Int. J. Oral Maxillofac. Surg. 2002; 31: 419–422
doi:10.1054/ijom.2002.0246, available online at http://www.idealibrary.com on IDEAL®

International Journal of Oral & Maxillofacial Surgery

Research and Emerging Technology Paper
Head and Neck Oncology

Increased expression of inducible nitric oxide synthase for human oral submucous fibrosis, verrucous hyperplasia, and verrucous carcinoma

Y.-K. Chen, S.-S. Hsuen, L.-M. Lin
Oral Pathology Department, School of Dentistry, Kaohsiung Medical University, Taiwan

Y.-K. Chen, S.-S. Hsuen, L.-M. Lin: Increased expression of inducible nitric oxide synthase for human oral submucous fibrosis, verrucous hyperplasia, and verrucous carcinoma. Int. J. Oral Maxillofac. Surg. 2002; 31: 419–422. © 2002 Published by Elsevier Science Ltd on behalf of the International Association of Oral and Maxillofacial Surgeons.

Abstract. Three isoforms of nitric oxide synthase (NOS), endothelial, neuronal and inducible NOS (iNOS), have been identified in humans. Enhanced expression of iNOS protein has been previously reported for human oral epithelial dysplasias, a human oral premalignant epithelial lesion; however, this expression has not been demonstrated for other premalignant epithelial lesions, namely, oral submucous fibrosis (SF) and verrucous hyperplasia (VH). On the other hand, iNOS protein expression has not been reported in human oral verrucous carcinoma (VCa). The aim of this current study was to determine whether iNOS protein also occurs for oral SF, VH and VCa lesions. We found that membranous stainings were observed chiefly in oral SF lesions (17/20, 85%), whereas cytoplasmic stainings were mainly found in the VH variants (16/20, 80%). By contrast, cytoplasmic and/or nuclear stainings were observed in the specimens of verrucous carcinoma (17/20, 85%). Since no iNOS activity could be detected for any of our specimens of normal buccal mucosa in the present immunohistochemical study, this suggests that an NOS-dependent mechanism may be involved in the malignant transformation of these two premalignant oral epithelial lesions.

Key words: iNOS; submucous fibrosis; verrucous hyperplasia; verrucous carcinoma; oral; human; premalignance.

Accepted for publication 3 February 2002

Introduction

In 1987 PALMER et al., first reported that nitric oxide (NO), a gaseous free radical, was equivalent to endothelium-derived relaxing factor[12]. Since then, many studies have been conducted elaborating the multifunctional aspects of NO *in vivo* and *in vitro*[2,11]. This radical is generated by the action of nitric oxide synthase (NOS) and requires several co-factors, including nicotinamide adenine dinucleotide phosphate, flavin adenine dinucloetide, and flavin mononucleotide, to produce NO and L-citrulline[2]. Nitric oxide synthase enzymes can be divided into two functional classes, constitutive and inducible, based on their sensitivity to calcium. One inducible isoform of nitric oxide synthase (iNOS) and two constitutively expressed isoforms, endothelial (eNOS) and neuronal (nNOS), have been identified and cloned[11]. It has been reported that iNOS is induced in various cell types, including keratinocytes and macrophages[3].

In two recent studies, immunohistochemical techniques have been used for expression of iNOS protein in human oral epithelial dysplasia[4,5], an overt premalignant lesion of the human oral mucosa. Given the fact that both oral submucous fibrosis (SF) and verrucous

0901-5027/02/040419+04 $35.00/0

hyperplasia (VH) are also premalignant epithelial lesions[13,14], we investigated the expression of iNOS protein for these two oral premalignant lesions. Furthermore, iNOS protein expression has not been reported in human oral verrucous carcinoma (VCa). Thus, the aim of the current study was to determine whether iNOS expression also occurs in SF, VH and VCa lesions of the human oral mucosa.

Material and methods

Specimens of the diseased buccal mucosa were obtained from archived tissue samples taken from 60 patients visiting the Oral Pathology Department of the Kaohsiung Medical University. The study population consisted of 55 men and five women, with a mean age of 56 years (SD ± 10.1). The diseased buccal mucosa samples consisted of oral submucous fibrosis (20), verrucous hyperplasia (20), and verrucous carcinoma (20). Normal buccal mucosa tissues, taken from five healthy individuals (mean age: 35 years; SD ± 10.2) without history of betel quid chewing and cigarette smoking, were included as controls.

Immunohistochemical staining for iNOS protein

Paraffin-embedded, 4 μm tissue sections from the 60 diseased and five normal buccal mucosa were stained for iNOS protein using a primary rabbit polyclonal anti-iNOS antibody obtained from Calbiochem-Novabiochem Corporation [Cat. no. 482728]. This antibody recognizes iNOS protein in humans, rats and mice and exhibits no cross-reactivities with eNOS and nNOS (information from manufacturer). Deparaffinization of all sections was performed through a series of xylene baths, with rehydration using graded alcohols. To retrieve the antigenicity, tissue sections were microwaved three times in 10 mM citrate buffer (pH 6.0) for 5 min per cycle. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 30 min, to block endogenous peroxidase activity, and were then incubated in normal goat serum to reduce non-specific binding. Sections were incubated for 30 min at room temperature with primary anti-iNOS antiserum (1:200). The sections were then processed using standard avidin-biotin immunohistochemistry, according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA, USA)[9]. Diaminobenzidine was used as a chromogen, and commercial haematoxylin was used for counterstaining. Immunohistochemical staining was classified as either negative (no staining or positive staining present for <25% of cells) or positive (>25% of cells positively stained)[4,5]. Negative controls for the specificity of anti-iNOS antisera were included by omitting the primary antisera.

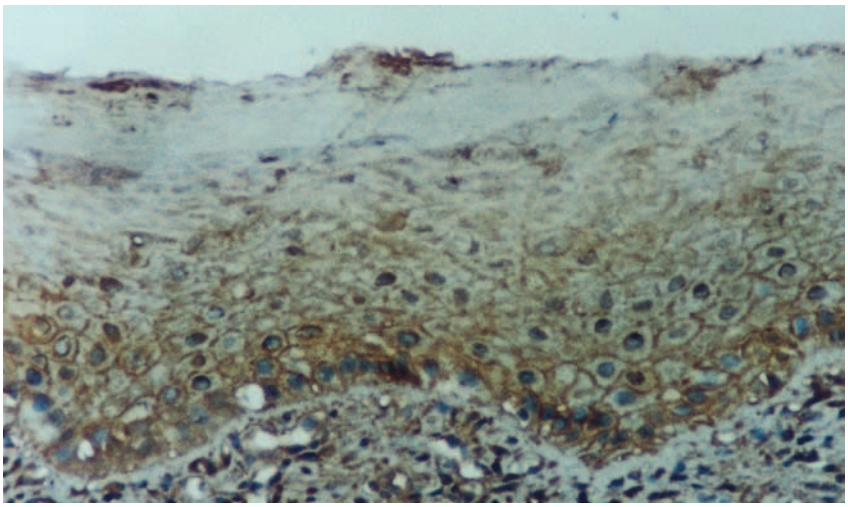

Fig. 1. Membranous staining for iNOS protein was predominantly observed in the lower portion of the epithelium of a representative section of oral submucous fibrosis with occasional positive staining of the keratin layer. Noting that nuclear staining is seen in some areas of the epithelium (ABC stain × 100).

Results

Membranous staining was observed chiefly for the oral SF lesions (17/20, 85%; Fig. 1), whereas cytoplasmic staining was mainly determined for the VH analogues (17/20, 85%; Fig. 2) and VCa lesions (16/20, 80%; Fig. 3). Amongst those OSF specimens showing membranous staining, nuclear staining could be noted in a few cases (5/17, 30%; Fig. 1). Inducible NOS staining was also determined for cells of the tumour stroma, presumed to be macrophages. No iNOS activity could be detected for any of the specimens of normal buccal mucosa. Omission of the primary antisera for control sections resulted in negative iNOS-activity findings for all specimens.

Discussion

Two previous immunohistochemistry-based studies have demonstrated enhanced iNOS-protein expression for oral epithelial dysplasia, a human oral premalignant epithelial lesion[4,5]; however, the corresponding expression of iNOS protein has not, to the best of our knowledge, been reported previously for other analogous premalignant epithelial lesions, such as the oral SF and VH variants. In this study, we demonstrate the presence of iNOS protein in oral SF and VH lesions. Therefore, it seems reasonable to propose that the escalated iNOS-protein activity may be associated with the risk for these two types of human oral premalignant epithelial lesions.

Notably, normal human hepatocytes, under the influence of high NO levels, trigger a series of events that rapidly induce both nuclear and mitochondral DNA damage, subsequently leading to cell-cycle arrest and mitochondral dysfunction, and ultimately associated with the development of hepatocellular carcinoma[8]. From immunohistochemical extrapolation, therefore, it is postulated that prolonged NO exposure for human oral keratinocytes, as revealed in this report from the enhanced iNOS activity demonstrated for oral SF and VH lesions, may contribute to the malignant transformation of these two human oral premalignant epithelial lesions.

It has been demonstrated that intensive iNOS activation and NO overproduction in tumour cells may induce apoptosis and suppress tumour growth[15,16], whereas low levels of NO may do the opposite[1,15]. This leads to the question of how high NO levels in the oral SF and VH lesions may promote malignant transformation. However, immunostaining intensity of iNOS in

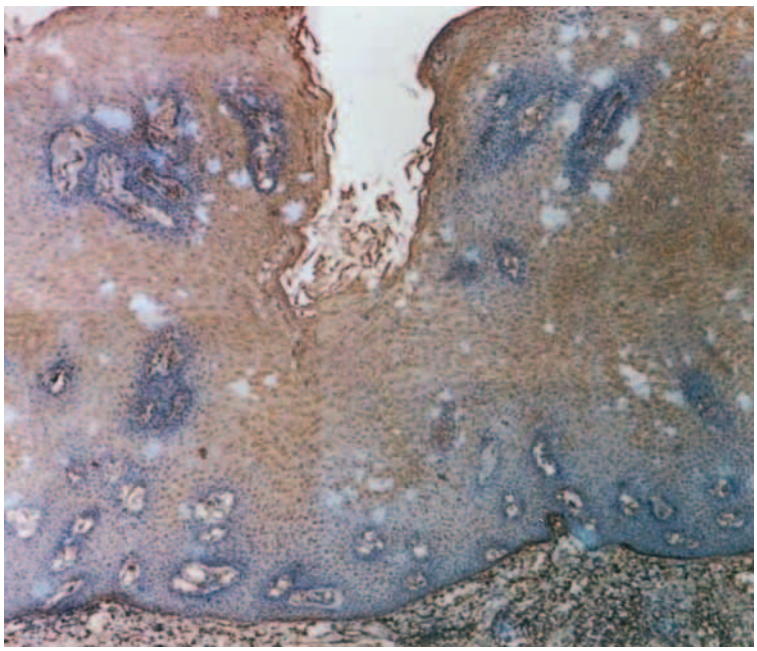

Fig. 2. Cytoplasmic staining for iNOS protein noted for almost the whole layer of the epithelium for a representative section of an oral verrucous hyperplasia. Marked staining in the basal layer is also observed. Positive staining was present in cells of the adjacent connective tissue area, presumed to be macrophages (ABC stain × 40).

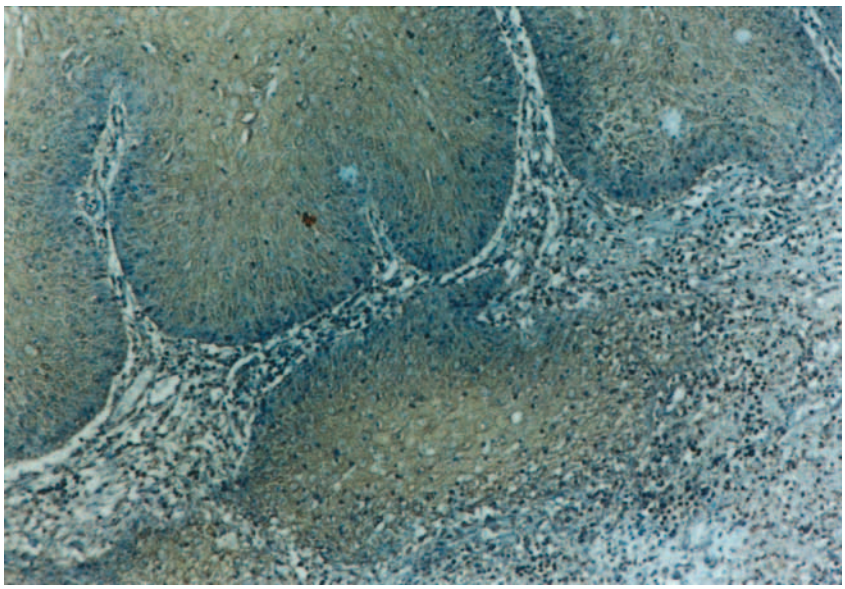

Fig. 3. Cytoplasmic staining for almost the whole layer of the epithelium for a representative section of an oral verrucous carcinoma (ABC stain × 100).

paraffin embedded tissue is quite variable and the intensity of staining is unable to quantify protein expression. Subsequent study on iNOS mRNA levels for lesions of OSF, VH and VCa using semi-quantitative reverse transcription polymerase chain reaction may provide a possible answer to this question.

It has been suggested that inducible NOS protein may be an immunohistochemical marker for malignant neoplasia of the prostate[10]. After about 2 years follow-up, four out of seventeen cases (24%) of verrucous hyperplasia showing iNOS positive staining have undergone malignant transformation to develop squamous cell carcinomas. On the other hand, two out of 16 cases (13%) of verrucous carcinoma showing iNOS positivity had recurred after treatment. These preliminary data suggest that iNOS expression may be a useful predictor of the outcome of oral (pre) cancer lesions. Further studies on the characteristics of iNOS-positive cells and more long-term clinical follow-up data are necessary before a confirmative conclusion and possible clinical application can be made.

The staining pattern for oral VH lesions is compatible with that for oral VCa analogues. By contrast, membranous staining was revealed predominantly for oral SF. The reasons for this difference in staining pattern are not clear. The results for iNOS staining of oral VCa in the current study are consistent with earlier investigations by Brennan et al.[6,7]. Furthermore, to the best of our knowledge, this is the first report identifying iNOS protein expression for lesions of oral VCa.

In conclusion, the present immunohistochemical study has demonstrated iNOS protein expression for a number of oral SF, VH and VCa lesions, compared with a lack of expression for samples from normal buccal mucosa. This suggests that iNOS protein expression is chiefly demonstrated for a subset of oral (pre)cancerous tissues, and, that an NOS-dependent mechanism may be involved in the malignant carcinogenesis of these oral epithelial (pre)malignant lesions.

Acknowledgments. We wish to acknowledge the technical assistance of Ms N. Y. Dai. This research was supported by a grant from the National Science Council, ROC (N.S.C. 89-2314-B-037-046).

References

1. 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**: 433–438.
2. Aoki E, Takeuchi IK, Shoji R. Nitric oxide: an attractive signaling molecule. Acta Histochem Cytochem 1995: **28**: 97–106.
3. Brennan PA, Downie IP, Langdon JD, Zaki GA. Emerging role of nitric oxide in cancer. Br J Oral Maxillofac Surg 1999: **37**: 370–373.
4. Brennan PA, Palacios-Callender M, Sinclair D, Spedding AV, Zaki GA. Does expression of inducible ntric oxide synthase correlate with severity of oral epithelial dysplasia? J Cranio-Maxillofac Surg 2000: **28**: 44–48.
5. 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**: 624–629.
6. 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**: 627–632.
7. 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**: 129–134.
8. D'ambrosio SM, Gibson-D'ambrosio RT, Brady T, Oberyszyn AS, Roberson FM. Mechanisms of nitric oxide-induced cytotoxicity in normal human hepatocytes. Environ Mol Mutagen 2001: **27**: 46–54.
9. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J Histochem Cytochem 1981: **29**: 577–580.
10. Klotz T, Bloch W, Volberg C, Engelmann U, Addicks K. Selective expression of inducible nitric oxide synthase in human prostate carcinoma. Cancer 1998: **82**: 1897–1903.
11. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. Cell 1994: **78**: 915–918.
12. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987: **327**: 524–526.
13. Pindborg JJ, Murti PR, Bhonsle RB, Gupta PC, Daftary DK, Mehta FS. Oral submucous fibrosis as a precancerous condition. Scand J Dent Res 1984: **89**: 270–274.
14. Shear M, Pindborg JJ. Verrucous hyperplasia of the oral mucosa. Cancer 1980: **46**: 1855–1862.
15. Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB. The multifaceted roles of nitric oxide in cancer. Carcinogenesis 1998: **19**: 711–721.
16. Xie K, Fidler IJ. Therapy of cancer metastasis by activation of the inducible nitric oxide synthase. Cancer Metastasis Rev 1998: **17**: 55–75.

Address:
Dr Li-Min Lin
Oral Pathology Department
School of Dentistry
Kaohsiung Medical University
100 Shih-Chuan 1st Road
Kaohsiung
Taiwan
Fax: +886-7-3210637
E-mail: k0285@ms22.hinet.net