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Original research

Expression of matrix metalloproteinase-1 and connexin-43 in oral cancer cell lines

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ABSTRACT

Objective: The aim of the present study was to investigate the molecular mechanisms underlying oral squamous cell carcinoma (OSCC) cell proliferation and invasion using a three-dimensional cell culture model.

Materials and methods: Two OSCC cell lines (BSC-OF and HSC-4) were cultured on type I collagen gel. Matrix metalloproteinase (MMP)-1 and connexin-43 expression levels were examined using reverse transcriptional-polymerase chain reaction (RT-PCR) and immunohistochemistry.

Results: Histologically, BSC-OF cells showed exophytic behavior with poor invasiveness, whereas HSC-4 cells showed marked invasion of the underlying collagen gel. RT-PCR revealed that MMP-1 expression was significantly higher in HSC-4 cells than in BSC-OF cells, while connexin expression was significantly higher in BSC-OF cells than in HSC-4 cells ($^*P < 0.05$). Immunohistochemically, marked expression of MMP-1 was observed in HSC-4 cells and aberrant expression of connexin-43 was detected in BSC-OF cells.

Conclusion: These results suggest that high expression of MMP-1 at the mRNA and protein levels in HSC-4 cells is a factor in the induction of cell invasion. They also showed low expression of MMP-1 and aberrant expression of connexin-43 at the mRNA and protein levels in BSC-OF cells, suggesting that this is a factor in the induction of exophytic growth.

1. Introduction

Oral squamous cell carcinoma (OSCC) accounts for approximately 90% of oral cancers [1]. Basaloid squamous cell carcinoma (BSCC) is a rare distinctive histological variant of OSCC with aggressive behavior [2]. OSCC cells invade the surrounding extracellular matrix (ECM), which acts as an essential medium for their growth, survival, and motility and a source of angiogenic factors, significantly affecting tumor biology and progression [3]. Type I collagen, the main component of the ECM, is widely used for cell culture. SAS cells cultured on a collagen gel showed higher gelatinase activity than when cultured on uncoated plastic dishes [4]. In a study using protein analysis, Ziober et al. [5] reported that HSC-3 cells cultured with epidermal growth factor (EGF) degraded type I collagen gel, and concluded that matrix metalloproteinase (MMP)-1 was essential in this process. OSCC cells were invasive when cultured with fibroblasts embedded in a collagen gel but were not invasive when cultured with a collagen gel without fibroblasts [6]. However, to our knowledge, no studies to date have investigated the behavior of BSCC cells cultured on type I collagen gel.

The monolayer cell culture system is commonly used to investigate the characteristics of cancer cell lines. With this system, however, it is impossible to observe three-dimensional (3-D) growth patterns such as invasion. Various *in vitro* invasion models such as spheroids, gel embedding, and advanced tissue-engineered models have been developed and used to analyze cancer biology under conditions similar to those in the living body [7]. Little is known, however, about the proliferation and invasion of OSCC cells when cultured in a 3-D system.

Matrix metalloproteinases are a family of secreted or transmembrane proteins that are capable of digesting the ECM and basement membrane components [8]. These ubiquitous proteases play roles in many major physiological processes such as embryonic development, organ morphogenesis, reproduction, tissue resorption and remodeling [9]. They also play significant roles in pathological processes such as arthritis, cancer, cardiovascular disease, nephritis, neurological disease and fibrotic lung disease [9].

☆ AsianAOMS: Asian Association of Oral and Maxillofacial Surgeons; ASOMP: Asian Society of Oral and Maxillofacial Pathology; JSOP: Japanese Society of Oral Pathology; JSOMS: Japanese Society of Oral and Maxillofacial Surgeons; JSOM: Japanese Society of Oral Medicine; JAMI: Japanese Academy of Maxillofacial Implants.

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Gap junctions are specific cell-to-cell channels formed by two hemichannels (connexons), each of which is composed of 6 proteins called connexins. Gap junctions allow the direct exchange of molecules smaller than 1 kDa such as metabolites, second messengers, and electrical signals between cells and regulate critical processes during cell proliferation, differentiation, and apoptosis [10,11].

The aim of this study was to investigate the molecular mechanisms underlying OSCC cell proliferation and invasion using a 3-D cell culture model employing type I collagen gel. Reverse transcriptional-polymerase chain reaction (RT-PCR) and immunohistochemical techniques were used to analyze expression of MMP-1 and connexin-43.

2. Materials and methods

2.1. Cell lines

Basaloid squamous cell carcinoma-mouth floor (BSC-OF) cells were established from an oral basaloid squamous cell carcinoma by Abiko et al. [12]. A well-differentiated human OSCC HSC-4 cell line was obtained from the Japanese Cancer Research Resources Bank. These cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA) and penicillin-streptomycin (100 U/ml, Invitrogen). Cells were cultured by incubation in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium was changed every 2 or 3 days. When the cells became confluent, they were detached using 0.25% trypsin/0.02% EDTA (pH 7.2, Invitrogen) and then resuspended in the supplemented culture medium described above.

2.2. Three-dimensional culture

The collagen solution was prepared according to the manufacturer's protocol (Cellmatrix Type I-A; Nitta Gelatin Inc, Osaka, Japan). Briefly, the collagen solution was produced by mixing 8 volumes of ice-cold 0.3% Cellmatrix Type I-A, 1 volume of 10× DMEM and 1 volume of 10× reconstitution solution. Twenty-four-well tissue culture plates (FALCON®, Franklin Lakes, NJ, USA) with round plastic cover slips 13 mm in diameter (Thermanox®, Rochester, NY, USA) were incubated with 200 μl mixed collagen solution and 6-well tissue culture plates (FALCON®) were incubated with 2 ml mixed collagen solution, each for 10 min at 37 °C, for morphological and PCR assays, respectively. After the collagen gel formed, the culture medium was added to avoid drying. The cells were then dispensed on the collagen gel at a density of 5×10^4 cells/ml and incubated. The culture media were renewed every other day throughout the experimental period.

2.3. Histological investigation

After 3, 5 or 7 days of culture, the collagen gels and cells were washed twice with 0.01% Phosphate Buffered Saline (PBS; Dainippon Sumitomo Pharma Co., Osaka, Japan) and fixed in 4% paraformaldehyde for 30 min at room temperature. After washing in PBS, the cells were incubated for 30 min with Alexa Fluor® 546 phalloidin (diluted 1:100 with PBS) (Invitrogen, Molecular Probes, Eugene, OR, USA) to stain F-actin and with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (diluted 1:100 with PBS) (Invitrogen, Molecular Probes) to counterstain the nuclei. The cells were then examined and photographed using a confocal laser scanning microscope (LSM 5 DUO, Carl Zeiss, Oberkochen, Germany; software, ZEN 2009). Horizontal sections of the cells were acquired by scanning the *x*–*y* axis of each specimen (0.2 μm in thickness) from the apical to the basal side. The total image was obtained by superimposing these images. The *x*–*z* axis images (vertical sections) of the cells were acquired by reconstructing the *x*–*y* images.

After fixation, samples were also vertically embedded in paraffin and cut into 2-μm thick sections, which were then stained with hematoxylin and eosin. The slides were examined under a light microscope coupled to a digital camera system (UPM Axiophoto2, Zeiss) and 5 random images of each sample were taken. Measurements were performed at the points at which the cell layers on or within the collagen gel were thickest to examine cell proliferation and invasion.

Table 1
Primers used for real-time reverse transcription-polymerase chain reaction.

Primer	Gene name	Assay ID
MMP-1	Matrix metalloproteinase-1, interstitial collagenase	Hs00899658_m1
Connexin	Connexin sorting protein	Hs00410932_m1
Beta-actin	Endogenous control	4333762F

2.4. Quantitative RT-PCR

After 3, 5 or 7 days of culture, total RNA was extracted from the cells using the acid guanidinium thiocyanate-phenol-chloroform method. Briefly, the cells were washed with PBS and homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and chloroform. Supernatants were then obtained by centrifugation at 12,000 rpm for 20 min at 4 °C. Each supernatant was then put into a tube containing 100% isopropanol and stored overnight at −20 °C. The precipitates were washed with 70% cold ethanol. RNA pellets were added to RNAse-free diethylpyrocarbonate-treated water. Total RNA was reverse-transcribed into complementary DNA (cDNA) using a Reverse Transcription Kit (Quanti Tect, Qiagen, Germantown, MD, USA). Quantitative RT-PCR (QRT-PCR) with TaqMan MGB probes (Applied Biosystems Inc., Foster City, CA, USA) was carried out using 1 μl each cDNA. The TaqMan MGB probes and primer sets for the genes (*MMP-1* and *Connexin*, with *β-actin* as an internal control) (Table 1) were purchased from Applied Biosystems. Quantification of mRNA expression was analyzed with the ABI 7500 system software (Applied Biosystems) and compared using the ΔΔCt method. The reaction conditions consisted of a primary denaturation at 95 °C for 20 s, then cycling for 40 cycles at 95 °C for 3 s and at 60 °C for 30 s. Each QRT-PCR analysis was reproduced 4 times.

2.5. Immunohistochemistry

For antigen retrieval, deparaffinized sections were incubated in 0.01 M buffered citric acid and microwaved at 60 °C for 20 min to block endogenous peroxidase activity, sections were exposed to 0.3% hydrogen peroxide in methanol and then incubated with 3% bovine serum albumin (BSA; Roche Applied Sciences, Indianapolis, IN, USA) for 10 min to avoid non-specific reaction. After removal of the blocking solution, the cells were incubated overnight at 4 °C with the primary antibodies. Anti-MMP-1 (ab52631, Abcam, Cambridge, USA) diluted 1:200 in 1% BSA and anti-connexin-43 (ab47368, Abcam) diluted 1:100 in 1% BSA were used. As a negative control, 0.01 M PBS was used instead of the primary antibody. Specimens were then washed and incubated with a biotinylated secondary antibody, Nichirei-Histofine simple-stain MAX-PO MULTI (Nichirei, Tokyo, Japan), for 30 min at room temperature. Staining was visualized using 3,3′-diaminobenzidine in 0.05 M Tris–HCl (pH 7.6) with hydrogen peroxide. The sections were then counterstained with hematoxylin. For the analysis, the slides were examined under a light microscope (UPM Axiophoto2).