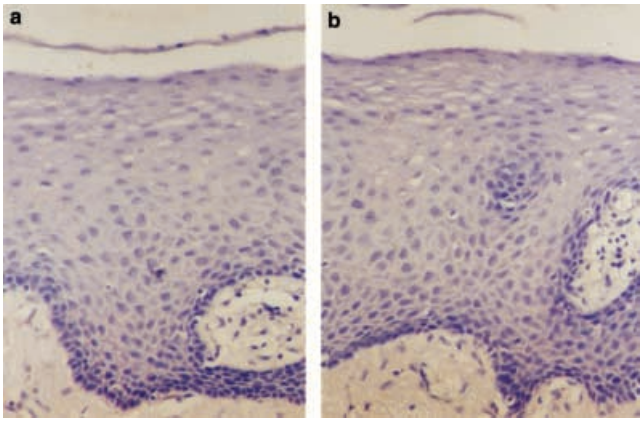

Figure 3 Neither iNOS (**a**) nor p53 (**b**) mRNA activities could be detected in the untreated pouches (×100)

mRNA expression (Table 2, Fisher's exact probability test, $P < 0.05$). No such bands (iNOS and p53 mRNA) were noted for the untreated animals, the mineral oil-treated tissues and the negative-control samples (Figure 1). All samples apart from the negative-control samples revealed bands of β-actin (350-bp; Figure 1).

In situ *reverse transcription–polymerase chain reaction*
Using IS RT–PCR, the proportional (percentage) expression of iNOS (13/13, 100%) and p53 (8/13, 62%) mRNA for the hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks was noted to be consistent with the findings using RT–PCR (Table 2). There was a significant association between iNOS and p53 mRNA expression (Table 2, Fisher's exact probability test, $P < 0.05$). We found some of the iNOS mRNA staining to be located around the keratin pearls (Figure 2a) whereas p53 mRNA staining was present randomly within the tumor islands (Figure 2b). Neither iNOS nor p53 mRNA activity could be detected in the untreated and the mineral oil-treated pouches (Figure 3). Omission of the primers for control sections revealed negative findings for iNOS mRNA activity amongst all specimens.

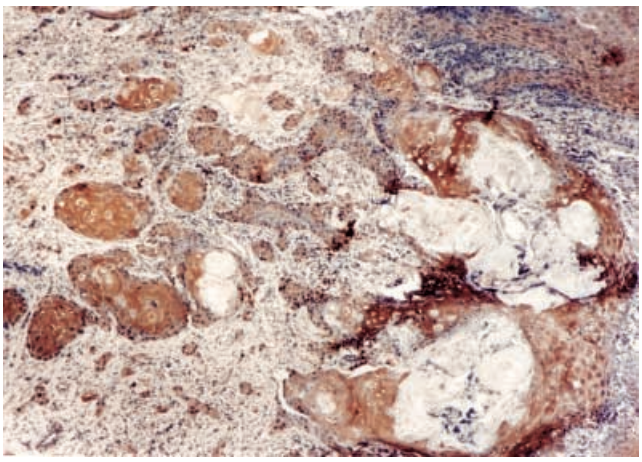

Figure 4 Cytoplasmic and/or nuclear stainings of iNOS protein in the tumor islands. Note the positive iNOS staining located in areas around the keratin pearls (ABC stain × 40)

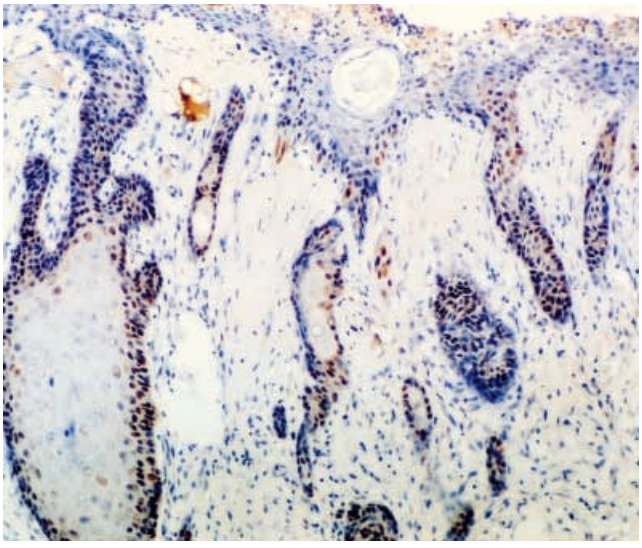

Figure 5 p53 protein (chiefly nuclear staining) was distributed randomly within the tumor islands (ABC stain × 40)

Immunohistochemical staining
The proportional (percentage) expression of iNOS (13/13, 100%) and p53 (8/13, 62%) proteins for the hamster buccal-pouch tissue specimens treated with DMBA for 15 weeks was noted to be consistent with the findings using RT–PCR and IS RT–PCR (Table 2). There was a significant association between iNOS and p53 protein expression (Table 2, Fisher's exact probability test, $P < 0.05$). Cytoplasmic and/or nuclear staining of iNOS protein was noted in the tumor islands for the 15-week DMBA-treated hamster buccal-pouch tissue specimens (Figure 4). We also noted that positive iNOS staining was also located in areas around the keratin pearls (Figure 4), whilst, by contrast, p53 protein staining (chiefly nuclear staining) was present randomly within the tumor islands (Figure 5). No iNOS and p53 activities could be detected in the untreated or mineral

oil-treated pouches. Omission of the primary antisera for control sections disclosed negative findings for iNOS and p53 activity for all specimens.

Discussion

In the current study, we demonstrated an enhanced expression of iNOS (protein and mRNA) for DMBA-induced hamster buccal-pouch carcinomas using immunohistochemistry, RT–PCR and IS RT–PCR. This finding is consistent with the earlier observations of iNOS expression (at both protein and mRNA levels) for chemically induced oral carcinomas located within hamster buccal-pouch mucosa (Chen and Lin, 2000; Chen *et al*, 2002a,b). Such a finding is also compatible with reports pertaining to human oral premalignant (Brennan *et al*, 2000a; Chen, Hsuen and Lin, 2002d,e) and malignant (Brennan *et al*, 2001a,b, 2002; Chen, Hsue and Lin, 2002f) epithelial lesions. Therefore, the present study further supports the hypothesis that iNOS may be associated with oral carcinogenesis.

The result of this study is in agreement with two earlier reports regarding p53 expression for chemically induced hamster-pouch carcinomas (Gimenez-Conti *et al*, 1996; Chang *et al*, 2000). Here, using RT–PCR, we report that p53 mRNA has been over-expressed amongst DMBA-induced hamster buccal-pouch carcinomas. We have also identified the cellular localization of p53 mRNA for these pouch carcinomas. To the best of our knowledge, this may be the first report demonstrating the presence of p53 mRNA within DMBA-induced hamster buccal-pouch carcinoma tissue. Compared with the observation of mRNA by RT–PCR, IS RT–PCR provides additional information about the cellular localization of mRNA. Furthermore, the IS RT–PCR technique was found suitable for localizing archival tissue and was not constrained by differing methods of tissue fixation.

We note that the protein expression of iNOS and p53 was well consistent with mRNA expression for almost all samples examined. Additionally, mRNA expression of iNOS and p53 using IS RT–PCR was consistent with mRNA expression using RT–PCR for nearly all samples examined. Only one sample proved to be negative on IS RT–PCR but revealed a positive response for p53 on RT–PCR. We speculate that this discrepancy between IS RT–PCR and RT–PCR could be due to mRNA degradation occurring during the tissue-sectioning processing or perhaps to topographical diversity of p53 expression within the same specimen.

To the best of our knowledge, no reports to date have studied the association of iNOS and p53 expression amongst DMBA-induced hamster buccal-pouch carcinomas. This may be the first report demonstrating the correlation between iNOS and p53 expression (at both protein and mRNA levels) using such an experimental model system for oral carcinogenesis, corroborating the findings of Brennan *et al* (2000b,c) for human oral squamous-cell carcinomas and dysplasias.

In the present study, iNOS expression was associated statistically significantly both with protein and mRNA levels of p53 expression. The apparent association between iNOS and p53 has been investigated for both carcinogenesis and tumor-progression cases (Ambs, Hussain and Harris, 1997; Lala and Chakraborty, 2001). The recent results reported by Ambs *et al* (1999) indicated that iNOS expression, and subsequent NO production, might impact upon DNA inducing p53 mutations for human colon tumors. Indeed, NO appears to be able to elicit DNA damage both directly and indirectly by inhibiting DNA-repair mechanisms (Wink *et al*, 1998). NO has also been found to induce cellular p53 accumulation in human cells *in vitro* (Messmer & Brune, 1995; Forrester *et al*, 1996). Moreover, Gallo *et al* (1998) suggested that elevated NOS activity and, consequently, induced tumor angiogenesis were attributable to p53 mutation for head and neck squamous-cell carcinomas. Indeed, the expression of iNOS appears to be positively associated with p53 mutation, as has also been reported for human lung (Ambs *et al*, 1998a) and colorectal (Ambs *et al*, 1999) cancers. Further, from the work by Forrester *et al* (1996), the cellular accumulation of wild-type p53 was found to suppress iNOS gene-promoter activity, resulting in the down-regulation of iNOS expression, thus suggesting a negative feedback loop between iNOS expression and wild-type p53. Thus, based on these findings, along with the appreciation of a rather high p53 mutation frequency for hamster buccal-pouch carcinomas (Gimenez-Conti *et al*, 1996; Chang *et al*, 2000) and the aforementioned results of this study, two possible mechanisms may be associated with the correlation between iNOS and p53 expression for DMBA-induced hamster buccal-pouch carcinogenesis.

First, following DMBA treatment, pouch keratinocytes appear to suffer from a high concentration of NO (produced by iNOS) which results in DNA damage, and leads to the accumulation of wild-type p53, and, as a consequence, it may be that the presence of wild-type p53 in the feedback loop suppresses the expression of NO (Ambs *et al*, 1997). Another potential mechanism for the correlation between iNOS and p53 may relate to the possibility that the p53-mutated pouch keratinocytes, as induced by a high concentration of NO and/or DMBA, could be responsible for the failure of the negative feedback loop that exists between NO production and wild-type p53 (Ambs *et al*, 1997). As a result of this failure, iNOS expression would therefore remain unchecked and hence an up-regulation of NO would subsequently occur, with the potential to lead to a cancerous state (Ambs *et al*, 1998b). Although the p53 oligoprimers and primary antibody used in this study were not able to differentiate wild-type p53 from mutant p53, a previous study (Wang *et al*, 1994) did suggest that the mutation of p53 could lead to an accumulation of p53. Furthermore, based on the finding that all samples in the present study revealed the over-expression of iNOS protein and mRNA, despite it being the subject of some conjecture, we hypothesize that the second-mentioned mechanism is likely to predominate. On the contrary, for the four samples revealing high levels of iNOS protein and mRNA, despite an absence of p53, such a situation may reflect the possibility that, lacking

the p53, the negative feedback loop between NO and the wild-type p53 would be disrupted, and iNOS expression would then continue uncontrolled and lead to an over-expression of NO for hamster buccal-pouch carcinomas.

The results of this study appear to suggest that iNOS is over-expressed in the hamster buccal-pouch carcinomas, resulting in p53 expression. However, it does not demonstrate whether or not the over-expression of iNOS and p53 in the early stages of DMBA treatment is a risk factor for the development of subsequent carcinomas. Further study pertaining to the sequential expression of iNOS and p53 during DMBA-induced hamster buccal-pouch carcinogenesis is clearly required in order to elucidate the causal relationship between induced expression of both iNOS and p53 for oral carcinogenesis.

In conclusion, we report what is, to the best of our knowledge, the first identification of the significant association between iNOS and p53 expression in this experimental model system for oral carcinogenesis, although their exact interactions remain to be clarified. The high expression of iNOS and p53 in hamster pouch carcinomas examined in this study may prompt us to speculate as to what possible impact a selective iNOS inhibitor such as L-N^6 (1-iminoethyl) lysine tetrazole-amide (SC-51) (Rao *et al*, 2002) may have upon tumor growth rate or hamster buccal-pouch carcinogenesis. Also, further study pertaining to what alterations to the NO-p53 pathway should arise if NO (produced by iNOS) is repressed should be both interesting and informative.

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