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Porphyromonas gingivalis-induced autophagy suppresses cell proliferation through G1 arrest in oral cancer cells

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ABSTRACT

Objectives: We investigated the response of oral cancer cells to intracellular invasion of *Porphyromonas gingivalis* to define changes in the biological characteristics of oral cancer cells evoked by the presence of oral pathogenic bacteria within a tumour microenvironment.

Designs: The proliferative activity, cell cycle, and autophagic response were evaluated in oral cancer cells infected with *P. gingivalis* 381. ROS generation was detected in these cells by DCFDA assay, and its role in the responses of oral cancer cells to *P. gingivalis* infection was further investigated.

Resutls: P. gingivalis inhibited proliferation of oral cancer cells by inducing G1 cell cycle arrest, but had no effect on apoptosis. Following infection with *P. gingivalis*, the expression of cyclin D1 and cdk4 was decreased in oral cancer cells, whereas p21, a Cdk inhibitor, was upregulated, in comparison with non-infected controls. Autophagy was prominently enhanced in these infected cells, presumably contributing to the suppressed proliferation. Further experiments revealed that such autophagic response was activated by the formation of reactive oxygen species, as evidenced by the lack of autophagic response and cell proliferation upon removal of reactive oxygen species.

Conclusions: These findings provide a novel insight into the mechanism by which cancer cells are influenced by tumour microenvironment including oral bacteria.

1. Introduction

Tumour cells are surrounded by the tissues termed as the tumour microenvironment that consist of stromal components, such as extracellular matrix, activated fibroblasts, immune cells, and vascular and lymphatic endothelial cells. Tumour cells continuously receive signals from and interact with diverse cellular elements and proteins in the complex networks of the tumour microenvironment.

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A growing body of studies has suggested that altered molecular elements and deregulated signalling pathways resulting from interactions between tumour cells and their microenvironment contribute to tumour progression.[1] Thus, gaining knowledge about the tumour environment and its control is becoming more important to better understand cancer biology and to devise novel therapeutic approaches.

Chronic periodontitis is one of the most prevalent chronic inflammatory diseases increasing with age. A previous study on the prevalence, severity and extent of periodontitis in adults aged 30 years and older estimated that 8.7%, 30.0% and 8.5% of adults had mild, moderate and severe periodontitis, respectively.[2] For adults aged 65 years and older, 64% had either moderate or severe periodontitis.[2] Coincidently, oral cancer, mostly squamous cell carcinoma, afflicts patients older than 40 years of age in the majority.[3] Therefore, chronic periodontitis may be a part of the microenvironment of oral cancer. Correlation between periodontitis and oral cancer has been evaluated in several epidemiological studies.[4,5] These studies found a significantly increased risk of oral cancer in patients with parameters of periodontal disease, suggesting that chronic periodontitis may be one of the most important contributing factors in oral carcinogenesis and/or the biological behavior of oral cancers. *Porphyromonas gingivalis*, a major etiologic micro-organism of chronic periodontitis, may colonize within the tumour microenvironment of aged patients with oral cancer.[6] The bacteria can invade many cell types by using membrane lipid rafts as a gateway for their internalization into host cells, and cellular invasion by *P. gingivalis* in oral epithelial cells is confirmed by high levels of intracellular *P. gingivalis*.[7–9] In addition, intracellular *P. gingivalis* remained viable for extended periods within the host cell.[10] Thus, it is expected that *P. gingivalis* in the tumour microenvironment can invade oral cancer cells to directly modulate their metabolism. However, little is known about the effect of *P. gingivalis* on oral cancer cells, in contrast to well-established association between *P. gingivalis* and systemic diseases occurring at remote locations from the main habitat of *P. gingivalis*, including cardiovascular lesions.[6,11–13]

In this study, we investigated the modulation of oral cancer cell fate by *P. gingivalis* and the underlying molecular mechanisms to reveal the effect of chronic infection and resultant inflammation on the biological behaviour of cancer cells, specifically oral cancer cells.

2. Materials and methods

2.1. Bacteria and cell culture

P. gingivalis strain 381 was cultured anaerobically at 37 °C in trypticase soy broth supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml) and menadione (1 μg/ml). The oral cancer cells, SCC25 and Ca9-22, were cultured in RPMI 1640 and DMEM, respectively, containing 10% fetal bovine serum (GIBCO-BRL, Rockville, MD, USA) at 37 °C with 5% CO_2. All other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2.2. Bacterial infection

Bacteria were washed twice and re-suspended in phosphate-buffered saline (PBS) before incubation with host cells. Oral cancer cells were infected with bacteria at a multiplicity of infection (MOI) of 100 for 2 h in a CO_2 incubator and then washed with PBS.

2.3. Bromodeoxyuridine incorporation assay

Cells were infected with *P. gingivalis* for 2 h. Subsequently, the cells were washed with PBS and grown for another 24 h before the bromodeoxyuridine (BrdU) incorporation assay (Calbiochem, La Jolla, CA, USA). BrdU assay was performed according to the manufacturer's protocol. Briefly, BrdU was added to plate wells and incubated for 24 h. The cells were fixed and permeabilized. Incorporated BrdU was detected by horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody and the HRP substrate, tetramethylbenzidine.

2.4. Cell cycle analysis

Infected or non-infected oral cancer cells were trypsinized, washed with cold PBS, and then fixed in 70% ethanol at −20 °C overnight. Ethanol-fixed cells were washed with PBS and then re-suspended in PBS containing 40 μg/mL propidium iodide, 0.5 mg/mL RNase and 0.1% Triton X-100. After resting for 30 min at 37 °C in the dark, the cells were analyzed with a flow cytometre (Beckman-Coulter Cytomics FC500, San Jose, CA, USA) equipped with an argon laser at 488 nm. The results were analyzed using the Wincycle software (Beckman-Coulter).

2.5. Acridine orange staining

For the detection of acidic cellular compartments, a characteristic feature of autophagy[14], we used acridine orange (AO), a pH-sensitive dye that emits bright red fluorescence in acidic vesicles, but green fluorescence in the cytoplasm and nucleus. AO was added to oral cancer cells at a final concentration of 1 μg/mL for a period of 15 min[15]. As a positive control for autophagy, cells were starved using Earle's balanced salt solution for 6 h before staining. For inhibition of autophagy, cells were pretreated with 0.5 mM of 3-methyladenine (3-MA; Calbiochem) for 1 h. The changes in cellular fluorescence were monitored using the Axioskop imaging system (Carl Zeiss, Germany).

2.6. Monodansylcadaverine staining

As an independent method to monitor autophagy, oral cancer cells were grown on coverslips, infected with bacteria for 2 h, and washed with PBS three times. After 24 h or 72 h, cells were stained with 0.05 mM monodansylcadaverine (MDC) at 37 °C for 1 h and monitored for their fluorescence. Pictures were obtained with the Axioskop imaging system (Carl Zeiss).

2.7. ROS measurement

To quantify the reactive oxygen species (ROS) generated by *P. gingivalis* invasion, oral cancer cells were treated with a