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ScienceDirect
Experimental and Molecular Pathology 81 (2006) 123–130
Experimental and Molecular Pathology
www.elsevier.com/locate/yexmp

NF-κB and COX-2 during oral tumorigenesis and in assessment of minimal residual disease in surgical margins

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Received 1 December 2005, and in revised form 1 December 2005
Available online 5 July 2006

Abstract

Oral cancer is a major health problem in many parts of the world including India. The molecular mechanisms involved in oral tumorigenesis are not completely understood. Although surgery continues to be the most common treatment modality for this cancer, survival rates of oral cancer patients have still not significantly improved over the last few decades. Classical diagnostic methods are still not sensitive enough in detecting completeness of surgery and assessing minimal residual disease. This study investigated the role of NF-κB and COX-2 both in oral cancer progression and assessment of minimal residual disease. Expression of NF-κB proteins and its inhibitory protein IκB-α was evaluated using immunohistochemistry, ELISA and EMSA, while RT-PCR was used to detect COX-2 expression. Cytoplasmic expression as well as nuclear translocation of NF-κB proteins increased with histological progression of oral cancer (from normal to leukoplakia to cancer). A similar pattern of expression was observed for COX-2 also. NF-κB proteins, both cytoplasmic and nuclear, had a significant negative correlation from tumor to surgical margin to extra margin; COX-2 paralleled the expression of NF-κB proteins. Our results thus point to NF-κB and COX-2 as participants in oral tumor progression and also to the validation of these two molecular markers in assessing minimal residual disease.

Keywords: Minimal residual disease; Oral cancer; NF-κB; COX-2

Introduction

Carcinomas of the oral cavity are among the 10 most common cancers in the world, accounting for about 3–5% of all malignancies (Parkin et al., 1993). There is an increase in the incidence of oral cancer globally, especially in developing countries. Oral cancer incidence varies strikingly around the world. In parts of India and Southeast Asia, it is one among the most common cancers owing to the use of chewing quids containing arecanut and tobacco. Squamous cell carcinomas (SCC) are the most common malignancies of the oral cavity (accounting for about 90% of oral tumors).

Malignancy of the oral cavity is often preceded by premalignant lesions, the most common of which is leukoplakia. The annual percentage of malignant transformation of leukoplakia varies in different parts of the world owing to the difference in the use of tobacco and dietary habits. The rate of malignant transformation varies from almost 0% to almost 20% in 1 to 30 years (Lodi et al., 2002).

Treatment modalities for oral cancer include surgery, chemotherapy and radiotherapy. Despite advances made in the management of oral cancer over the last few decades, the survival rate of patients remains unacceptably low. The most important factor in surgical management of oral cancer is the completeness of surgical removal of tumor. Surgical oncologists rely heavily on histopathological assessment of surgical margins to ensure total excision of the tumor. Even when the surgical margins are diagnosed as tumor-free by histopathological examination, local recurrence rate is still 10–30% (Leemans et al., 1994). The success rate of many cancer treatments is largely dependent on the often microscopically undetectable tumor cells that remain in the body (minimal residual disease or MRD). Classical diagnostic modalities such as histopathology and radiology are not sensitive enough to detect these small numbers of cells that remain in the body. However, tumor cells harbor specific clonal genetic

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doi:10.1016/j.yexmp.2006.05.001

changes. These genetic alterations can be used as molecular markers (van Houten et al., 2000) for the detection of oral tumorigenesis and in the assessment of MRD.

Nuclear factor kappa B (NF-κB) is an inducible transcription factor that mediates signal transduction between cytoplasm and nucleus (Baeuerle and Henkel, 1994). There are five mammalian REL/NF-κB proteins that belong to two groups: those that do not need proteolytic processing and those that do require proteolytic processing. The first group consists of RELA (also known as p65), c-REL and RELB. The second group includes NF-κB1 (also known as p105) and NF-κB2 (also known as p100), which are processed to produce the mature p50 and p52 proteins respectively. Members of these two groups of proteins can form homodimers and heterodimers—the most commonly detected NF-κB dimer is the heterodimer p50-RELA (p50–p65) (Karin et al., 2002). In most of the resting cells, NF-κB complex exists in a latent, inactive form in the cytoplasm bound with their inhibitory proteins (IκBs) (Barkett and Gilmore, 1999), of which IκB-α is the best characterized. Upon stimulation with a variety of pathogenic inducers such as viruses, mitogens, bacteria, agents providing oxygen-free radicals and inflammatory cytokines, IκB is phosphorylated, ubiquitinated and degraded in the cytoplasm. This NF-κB complex can migrate to the nucleus and binds to DNA recognition sites in the regulatory regions of target genes (Thanos and Maniatis, 1995). There are several reports indicating the role of the gene products of this transcription factor in cell proliferation, transformation and tumor development (Pahl, 1999; de Martin et al., 1999; Sonenshein, 1997; La Rosa et al., 1994). Several studies have indicated that NF-κB is constitutively activated in several tumors (Zhu et al., 2002; Guo et al., 2001; Wang et al., 1999; Sovak et al., 1997) including oral tumors (Nakayama et al., 2001).

Cyclooxygenase (COX) enzymes catalyze the synthesis of prostaglandins from arachidonic acid. The two isomers of COX, COX-1 and COX-2, differ in many respects. COX-1 is expressed constitutively in most tissues. This isoenzyme is responsible for the production of prostaglandins that control normal physiological functions (Subbaramaiah and Dannenberg, 2003). COX-2 is not detected in most normal tissues and is induced by most mitogenic and inflammatory stimuli, which results in enhanced synthesis of prostaglandins in neoplastic and inflamed tissues (Smith et al., 2000; Subbaramaiah et al., 1996). Multiple lines of evidence show that COX-2 has a significant role in carcinogenesis and is overexpressed in transformed cells (Subbaramaiah et al., 1996), premalignant and malignant tissues (Kulkarni et al., 2001; Tucker et al., 1999; Ristimaki et al., 1997; Eberhart et al., 1994) including that of head and neck (Chan et al., 1999).

The promoter region of human COX-2 gene contains two NF-κB consensus sites, and the expression of COX-2 is largely regulated by NF-κB in several cells (Hla and Neilson, 1992). A large amount of evidence also supports a role of NF-κB in the expression of COX-2 that regulates cell proliferation (Kojima et al., 2000; Gallois et al., 1998). This investigation looked at the role of these two molecular markers—NF-κB and COX-2 in oral tumorigenesis and in the assessment of MRD. To our present day knowledge, this is the first study comparing the expression of a transcription factor NF-κB and one among its target gene COX-2 in oral tumor progression and in the assessment of MRD.

Materials and methods

Study subjects and tissue specimens

Two sets of samples were used for this study. The first set included tissues from various phases of oral cancer progression, namely 40 normal oral mucosa, 54 leukoplakia and 54 oral cancer. The second set was obtained from 34 patients undergoing surgical resection for oral cancer and included the primary tumor, surgical margin and an extra margin tissue (2 cm away from the actual surgical margin). The Institutional Review Board and Human Ethics Committee of the Regional Cancer Centre approved the study. Informed consent was obtained from all study subjects. All clinicopathological data were entered into a study database. Normal oral mucosa was taken from persons undergoing oral and maxillofacial reconstructive surgery and specifically excluded any subject with a history of tobacco or alcohol use. None of the leukoplakia patients had received any treatment. Oral cancer patients, who received preoperative chemotherapy or radiotherapy, were excluded from this study.

Immunohistochemistry

Immunohistochemical reactions were carried out as described by us earlier (Lakshmi et al., 1997). Briefly, sections were deparaffinized in xylene and rehydrated through graded alcohol to water. Endogenous peroxidase activity was blocked with 1.5% H_2O_2 in methanol for 15 min. Sections were then incubated with 3% bovine serum albumin (BSA) for 30 min followed by incubation at 4°C overnight with primary antibodies specific for p50, p65 and IκB-α (IMGENEX, USA) at dilutions 1:150, 1:50 and 1:50 respectively. The reactions were visualized using DAKO ABC kit (DAKO, Denmark) following manufacturer's protocol. The color developed with diaminobenzidine (DAB-Sigma, USA) was counterstained with Mayer's hematoxylin. The sections were dehydrated in graded alcohol, cleared in xylene and mounted.

Preparation of nuclear extract

To get a homogenous cellular suspension, the tissues were minced and homogenized in 1 × PBS on ice, strained through cheesecloth and centrifuged at 4000 rpm. The pellet was treated with Buffer A (10 mM HEPES pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF with 2 μg each of aprotinin, leupeptin and benzamidine), the mixture was incubated on ice for 30 min and centrifuged at 10,000 rpm to get the intact nuclei. Buffer C (10 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and 1 mM PMSF with 2 μg each of aprotinin, leupeptin and benzamidine) was added and incubated on ice for 3 h with occasional mixing. The extract was centrifuged at 12,000 rpm, and the supernatant was stored at −80°C until use.

Electrophoretic mobility shift assay (EMSA)

The NF-κB gel shift oligonucleotide (Santa Cruz Biotechnology, USA) was used as DNA probe for gel shift assay following manufacturer's protocol. In brief, the DNA probe was labeled with [γ-p^{32}] ATP, using a T4 Polynucleotide kinases (50,000 cpm/ng) (Promega, USA). Binding reaction mixture containing 0.5 ng of labeled probe and 10 μg nuclear extracts in 10 mM Tris (pH 7.5) buffer with 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol and 1 μg of ploy dI–dC was incubated on ice for 40 min. DNA–protein complexes were resolved by electrophoresis through a non-denaturing 4% PAGE containing 2.5% glycerol and 0.25× TBE. The gels were subsequently dried and developed by autoradiography.

Enzyme linked immunosorbent assay (ELISA)

Nuclear translocation of NF-κB in tissue samples was detected using NF-κB active ELISA™ kit (Imgenex, USA). This kit measured free p65 in the nuclear extract. NF-κB active ELISA™ was carried out following manufacturer's