INDUCIBLE NITRIC OXIDE SYNTHASE IMMUNOREACTIVITY IN DENTURE INDUCED FIBROUS INFLAMMATORY HYPERPLASIA AND HEALTHY ORAL MUCOSA: AN IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Excessive production of nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS) has been implicated in the pathogenesis of numerous inflammatory processes. The exact pathogenesis of denture-induced fibrous inflammatory hyperplasia (DIFIH) remains unclear. The aim of this study was to assess iNOS expression in healthy oral mucosa (HOM) and DIFIH by immunohistochemical (IHC) method. The expression of iNOS protein in HOM has been reported in numerous publications; particularly these tissues were used as a control group. However, there are some conflicting results whether healthy oral tissues express iNOS. In this study performing IHC method, DIFIH (n= 26) and HOM (n= 28) in formalin-fixed, paraffin-embedded sections were evaluated in terms of iNOS expression. The statistical analysis showed that there were no significant differences between DIFIH and HOM regarding iNOS immunoreactivity (p>0.05). The results in this study indicate that iNOS does not have a determining role in pathogenesis of DIFIH. The constitutive iNOS expression in HOM under physiological conditions needs to be further evaluated.

Keywords: inducible nitric oxide synthase, iNOS, denture induced fibrous inflammatory hyperplasia, healthy oral mucosa

Introduction

Nitric oxide (NO) is a free radical which has complex roles in both physiological and pathological conditions (22, 29, 30). When discovered, it was thought to be responsible for vascular smooth muscle relaxation, and physiological and pathological effects of this simple molecule were not expected to be significant to this extend (31). In the following research it has been reported that NO, being an important intercellular and extracellular messenger molecule, has both homeostatic functions like vasodilatation, neurotransmission, inhibition of platelet adhesion and aggregation, and also host defense functions against infectious agents such as bacteria, fungi and parasites, and tumor cells (29). Although NO is important in host defense and homeostasis, it is also regarded as harmful and has been implicated in the pathogenesis of a wide variety of inflammatory conditions (1, 15, 19, 21, 35, 37, 38, 39). NO is synthesized by a group of isoenzymes collectively named NO synthase (NOS). Three different isoforms of NOS have been identified (13). Type I (neuronal NOS; nNOS) and Type III (endothelial NOS; eNOS) were discovered first, and are Ca²⁺/calmodulin-dependent. Ca²⁺/calmodulin-independent Type II (macrophage or inducible NOS; iNOS) is produced during inflammatory process when cytokines are secreted. Once the production commences it lasts much longer compared to eNOS and nNOS, sometimes up to several days (22). Expression of iNOS from macrophages occurs by stimulation of some proinflammatory mediators (3, 16). nNOS and eNOS are also called constitutive NOS (cNOS). While cNOS in constitutive form is basically active in regulation of normal physiological processes, increased iNOS expression occurs primarily in pathological processes. Presence of iNOS which causes increased amount of NO in tissues is encountered in complex events, including inflammatory process and premalign and malign transformation (2, 3, 8, 14, 15, 23, 34, 37, 38, 39).

Oral mucosal lesions resulting from ill-fitting removable dentures can be acute or chronic and they form a heterogeneous group based on pathogenesis (6, 9). The development of these lesions can be caused by microbial denture plaque, base material of denture or mechanical trauma of denture. It is reported that denture induced fibrous inflammatory hyperplasia (DIFIH), commonly caused by mechanical trauma of ill-fitting denture, is encountered in 10%-15% of denture users (6, 40).

DIFIH is of inflammatory character, thus it is a reactive lesion. Formerly it was named with outdated synonyms as epulis fissuratum/fissurata, denture epulis, denture-induced fibrous hyperplasia, denture irritation hyperplasia, currently DIFIH or the slight variation of it, (denture – induced) inflammatory fibrous hyperplasia (IFH) is used (26, 40). In a recent clinical research on the biopsies from patients older than 60 years of age it has been shown that inflammatory fibrous hyperplasia lesions are one of the three most common pathologies encountered in elderly people, along with squamous cell carcinoma (SCC) and fibroma (12). According to some researchers chronic injury of the oral mucosa by dentures in rare instances may predispose to development of carcinomas (6, 41). DIFIH is mostly encountered often in the elderly, women and on the anterior regions of the jaws (9, 10, 26). It is also encountered more often in patients using dentures...
for a long time (15-20 years) and in patients using immediate complete dentures (9). While the role of denture usage related to chronic traumatic ulcer in development of these lesions is not clearly understood, alveolar resorption is thought to have an effect on lesion progression (40). As much as 70% of the patients with these lesions are more than 40 years of age (26).

The exact mechanism by which DIFIH occurs remains unclear. The aim of study was to assess the iNOS protein expression in healthy oral mucosa and denture-induced fibrous inflammatory hyperplasia by immunohistochemical technique. As a result, the presence of iNOS expression in both DIFIH and HOM has been analyzed.

Materials and Methods
Tissue samples were obtained from 54 patients, of whom 40 were female and 14 were male. 26 of these samples were pathologic tissues from DIFIH, while 28 were HOM. Before starting operations, all patients were informed regarding the surgical procedure, histopathological and immunohistochemical (IHC) methods and their consents were obtained. The study followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants.

DIFIH tissue samples were obtained from patients who needed DIFIH excision for preprosthetic reasons. The ages of the patients were in the range 48-75 (median 61). HOM samples were obtained during surgical removal of unerupted uninflamed tooth or during simple alveolar recountsouring procedure in preprosthetic surgery stage, before flap readaptation from clinically healthy mucosal remnant tissues. Tissues obtained from patients with systemic diseases and from patients who were under medication in the last 6 months were excluded from this research. Each tissue sample obtained during surgical procedure was separated in two parts, one part was used for histopathological evaluation according to a protocol. IHC methods were applied to DIFIH (n=26) and HOM (n=28) parts which were pathologically identified.

Immunoreactivity of iNOS (iNOS-IR) was analysed in formalin-fixed, paraffin-embedded sections by IHC methods. Tissue samples were fixed in 10% buffered formalin for 18 hours. After tissues were fixed, samples were rinsed with phosphate buffered saline (PBS) and the specimens were subsequently dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections (5-7 μm) were cut and mounted on glass slides. The sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, covered with 10 mM sodium citrate buffer (pH 6.0) and heated at 95°C for 5 min, for antigen retrieval. Endogenous peroxidase activity was quenched by 5 min incubation in peroxidase blocking solution (DBS Universal Immunostaining Kit, KP-50L, CA).

Tissues were then incubated with the rabbit polyclonal antibody against iNOS (Chemicon, AB5384, CA) for 2 hours and then in the biotinylated secondary antibody for 10 minutes (DBS Universal Immunostaining Kit, KP-50L, CA). Negative controls were performed by substituting the primary antibody with nonimmune rabbit serum. Immunoreactivity was detected by means of horseradish-peroxidase (HRP)-streptavidin complex using diaminobenzidine (DAB) chromogen as a marker (DBS Universal Immunostaining Kit, KP-50L, CA). Sections were counterstained in Mayer’s hematoxylin for 30 seconds. Subsequently, the sections were rinsed and finally mounted (DBS CC/Mount, K002, CA). All steps were carried out at room temperature in a humidified chamber. Assessments, counts and photography were performed by a Laborlux K (Leitz, Germany) light microscope.

The results of immunohistochemical staining were evaluated semiquantitatively on the basis of a four-point scale: - negative staining; + low expression, less than 10% of positive cells; ++ moderate expression 10-50% of positive cells; +++ diffuse expression, more than 50% of positive cells.

Statistical analysis was performed using Pearson chi-square test. P values of <0.05 were considered significant.

Results and Discussion
Different levels of iNOS-IR positivity were detected in two groups, namely DIFIH and HOM. Sections serving as negative controls were all unstained. Cytoplasmic staining was observed in all immunoreactive cell types. The intensity of iNOS-IR in cells was observed to be similar or not differentiable with the method used. Therefore, taking all cell types into account, immunoreactive (iNOS-IR-positive) cells in randomly selected microscopic areas, were counted and their percentages in all cells counted in every area was calculated. In order to prevent mistakes resulting from the manual method, immunoreactivity in tissues were analyzed without cell type discrimination.

iNOS-IR was diffuse in 23.08%, moderate in 73.08% and low in 3.84% of DIFIH (Fig.1); diffuse in 28.57% and moderate in 71.43% of HOM (Fig. 2), (Table 1).

Fig. 1. Immunoreactivity for iNOS in denture induced fibrous inflammatory hyperplasia, x160
The statistical analyses (Pearson chi-square test) showed that there were no significant differences between the DIFIH and HOM group regarding iNOS-IR ($p>0.05$).

Fig. 2. Immunoreactivity for iNOS in healthy oral mucosa, x63

TABLE 1
Expression of iNOS in denture induced fibrous inflammatory hyperplasia (DIFIH) and healthy oral mucosa (HOM)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>iNOS staining score</th>
<th>- n (%)</th>
<th>+ n (%)</th>
<th>++ n (%)</th>
<th>+++ n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIFIH</td>
<td></td>
<td>0</td>
<td>1 (3.84)</td>
<td>19 (73.08)</td>
<td>6 (23.08)</td>
</tr>
<tr>
<td>HOM</td>
<td></td>
<td>0</td>
<td>0</td>
<td>20 (71.43)</td>
<td>8 (28.57)</td>
</tr>
</tbody>
</table>

DIFIH: Denture induced fibrous inflammatory hyperplasia; HOM: Healthy oral mucosa

The expression of iNOS protein in healthy oral tissues such as gingiva and oral mucosa have been reported in numerous publications. Particularly these tissues were used as a control group. However, there are some conflicting results regarding whether healthy oral tissues express iNOS. Oral cavity is a niche of commensal bacteria. At certain levels, continuous exposure to these pathogens would induce iNOS activity and, thus, expression. Yet, some immunohistochemical studies showed that healthy oral tissues do not express iNOS. These conflicting results were suggested due to tissue preparation (27). In contrast to cryostat preparation, paraffin-embedded tissue section tended to give low or even no iNOS activity. Of interest, the present study showed that iNOS protein was relatively high expressed.

The possible role of iNOS in cancer transformation from dysplasia has been published (2). In this regard, iNOS determination in DIFIH lesions, which are known to show about 4% dysplastic formation may be important (10, 11). DIFIH occurrence in about 15% of the denture users (6, 40) and dysplasia incidence in DIFIH being 4% are important data (10, 11). For that reason, denture using patients should be checked regularly (6, 41). The risk of malign potential of DIFIH has been the source of various immunohistochemical researches. In a study evaluating epithelial proliferative activity level of DIFIH lesions by using proliferating cell nuclear antigen (PCNA), surgical treatment of DIFIH by large excisions has been recommended because normal tissues are only detected beyond 2cm of the lesion (11). It has been determined that oral mucosal lesions are more likely to be benign in the gingival area and more likely to be malign in the alveolar mucosa (36). In this regard, detection of DIFIH lesions in alveolar mucosa may be important. Moreover, constant trauma and inflammation prevents total recovery in DIFIH lesions which results in an extremely complex histological picture in repeating injury-healing-reinjury cycle (13, 40). In some research, it has been suggested that failure to treat chronic trauma-based lesions in time may cause these to behave as a precancerous lesion (32, 41). In some oral lesions containing DIFIH, tumour markers like Ca1 have been detected specifically in the epithelial layer on areas infiltrated by inflammatory cells. However, comparisons to carcinomas lead to the conclusion that Ca1 is not reliable in discriminating malignant and premalignant lesions (33).

Presence of iNOS-IR in DIFIH may be a mediator in the inflammatory process or it may be responsible for the activation of proliferative potential. There is a hypothesis claiming that chronic trauma may aid development of squamous cell carcinomas (SSC), which is commonly encountered on sides of the tongue (41). In another research, it has been shown that oral chronic traumatic lesions do not affect tumor growth themselves. Combined with chemical carcinogens, they act as promoter agents on patients with subclinic tumor initiation (17, 32). In contrast to research reports, mechanical trauma is more active in oropharyngeal cancer etiology than anticipated before (41), some other works indicate that denture usage, denture irritation, irregular teeth and habits like “cheek biting” have no important role in oral carcinoma development (5, 18, 28).

The most considered NOS type in many inflammatory processes is iNOS (1, 21, 35, 37, 38, 39). The important effects of iNOS produced NO in pulpa (15), periapical tissue (19, 21, 37, 38, 39) and periodontal inflammatory lesions (1, 20, 24) have been shown. The iNOS based proliferative potential in epithelial tissues on periapical inflammatory lesions has been emphasized (37). Similarly, iNOS expression may be related to proliferative potential of the epithelial components of DIFIH lesions, which are characterized by epithelial hyperplasia.

The possibility of chronic inflammation mediating oral cancer growth from oral premalign lesions has been emphasized (25). It is important to determine the presence and level of dysplasia in malignant transformations. Brennan et al, has pointed out that in oral epithelial dysplasia, iNOS expression increases with the amount of dysplasia (2). It is claimed that iNOS may have a role in invasive oral cancer transformation from dysplasia (2). This leads to the conclusion that in DIFIH lesions, for which dysplastic formation is possible, iNOS-IR should be taken into consideration. Moreover, the importance
of NOS-related mechanism in oral premalign to malignant lesion transformation has been shown (7, 8). In the publications on malign pathologies, iNOS is considered almost like a tumour marker (8); at the very least it was thought to be related to malignant potential (23). At this point, it is necessary to consider the complex effects of NO on the body. NO generated by NOS (synthesized from the amino acid L-arginin) has both positive and negative effects on the body. Thus acting like a “double edged sword” (29, 34).

Our results show that iNOS expression may be present in HOM constitutively. Although the importance of iNOS in malign transformation has been emphasized, sometimes iNOS expression, even in neoplastic epithelial, may be similar to normal epithelial tissues (4, 23, 29).

Conclusions
In this study, authors took notice of iNOS implicated in the pathogenesis of inflammatory process and investigated the relationship between iNOS and DIFIH. The results indicate that iNOS-IR cannot be determining factor in pathogenesis of DIFIH. However, HOM and DIFIH both being iNOS-IR positive, is not surprising since there is lack of statistical difference between them. This may lead to the conclusion that, oral mucosa is already susceptible for iNOS-based inflammatory process and proliferative potential activation caused by mechanical trauma. However, there is need for further research on constitutively iNOS expression of HOM under physiological conditions.

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REFERENCES