The expression of the receptor for glycation endproducts (RAGE) in oral squamous cell carcinomas

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Advanced glycation endproducts (AGEs) and their receptors, receptor for advanced glycation endproducts (RAGE), are novel groups of molecules with roles in inflammation, cytokine activation, and promotion of cell growth. Recently, RAGE has been implicated in the progression and metastasis of several epithelial tumors. The expression of RAGE was examined in 38 oral squamous cell carcinoma (OSCC) cases by immunohistochemistry. In the OSCCs, RAGE positivity, interpreted as more than 25% positive cells, was detected in 10 of 10 well-differentiated, 3 of 4 well-to-moderately differentiated, 3 of 9 moderately differentiated, 1 of 7 moderate-to-poorly differentiated, and 0 of 8 poorly differentiated tumors. The staining percentage was significantly higher in well-differentiated tumors compared to moderately ($P < .05$) and poorly differentiated ($P < .05$) tumors. All normal mucosa samples were RAGE-positive. Western blot analysis for RAGE was performed on 2 OSCCs and 2 normal oral mucosa samples. Higher expression was observed in the normal tissues compared to the OSCCs. Our results show that RAGE immunoreactivity correlates with histologic differentiation in OSCC. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;105:617-24)**

Oral squamous cell carcinoma (OSCC) is the eighth most common cancer worldwide,[1,2] accounting for 2% to 4% of all newly diagnosed malignancies in the United States[3] and 90% of all oral malignancies.[4,5] Although OSCC is a potentially curable disease in its early stages, treatment may be associated with a plethora of long-term side effects, post-therapy complications, and significant decreases in quality of life.[6] Furthermore, it is well recognized that the mortality rates associated with OSCC have remained largely unchanged over the past several decades,[5,7-9] even increasing in certain regions of the world.[10] Consequently, continued focus on improving prevention strategies as well as identifying superior predictors of disease appear necessary.

OSCC is a multifactorial condition with etiologic links to a wide array of external carcinogens, including tobacco, alcohol, betel quid (paan), and certain viral and bacterial infections.[7] Carcinogenesis likely involves a complex interplay of environmental and hereditary predisposition factors, which together promote the development of specific genetic aberrations.[2,11] In particular, the activation of certain oncogenes and abrogation of tumor suppressor genes has garnered considerable interest in recent years. To date, investigators have successfully elucidated roles for several genes including epidermal growth factor (EGFR), cyclin D1, cyclin A1, p53, p16,[9,12-15] and many others in the development and progression of OSCC.

Advanced glycation endproducts (AGEs) and their receptors—receptor for advanced glycation endproducts (RAGE) represent classes of molecules that are formed by nonenzymatic glycation of proteins. Increased expression of AGEs and RAGE are seen in a number of different pathologic and nonpathologic states including inflammation, diabetes, renal failure, Alzheimer's disease, and aging.[16-19] Interestingly, recent evidence supports a role for RAGE and its ligands in the development and metastasis of a subset of carcinomas.[20-24]

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In this study, we aim to examine the expression of RAGE in OSCCs of varying histologic differentiations.

MATERIALS AND METHODS

Data retrieval

Thirty-eight biopsies of OSCC were retrieved from the files at the Oral Diagnostics Biopsy service and the Department of Pathology at Columbia University Medical Center (CUMC). The histological diagnosis and grading of each case was verified independently by 2 oral pathologists (V.W., C.P.) according to the World Health Organization (WHO) criteria.[1] A total of 10 well-differentiated, 4 well-to-moderately differentiated, 9 moderately differentiated, 7 moderate-to-poorly differentiated, and 8 poorly differentiated tumors were selected for the immunohistochemical staining studies. Tissue from 2 OSCCs, 1 well-to-moderately differentiated and 1 moderate-to-poorly differentiated, was snap-frozen for subsequent western blot analysis. Normal human oral mucosa served as controls (n = 12 for immunohistochemistry, n = 2 for western blot). This study was conducted in accordance with CUMC Institutional Review Board (IRB) regulations.

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tissue sections were subjected to microwave heat-induced epitope retrieval using citrate buffer for 10 minutes. A 3% hydrogen peroxide solution was used to eliminate endogenous peroxides. Nonspecific ligands were blocked with CAS block solution (Zymed, San Francisco, CA). The sections were then exposed to polyclonal anti-RAGE immunoglobulin G (IgG) (dilution 1:20, R&D, Minneapolis, MN) or monoclonal anti–proliferating cell nuclear antigen (anti-PCNA) IgG (dilution 1:50, Dako, Glostrup, Denmark). Detection of the antibody-antigen complexes was achieved by an indirect biotin-avidin system as per the manufacturer's specifications (Vector, Burlingame, CA).

RAGE immunoreactivity was assessed independently by both oral pathologists with consensus achieved on all 38 tumors. A standard 4-point scoring system based on the percentage of positive cells was employed as follows: score 1, 5% or fewer cells positive; score 2, 6% to 25% cells positive; score 3, 26% to 50% cells positive; and score 4, more than 50% cells positive. Staining intensity was also evaluated and graded as negative, weak, moderate, or strong as per Katz et al.[25] A tumor was designated as overall RAGE-positive if greater than 25% of the epithelial cells (RAGE score of 3 or 4) exhibited immunoreactivity.

Proliferating cell nuclear antigen (PCNA) is a cell cycle-regulated protein essential for DNA replication and repair, with purported roles in chromatin assembly and RNA transcription.[26] Nuclear localization of this protein is seen at sites of DNA synthesis and can serve as a useful marker of active cell turnover.[27] We performed PCNA staining in all our samples to determine the fraction of proliferating cells. Furthermore, we were interested in confirming that any lack of RAGE expression was not related to a decrease in proliferation rate and/or tumor necrosis or infarction. PCNA reactivity was assessed independently by both oral pathologists with consensus achieved on all 38 tumors. A 4-point scoring system based on percentage positive cells was used as follows: score 1, 10% or fewer cells positive; score 2, 11% to 25%; score 3, 26% to 50%; and score 4, more than 50%. Tumor cells were required to exhibit nuclear staining to be considered positive. Appropriate positive and negative controls accompanied all immunohistochemical procedures.

Detection of RAGE antigen by western blot analysis

Western blot analysis of 2 samples of normal oral epithelium, 1 case of well-to-moderately differentiated OSCC, and 1 case of moderate-to-poorly differentiated OSCC was performed in parallel with the RAGE immunohistochemistry studies. All samples were homogenized in lysis buffer, loaded onto a 4% to 12% PAGE gel (8 μg protein/lane), transferred to a nitrocellulose membrane, and blocked with 5% nonfat dry milk/tris buffered saline (TBS) at 37°C. The membranes were incubated with the primary antibody (anti-human RAGE monoclonal antibody, dilution 1:500 [R&D, Minneapolis, MN]) overnight at 4°C. Following 3 washes with TBS, the secondary antibody was applied (Ab-HRP, dilution 1:10,000 [Sigma, St. Louis, MO]) for 30 minutes at 37°C. The membranes were then developed with Enhanced Chemiluminescence (Amersham, Piscataway, NJ) and exposed to film for 30 minutes.

To further verify that the antibody used for our studies was specific for RAGE, antibody depletion studies with soluble human RAGE (shRAGE) were performed. Two samples of normal human oral epithelium were run on 4% to 12% PAGE gels (8 μg protein/lane), transferred onto nitrocellulose paper, blocked with 5% nonfat dry milk/TBS for 30 minutes at 37°C, and washed with TBS solution for 5 minutes. The "absorbed" anti-RAGE antibody consisted of antibody previously incubated overnight at 4°C with shRAGE in a 1-mM $CaCl_2$ solution to enhance binding. The anti-RAGE antibody and the absorbed anti-RAGE antibody solutions were incubated with the membranes overnight at 4°C. Following 3 washes with TBS, the secondary antibody was applied and the membrane was developed as described above.