Impact of Smoking on p65 Nuclear Factor κB, p38 Mitogen-Activated Protein Kinase, and Inducible Nitric Oxide Synthase Expression Levels in Oral Mucosa

Aysenur Meric, MD, Berke Ozucer, MD,* Orhan Gedikli, MD,† Yalcin Yildirim, MD,* Arzu Yasemin Korkut, MD,‡ Volkan Kahya, MD,* Sabri Baki Eren, MD,* and Adnan Somay, MD§*

Objectives: Smoking plays an important role in oral cancer development; however, the molecular carcinogenesis mechanism in oral mucosa is not well understood. The aim of this study was to examine and compare the levels of p65 nuclear factor κB (NF-κB), p38 mitogen-activated protein kinase (MAPK), and inducible nitric oxide synthase (iNOS) expressions between oral mucosa of nonsmoker and smoker volunteers.

Methods: Oral cheek mucosa was collected from 78 volunteers. Smokers were divided into 2 subgroups: light smokers (<40 pack years) and heavy smokers (≥40 pack years). Paraffinized tissue immunochemistry was carried out for p65 NF-κB, p38 MAPK, and iNOS expression with specific antibodies. Results were evaluated based on diffuseness and intensity of staining.

Results: Group 1 composed of 40 nonsmokers: 52.5% were female and 47.5% were male, with a mean age of 46.4 years. Group 2 composed of 38 smokers (20 light smokers, 18 heavy smokers): 39.5% were female and 60.5% were male, with a mean age of 48.9 years. Total immunohistochemical staining scores of smokers were significantly higher compared with those of nonsmokers in p65 NF-κB, p38 MAPK, and iNOS expression ($P < 0.001$). The highest p65 NF-κB, p38 MAPK, and iNOS expression levels were detected in the oral mucosa of heavy smokers. The expression of iNOS and p65 NF-κB in heavy smokers was significantly higher compared to that in light smokers ($P < 0.01$ and $P < 0.05$, respectively). Although p38 MAPK expressions were higher in heavy smokers compared with light smokers, the difference was not statistically significant ($P > 0.05$).

Conclusions: Our results show for the first time the significant increase in the expression of p65 NF-κB and p38 MAPK in the oral mucosa of smokers. Levels of p65 NF-κB, p38 MAPK, and iNOS expression in the oral mucosa of smokers were related to the number of pack years.

Key Words: Smoking, oral mucosa, nuclear factor κB, mitogen-activated protein kinase, inducible nitric oxide synthase

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From the *Otolaryngology Department, Bezmialem Vakif University Medical Faculty, Fatih, Istanbul; †Otolaryngology Department, Kirklareli University Medical Faculty, Kirklareli; ‡Otolaryngology Department, Bagcilar Training and Research Hospital, Bagcilar; and §Pathology Department, Bezmialem Vakif University Medical Faculty, Fatih, Istanbul, Turkey.

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Address correspondence and reprint requests to Berke Ozucer, MD, Department of Otolaryngology, Bezmialem Vakif University Medical Faculty, Fatih, Istanbul, Turkey; E-mail: berkeozucer@gmail.com

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Oral cancer is the fifth most common malignancy worldwide. Epidemiologic studies have identified the major role of smoking on the development of head and neck cancers.[1,2] It has been currently shown that as smoke passes through the upper respiratory airways; nicotine, along with other components, causes oxidative damage, induces cytotoxicity, causes necrosis of keratinocytes, and leads to the formation of premalignant and malignant lesions on oral mucosa.[3] However, the molecular mechanisms of this malignant transformation are not well understood.

p65 nuclear factor κB (p65 NF-κB) is a ubiquitous transcriptional factor for the induction of gene expressions of inflammatory and host defense processes.[4] p65 NF-κB also has the ability to inhibit apoptosis, maintain survival, and promote cell growth. Free radicals and oxidative damage results in NF-κB translocation, leading to activation of genes such as inducible nitric oxide synthase (iNOS). p38 mitogen-activated protein kinase (MAPK) is involved in the activation of NF-κB and is shown to increase iNOS expression in in vitro experiments.[5,6] Activation of iNOS results in high levels of nitric oxide (NO), which is cytotoxic to mucosa and plays an important role in carcinogenesis and tumor progression.[7] Previous studies have shown the role of iNOS, p65 NF-κB, and p38 MAPK pathways in DNA injury.[5–10]

In this study, we studied p65 NF-κB, p38 MAPK, and iNOS expression levels in the oral mucosa of smokers and nonsmokers to investigate the effects of smoking on the expression of these factors that are closely related to cytotoxicity, DNA injury, and carcinogenesis.

METHODS

Collection of Tissues

The present study was conducted at the Otolaryngology Department, Medical Faculty, Bezmialem Vakıf University, according to the Declaration of Helsinki. All procedures were carried out under informed consent, with approval from the ethics committee and in

accordance with the guidelines of the National Health and Medical Research. Study design was prospective, randomized, and double-blind. Participants were questioned in detail for history of alcohol consumption, use of local oral agents, intraoral infections, oral lesions, dental diseases, systemic diseases, and tumoral lesions in the oral mucosa; any positive history was a criterion for exclusion from the study.

Each volunteer was questioned for age, smoking history, duration, and pack years of smoking. Smokers were classified into 2 subgroups: light smokers had a smoking history of less than 40 pack years and heavy smokers had a smoking history of more than 40 pack years.

Tissue samples of the oral mucosa were collected by doing a punch biopsy on the cheek after 1% lidocaine infiltration anesthesia.

Immunohistochemical Analysis

Preparations were fixated with formol and incubated in paraffin wax blocks. Each preparation was examined with light microscopy and incubated at 60°C overnight. After rehydrating in a decreasing series of ethanol, specimens were washed with distilled water and phosphate-buffered saline for 10 minutes. Specimens were then treated with 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37°C for 15 minutes and again washed with phosphate-buffered saline.

Specimens were delineated with a Dako pen (Dako, Glostrup, Denmark), incubated in a solution of 3% H_2O_2 for 15 minutes to inhibit endogenous peroxidase activity, and incubated with antibodies afterward. Antibodies against p65 NF-κB and iNOS (RB1639R7 and RB1605R7, respectively) were purchased from Neomarkers Lab Vision (Fremont, CA). p38 MAPK antibody was obtained from Vector Laboratories (Burlingame, CA). Ultra Vision (Lab Vision) horseradish peroxidase/3-amino-9-ethylcarbazole staining protocol was used.

Specimens prepared for each individual were examined with light microscopy. Specimens of oral mucosa were used as control according to data provided by the antibody manufacturer. Positive and negative controls were conducted along with p65 NF-κB–, p38 MAPK–, and iNOS-stained specimens. Commercially available antibody-stained specimens served as the positive control; negative control included staining tissue specimens with omission of the primary antibody.

On the basis of the diffuseness of the staining specimens were scored as follows: 0, no staining; 1, staining less than 25%; 2, staining between 25% and 50%; 3, staining between 50% and 75%; or 4, staining 75%. Another scoring system was based on the intensity of the staining: 0, no staining; 1, weak but detectable staining; 2, distinct staining; 3, intense staining as explained elsewhere.[11] Total score was obtained by adding the diffuseness and intensity scores (level 0–7).

Statistical Analyses

Statistical analyses of data were carried out with the χ^2 test and one-way analysis of variance followed by Tukey multiple comparison tests. Results of all groups were calculated as mean (SD). $P < 0.05$ was accepted as statistically significant.

RESULTS

A total of 78 subjects in the study were divided into 2 main groups: group 1 was composed of 40 nonsmokers (21 women [52.5%] and 19 men [47.5%]), with a mean (SD) age of 46.45 (5.7) years; and group 2 was composed of 38 smokers (15 women [39.5%] and 23 men [60.5%]), with a mean (SD) age of 48.92 (5.8) years. Group 2 was further divided into 2 subgroups: 20 smokers with a smoking history of less than 40 pack years (20.95 [3.4]) and 18 subjects with smoking history of more than 40 pack years. There was no statistically significant difference between the 2 groups in age and sex ($P > 0.05$).

TABLE 1. Detailed Immunohistochemical Staining Scores of Groups 1 and 2

Total Score	Group 1 (n = 40)	Group 2 (<40 Pack Years; n = 20)	Group 2 (>40 Pack Years; n = 18)
iNOS			
0	14	—	—
1	8	—	—
2	10	—	—
3	7	—	—
4	1	7	1
5	—	5	4
6	—	6	7
7	—	2	6
NF-κB/p65			
0	13	—	—
1	6	—	—
2	13	3	—
3	5	2	—
4	3	7	3
5	—	2	4
6	—	4	6
7	—	2	5
p38			
0	15	—	—
1	5	1	—
2	8	2	—
3	6	1	—
4	2	4	3
5	4	2	3
6	—	5	5
7	—	5	7

Total immunohistochemical staining scores of subjects are detailed in Table 1: The iNOS protein was detected as level 0 or 1 in 55% of nonsmokers (Fig. 1A). Conversely, iNOS was detected as level 6 or 7 in 55.2% of smokers (Fig. 1D). p38 MAPK was expressed as level 0 or 1 in 50% nonsmokers (Fig. 1B) and as level 6 or 7 in 57.9% of smokers (Fig. 1E). Nuclear factor κB staining was mainly detected as level 0 or 1 in 47.5% of nonsmokers (Fig. 1C) and as level 6 or 7 in 44.7% of smokers (Fig. 1F).

Mean immunohistochemical staining scores are compared in Figure 2. Mean staining scores of p65 NF-κB, p38 MAPK, and iNOS expression in group 2 (smokers) were found to be significantly higher compared with group 1 ($P < 0.001$). The highest p65 NF-κB, p38 MAPK, and iNOS expression levels were detected in the oral mucosa of smokers with a smoking history of more than 40 pack years. The expression of iNOS and p65 NF-κB in heavy smokers was significantly higher compared with that in light smokers ($P < 0.01$ and $P < 0.05$, respectively). Although p38 MAPK expressions were higher in heavy smokers compared with light smokers, it was not statistically significant ($P > 0.05$).

DISCUSSION

Oral cancers are associated both with genetic and environmental factors such as smoking and alcohol consumption. The oral mucosa is a candidate tissue for smoke-induced carcinogenesis owing to its direct exposure to high concentrations of compounds contained within cigarette smoke. The oral mucosa can be used to evaluate the response to cigarette smoke. Taking a sample from the

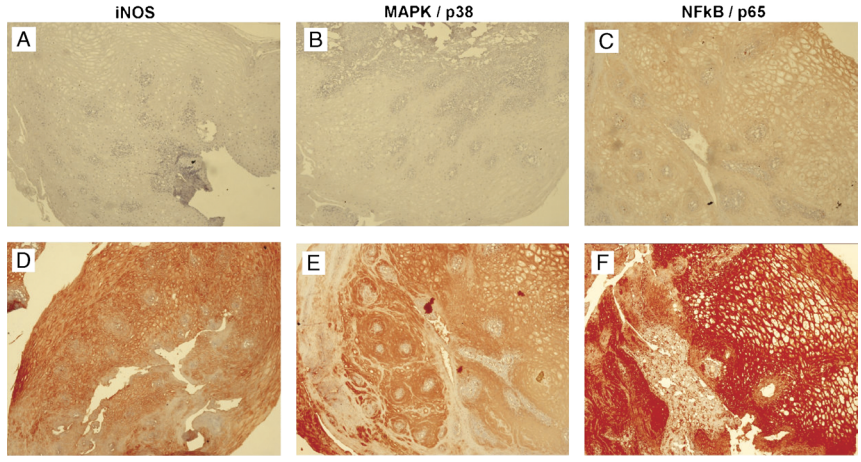

FIGURE 1. Immunohistochemical staining showing p65 NF-κB, p38 MAPK, and iNOS expressions. Original magnification, ×100. A, Group 1: low staining (score 1) with iNOS. B, Group 1: low positivity (score 1) with MAPK/p38. C, Group 1: low NF-κB/p65 positivity (score 2). D, Group 2: diffuse iNOS staining (score 6). E, Group 2: intensive MAPK/p38 positivity (score 6). F, Group 2: intensive NF-κB/p65 positivity (score 7).

oral cavity is practical and less invasive, and it would allow for the use of larger cohorts for developing and validating biomarkers of cigarette exposure and susceptibility to cigarette-related disease.[12,13]

Increasing evidence suggests that NF-κB plays a role in carcinogenesis. Du et al[14] demonstrated that the expression of p65 NF-κB was significantly increased in the tumor tissue and that the expression was mainly confined to the nucleus. Duffey et al[15] observed that nuclear localization of p65 NF-κB were detected in squamous cell carcinoma cell lines of human pharyngeal origin. Human cell lines have been used to demonstrate the increased level of NF-κB–dependent cytokines that have roles in cell growth, tumor invasion, and interruption of tumor suppression in oral-pharyngeal cancers in vitro.[16] In our study, we showed that p65 NF-κB expression increased in parallel with the duration of smoking. This may help to explain the mechanism of transformation of oral mucosa to carcinoma.

Mitogen-activated protein kinase pathways are important in cancer pathogenesis because they control processes that are central to malignant progression such as cell growth, apoptosis, and cellular migration.[17] Mishima et al[18] observed the overexpression of the MAPK in human oral squamous cell carcinoma. In our study, we observed that the staining intensity of heavy smokers was severe for p38 MAPK, which may have a role in the malign transformation of the oral mucosa.

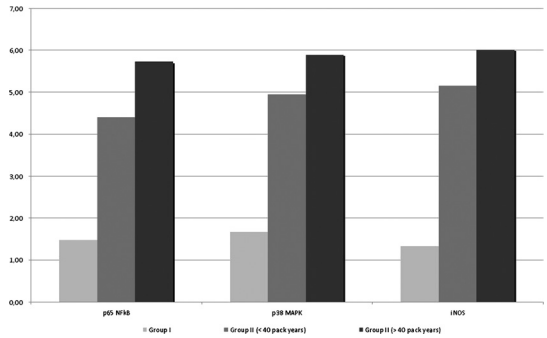

FIGURE 2. Immunohistochemical staining score in groups 1 and 2. Values are expressed as mean staining score. Total staining scores of both group 2 (<40 pack years) and group 2 (>40 pack years) are higher compared with those of group 1 ($P < 0.001$) for p65 NF-κB, p38 MAPK, and iNOS ($P < 0.001$). The expression of iNOS and p65 NF-κB in heavy smokers was significantly higher compared with that in light smokers ($P < 0.01$ and $P < 0.05$, respectively). Although p38 MAPK expressions were higher in heavy smokers compared with light smokers, it was not statistically significant ($P > 0.05$).

Bentz et al[19] reported the immunohistochemical expression of iNOS in cases of hyperplasia, dysplasia, and invasive cancer and found significant increase in iNOS staining intensity in cancer cases compared with the normal oral mucosa. A different study showed that the expression level of iNOS was higher in head and neck squamous cell carcinoma specimens as compared with that in normal mucosa.[20] Increased iNOS expression and the generation of high NO levels might have a role in oral squamous cell carcinoma development.[21] Sappayatosok et al[22] found that the expression of iNOS is associated with carcinogenesis and angiogenesis in oral squamous cell carcinoma. In our study, we observed that iNOS expression in the oral mucosa is increased by cigarette smoke in a dose-dependent manner. This suggests that iNOS may have a role in dysplastic degeneration caused by smoking in human oral mucosa.

Uffort et al[23] showed that the constitutively activated MAPK pathway stimulates activation of NF-κB, which, in turn, drives iNOS expression and aggravates carcinogenesis. Previous studies have suggested that p65 NF-κB, p38 MAPK, and iNOS may have a role in tumorigenesis by DNA damaging.[24]

In a study, it was observed that NF-κB is dose-dependently activated by subchronic exposure to cigarette smoke in vivo.[25] In our study, there was a significant difference in p65 NF-κB and iNOS expression between light smokers and heavy smokers. In heavy smokers, staining intensity was severe for p65 NF-κB, p38 MAPK, and iNOS expression. Therefore, our findings show that p65 NF-κB, p38 MAPK, and iNOS expression increases in parallel with the years and intensity of the smoking. p65 NF-κB, p38 MAPK, and iNOS were also expressed in the oral mucosa of healthy nonsmokers but in lower degrees. It has previously been shown that normal cell growth may require the maintenance of these 3 proteins' expression within a narrow range.[4,5]

In our study, we did not confirm the relation and clinicopathologic correlation of p65 NF-κB, p38 MAPK, and iNOS; therefore, further research is required to establish the relationship among these proteins. It would be interesting to determine whether one or more of these proteins are involved in malign transformation process of the oral mucosa. Further studies will be necessary to address how much and by what kind of mechanisms the p65 NF-κB, p38 MAPK, and iNOS pathway may contribute to oral carcinogenesis of smokers.

CONCLUSIONS

Our results show for the first time the differential expression of p65 NF-κB, p38 MAPK, and iNOS in nonsmoker and smoker oral mucosa. A gradual increase in p65 NF-κB, p38 MAPK, and iNOS expression in smokers' oral mucosa was found in parallel to the increase in years of smoking. The p65 NF-κB, p38 MAPK, and iNOS pathway may be responsible for smoking induced damage. Further studies are required to confirm this proposal using damaged oral mucosa samples of smokers.

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