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Expression of inducible nitric oxide synthase in human oral premalignant epithelial lesions

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Abstract

Increased expression of inducible nitric oxide synthase (iNOS) has been found at the protein level in human oral epithelial dysplasias, but, a corresponding expression at the mRNA level has not been demonstrated. The purpose here was to assess the expression of iNOS mRNA and its correlation with the expression of the enzyme protein in human buccal premalignant epithelial lesions. Activities for iNOS protein (57/80, 64%) and mRNA (53/80, 53%) were detected in the specimens examined. Cytoplasmic and/or nuclear staining for iNOS protein was detected immunohistochemically in a number of mild oral epithelial dysplasias (16/20, 80%), moderate to severe oral epithelial dysplasias (14/20, 70%), submucous fibrosis (14/20, 70%) and verrucous hyperplasia (13/20, 65%). Upon in situ reverse transcription-polymerase chain reaction (RT-PCR), the cellular location of iNOS mRNA was compatible with the immunohistochemical findings. Furthermore, iNOS mRNA was found in 15 specimens of mild oral epithelial dysplasia (15/20, 75%), 13 specimens of moderate to severe oral epithelial dysplasia (13/20, 65%), 13 specimens of submucous fibrosis (13/20, 65%) and 12 specimens of verrucous hyperplasia (12/20, 60%). No iNOS protein or mRNA was found in samples of normal buccal mucosa, or in negative controls. Further studies on the characteristics of iNOS-positive cells and more long-term clinical follow-up data are needed.

Keywords: iNOS; Oral; Premalignancy; Immunohistochemistry; In situ RT-PCR

1. Introduction

Nitric oxide mediates a diverse array of pathological phenomena associated with inflammatory processes and cancer formation (Nathan and Xie, 1994). It is a small, highly reactive radical produced from the amino acid L-arginine by the enzyme nitric oxide synthase (E.C.1.14.13.39) (Palmer et al., 1987; Bredt and Snyder, 1990). Nitric oxide synthase exists in three different isoforms, two constitutively produced and one inducible (Knowles and Moncada, 1994). The constitutive forms (endothelial and neuronal) contribute to the maintenance of normal physiology in the nervous and cardiovascular systems and are both calcium- and calmodulin-dependent (Knowles and Moncada, 1994). The inducible form is involved principally in inflammation and carcinogenesis, and is both calcium- and calmodulin-independent (Palmer et al., 1998).

Nitric oxide may have both beneficial and detrimental actions (Hevel et al., 1991). In neoplastic tissues, it has been implicated as having a positive role in permitting tumour growth, including mutagenicity, angiogenesis and metastasis, although it has also been implicated in the cytotoxicity of macrophages toward tumour cells and in immunosuppression (Hevel et al., 1991). Furthermore, nitric oxide reacts with the superoxide anion to form a peroxynitrite anion, a highly toxic molecule causing DNA damage and protein modifications (Nguyen et al., 1992).

Enhanced expression of inducible nitric oxide synthase (iNOS) at the protein level has been demonstrated in oral epithelial dysplasias (Brennan et al., 2000a,b); a corresponding expression at the mRNA level has not, to the best of our knowledge, been shown in human oral premalignant epithelial lesions (oral epithelial dysplasia, submucous fibrosis, and verrucous hyperplasia). Whether there is any

Abbreviations: Phosphate-buffered saline (PBS); Reverse transcription-polymerase chain reaction (RT-PCR)

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discrepancy between the amount of mRNA expressed and the expressed protein in human oral precancerous tissues also remains to be determined. Therefore, we have now investigated the correlation between the expression of the mRNA and the protein of iNOS in human buccal premalignant epithelial lesions. We also investigated the cellular localization of the protein and the mRNA.

2. Materials and methods

2.1. Source of tissue

Specimens of premalignant epithelial lesions of the buccal mucosa were obtained from archival samples from 80 male patients aged between 34 and 75 years (mean age, 54 years), who had visited the Oral Pathology Department at the Kaohsiung Medical University Hospital. All of the patients were betel-quid chewers. The buccal lesions comprised mild oral epithelial dysplasia (20 samples), moderate to severe epithelial dysplasia (20 samples), submucous fibrosis (20 samples) and verrucous hyperplasia (20 samples). Normal buccal mucosal tissue was taken from five healthy individuals between 22 and 65 years old (mean age, 47 years), none of whom chewed betel-quid or smoked cigarettes, and was included as a control tissue. In all cases, both test and control, no treatment had been undertaken before the removal of any of the oral tissue. All tissues (including the normal) were obtained with the patients' informed consent and with the approval of the Ethics Committee for Scientific Research on Human Beings of this Institution. The buccal tissue was fixed in 10% neutral buffered formalin solution for about 24 h, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin for immunohistochemistry and in situ reverse transcription-polymerase chain reaction (RT-PCR).

2.2. Immunohistochemistry

Paraffin-embedded, 4 μm-thick tissue sections were stained for iNOS protein using a primary rabbit polyclonal antibody against the enzyme obtained from Calbiochem-Novabiochem Corporation (cat. no. 482728). This antibody recognizes the protein of the inducible enzyme in man, rat and mouse, and exhibits no cross-reactivities with endothelial and neuronal nitric oxide synthase (information from manufacturer). The specificity of the antibody was established in a previous study (Chen and Lin, 2000). All sections were deparafffinized through a series of xylene baths, and rehydrated through graded alcohols. To retrieve the antigenicity, sections were treated three times with microwaves in 10 mM citrate buffer (pH 6.0) for 5 min each. They were immersed in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity, then incubated in normal goat serum to reduce non-specific binding. Sections were incubated for 30 min at room temperature with the primary antibody (1:200). They were then processed using standard avidin–biotin immunohistochemistry, according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA) (Hsu et al., 1981). Diaminobenzidine was used as a chromogen and commercial haematoxylin for counterstaining. Immunohistochemical staining was classified as either negative, if no staining or positive staining was present in <25% of the cells, or positive, if >25% of the cells stained positively (Brennan et al., 2000b). Negative controls for the specificity of the antibody were included by omitting the primary antibody.

2.3. In situ RT-PCR

RNase-free conditions were used throughout slide preparation and the in situ 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 phosphate-buffered saline (PBS). The Titan one-tube RT-PCR system (Boehringer Mannheim, Indianapolis, IN) was used. The oligonucleotide primers specific for iNOS were purchased from Genset (La Jolla, USA) (Table 1). The primer pairs were chosen from the published cDNA sequences of the enzyme (Zhang et al., 1998) (GenBank accession no. D14051). The specificity of the primers had already been established by Ribbons et al. (1997). Reverse transcription was carried out at 50 °C for 30 min on the block of a thermal cycler (TaKaRa MP, Tokyo, Japan). Then, 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 DIG nucleic acid detection kit (Boehringer Mannheim) was used to detect the digoxigenin-tagged, in situ RT-PCR-amplified products. The slides, after rinsing with PBS, were incubated in antidigoxigenin antibody conjugated with alkaline phosphatase diluted at 1:500 for 2 h. An enzyme-catalysed colour reaction solution with

Table 1
Oligonucleotide primers used to amplify iNOS cDNAs

Oligonucleotide primers	cDNA positions	Sequences	PCR products
iNOS sense	529–550	5′-TCG AAA CAA CAG GAA CCT ACC-3′	907 bp
iNOS antisense	1414–1435	5′-ACA GGG GTG ATG CTC CCG GAC-3′	

5-bromo-4-chloryl-3-indolyl phosphate and nitrobluetetrazolium salt was then applied to the slides to produce an insoluble purple coloration. Colour development was checked under the microscope, slides were washed with Tris buffer containing EDTA to terminate the reaction, and counterstained with nuclear Fast Red. The positivity of iNOS mRNA in each section was observed by light microscopy. Negative control experiments were performed for each specimen on the same slide by omitting the primers in order to exclude endogenous priming of degraded DNA.

3. Results

Protein (57/80, 64%) and mRNA (53/80, 53%) activities for iNOS were detected in a subset of buccal premalignant epithelial specimens. Cytoplasmic and/or nuclear staining for the protein was detected immunohistochemically in a number of mild oral epithelial dysplasias (16/20, 80%; Fig. 1A), moderate to severe oral epithelial dysplasias (14/20, 70%; Fig. 2A), submucous fibrosis (14/20, 70%) and verrucous hyperplasia (13/20, 65%, Fig. 3A). Staining for the inducible enzyme was also found in stromal cells that were assumed to be macrophages. Upon in situ RT-PCR, the cellular location of mRNA for iNOS was found to be compatible with the immunohistochemical findings (Figs. 1–3B). Furthermore, the mRNA was found in 15 specimens of mild oral epithelial dysplasia (15/20, 75%; Fig. 1B), 13 specimens of moderate to severe oral epithelial dysplasia (13/20, 65%; Fig. 2B), 13 specimens of submucous fibrosis (13/20, 65%) and 12 specimens of verrucous hyperplasia (12/20, 60%; Fig. 3B). No protein or mRNA for the indicuble

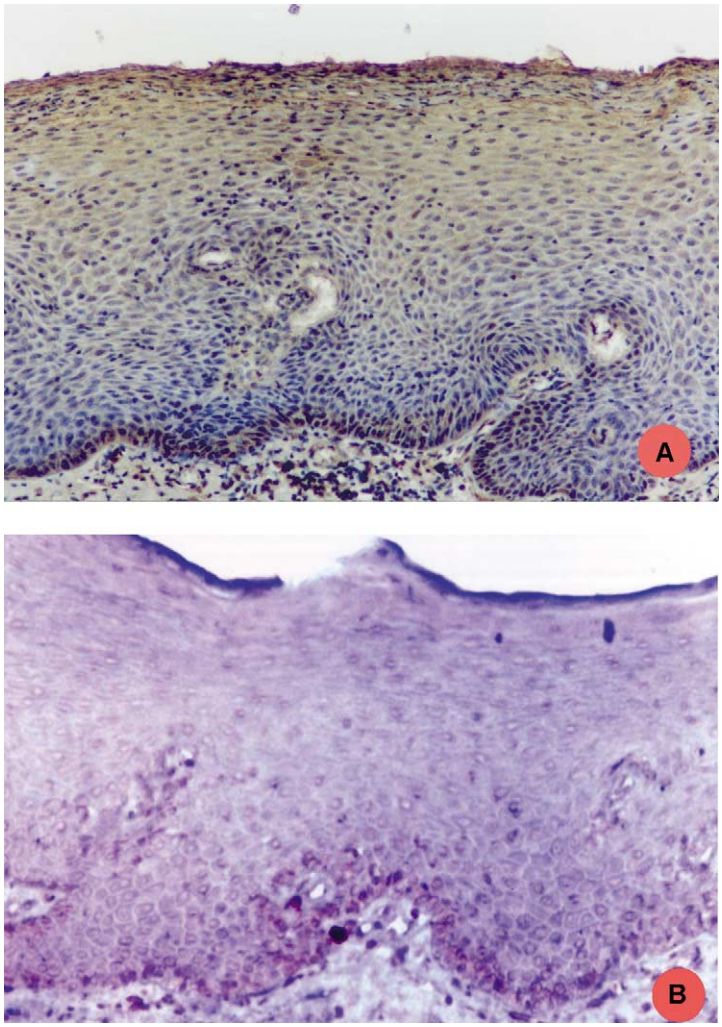

Fig. 1. iNOS protein in a representative sample of epithelial hyperplasia with mild oral epithelial dysplasia (A), and the expression of the enzyme's mRNA in the same sample (B). 100×.

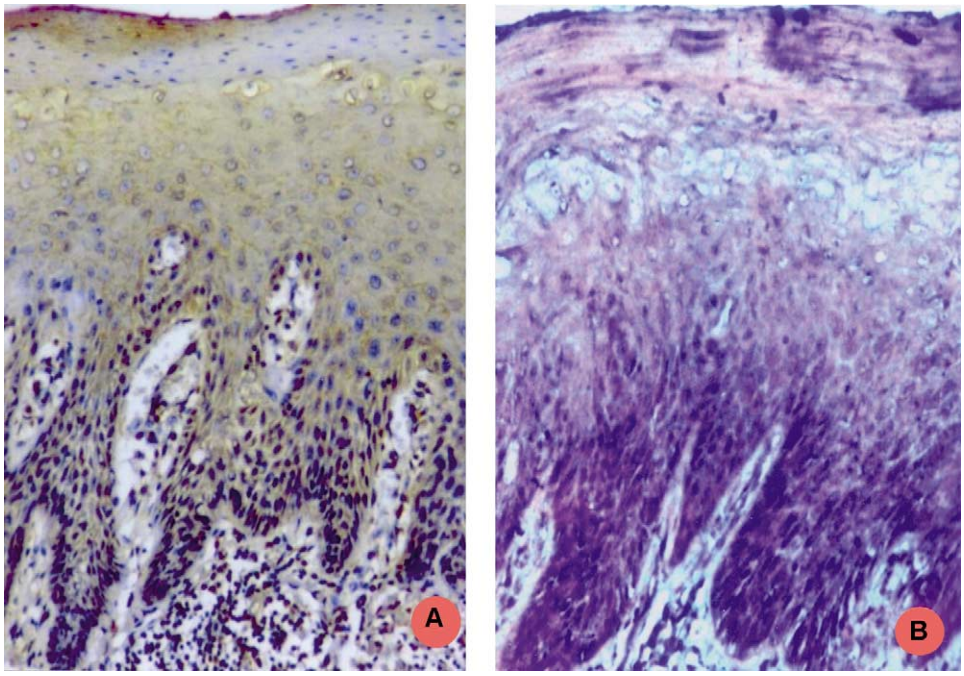

Fig. 2. iNOS protein (A) and mRNA (B) in a representative sample of moderate oral epithelial dysplasia. 100×.

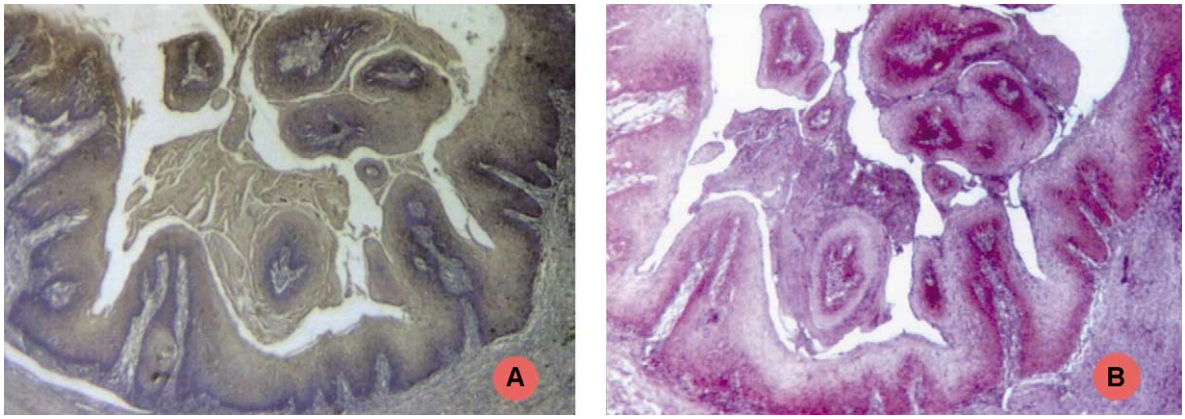

Fig. 3. iNOS protein (A) and mRNA (B) in a representative sample of verrucous hyperplasia. 40×.

enzyme was seen in any sample of normal buccal mucosa, or the negative controls.

4. Discussion

Our results are consistent with those of previous reports on the expression of iNOS protein in human oral epithelial dysplasias (Brennan et al., 2000a,b). To the best of our knowledge, there have been no published reports on the expression of that enzyme's mRNA in human oral premalignant epithelial lesions. Here, using in situ RT-PCR, we report the presence of mRNA for iNOS among a number of human buccal premalignant epithelial lesions. We also identify the cellular localization of the enzyme's mRNA in those lesions. The protein expression was consistent with the mRNA expression in most of our samples. Only four cases were negative on in situ RT-PCR but positive for iNOS on immunohistochemistry. We think that this discrepancy could be due to mRNA degradation during tissue processing or to topographical diversity in the enzyme's expression within the same specimen.

Although, at present we cannot completely confirm whether the overexpression of iNOS protein and mRNA in human oral premalignant epithelial lesions is a result of malignant transformation or due to interaction with cytokines, or is mediated via hypoxia in precancerous tissue, it appears reasonable to suspect that the increased activity of the enzyme may, at least in part, indicate that nitric oxide has a pathophysiological role in human oral carcinogenesis, which may possibly be p53-dependent (Ambs et al., 1998).

There may be a feedback loop between the production of nitric oxide and the p53 tumour-suppressor gene (Ambs et al., 1997). If inducible synthase produces high concentration of nitric oxide resulting in DNA damage, such activity could lead to the accumulation of p53, p53-mediated growth arrest or apoptosis (Messmer and Brune, 1996). Then, the presence of the wild-type p53 gene in a feedback loop might suppress the expression of nitric oxide (Ambs et al., 1997). For p53-null or p53-mutant cells, however, the expression of the synthase would be unchecked and hence an upregulation of nitric oxide would subsequently occur, with the potential to lead to a cancerous state (Ambs et al., 1998). Given that mutation of the p53 gene is a frequent event in human oral cancerous tissues (Nylander et al., 2000), it therefore appears reasonable to suggest that oral keratinocytes with mutant p53 could be implicated in the development of cancer in the presence of high concentrations of nitric oxide.

All our patients were betel-quid chewers; in particular, a higher number of heavy betel-quid chewers showed positive staining for the inducible enzyme (data not shown). It would be interesting to know if betel-quid has any effect on the expression of nitric oxide and its synthase. Confirmation of such an effect could only be achieved by investigating the nitric oxide activity and its synthase in patients with oral precancer who are not habitual of betel-quid chewers and those who do chew betel-quid but have no disease in their buccal mucosa. On the other hand, in about 2 years of follow-up, two out of 13 cases (16%) of moderate to severe epithelial dysplasia and four of the 12 cases (33%) of verrucous hyperplasia showing both iNOS protein and mRNA-positive staining have undergone malignant transformation to squamous-cell carcinoma. These preliminary data suggest that the expression of iNOS could be a useful tumour marker (Klotz et al., 1998). However, further studies on the characteristics of synthase-positive cells and more long-term clinical follow-up data are needed before firm conclusions and possible clinical applications can be made.

In conclusion, we report what is, to the best of our knowledge, the first identification of an enhanced expression of both protein and mRNA for iNOS in a number of human buccal premalignant epithelial lesions compared with normal buccal mucosa. Insight into the potential role of this enzyme in human oral carcinogenesis will require further investigation of whether its origin is a result of malignant transformation, and of the direct or indirect part it might play in human oral carcinogenesis.

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