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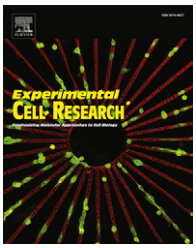

Research Article

The hypoxic tumor microenvironment regulates invasion of aggressive oral carcinoma cells

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ABSTRACT

Invasion is an important hallmark of cancer involving interactions between the tumor microenvironment and the cancer cells. Hypoxia, low oxygen level, is related to increased invasion and metastasis in many cancers. The aim was to elucidate the effect of hypoxia on invasion of oral squamous cell carcinoma cells (OSCCs), and the applicability of a novel 3-dimentional myoma organotypic invasion model in hypoxia experiments. OSCC cell lines (primary oral carcinoma derived cells UT-SCC-43A, recurrent oral carcinoma cells UT-SCC-43B and aggressive tongue carcinoma cells HSC-3) were studied for their migration and invasion capabilities under normoxia, hypoxia, and in the presence a hypoxia-mimicker cobalt chloride. As expected, the recurrent UT-SCC-43B cells were significantly more aggressive than the primary tumor derived cells. In contrast to tongue carcinoma HSC-3 cells, they only mildly responded to hypoxia in the migration or invasion assays, indicating a cell line specific response of hypoxia on the invasive potential. The modification of the organotypic human tissue-derived matrix via the removal of various yet unidentified soluble factors by rinsing the tissue resulting in stripped matrix substantially changed the invasion pattern of HSC-3 cells and the outcomes of hypoxic treatments. Only in the stripped tissue hypoxia significantly increased invasion, whereas in native intact tissue the induced invasion was not observed. This demonstrates the importance of the soluble factors to the invasion pattern and to the hypoxia response. A metastasis and poor prognosis marker, hypoxia-regulated lysyl oxidase (LOX), was present in the myoma tissue, but could be removed by rinsing. The inhibition of LOX resulted in a decrease in invasion area, but

Abbreviations: 3D, Three-dimensional; CAIX, Carbonic anhydrase 9; ECM, Extracellular matrix; EMT, Epithelial–mesenchymal transition; HIF, Hypoxia-inducible factor; IIICTP, Carboxyterminal telopeptide of type III collagen; LOX, Lysyl oxidase; OSCC, Oral squamous cell carcinoma; RIA, Radioimmunoassay; SE, Standard error

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only very mildly in invasion depth. Thus, it may have a role in the modulation of the invasion pattern. Another hypoxia-related poor prognosis marker carbonic anhydrase 9 (CAIX) was induced in HSC-3 cells both by the hypoxic exposure and interestingly in invading HSC-3 cells inside the tissue even in normoxic conditions. In conclusion, this suggests that the intact myoma organotypic model offers optimally hypoxic surroundings, thus being an excellent human tumor microenvironment mimicker.

Introduction

Invasion is a complex process involving interactions between the microenvironment and the cancer cells. Changes in the microenvironment have major consequences on the signaling between the stroma and the cancer cells, allowing abnormal proliferation, migration and invasion [1–3].Therefore, cancer progression cannot properly be studied focusing simply on the cancer cells.

Tumors often contain areas with low oxygen level. Hypoxia is extensively related to the aggressiveness of the disease, with many links to increased invasion. Hypoxia alters gene transcription affecting cell cycle, metabolism, angiogenesis and invasion rate. The abundance of hypoxic areas in tumors is found to correlate positively with increased metastasis formation that results in poor patient outcome [4–7]. Cellular hypoxia is usually considered to be 0.5–1% (7 mmHg) of oxygen [8]. The lack of oxygen in a tumor is primarily due to the enlargement of oxygen diffusion distances, partially because of less ordered vasculature unable to deliver enough oxygen. Tumor areas may also undergo transient changes of oxygen levels or chronic hypoxia after the generation of malformed leaky tumor vessels [9].

The molecular basis for the ability of cancer cells to migrate, invade and metastasize in hypoxic conditions is still unclear, although some important molecules have been elucidated. The major regulator of hypoxic response is hypoxia-inducible factor-1 (HIF-1α), which accumulates under low oxygen level and acts as a transcription factor for over 100 target genes [7,10]. In addition, other signaling pathways regulated by hypoxia are known to take part in tumor progression. Expression of carbonic anhydrase 9 (CAIX), a downstream target of HIF-1α is seen ubiquitously in cancers, including head and neck cancers [11,12]. Carbonic anhydrases are enzymes that function in the regulation of pH homeostasis. CAIX is closely associated with tumor hypoxia. In addition to pH homeostasis, CAIX is implemented in the cell proliferation and transformation, cell adhesion, and tumorigenesis. The expression of CAIX in cancers, including head and neck cancers, is restricted to perinecrotic or hypoxic areas of the tumors [11–15].

Lysyl oxidase (LOX) is another enzyme regulated by HIF-1α that catalyzes the crosslinking of collagens or elastins. LOX is implicated in migration, cell signaling, transcriptional gene regulation, and the regulation of cell adhesion [16] as well as in metastasis promotion [17]. Expression of LOX was reported as a predictor of poor prognosis in lung adenocarcinoma [18], and a predictor of lymph node metastasis in OSCC [19], whereas the downregulation of LOX is reported to increase cell proliferation and the loss of growth factor control [20].

As the hypoxic tumor microenvironment and cancer cell invasion process are interconnected, and the hypoxic areas in tumors are highly related to increased invasion and poor prognosis, we wished to elucidate the role of hypoxia in OSCC migration and invasion utilizing a novel organotypicmyoma invasion model [21]. The myoma model provides an unique human tissue-based tool to study human cancer–human stroma interactions. The specific aims were to characterize the migration and invasion efficacies of various OSCC cell lines; to find out the applicability of the myoma organotypic invasion model to hypoxia experiments; to clarify the effects of microenvironmental factors in the invasion process under normoxic and hypoxic conditions utilizing modifications of the myoma organotypic model; and to study LOX and CAIX in the organotypic-stromal tissue and in the cancer cells. The ultimate aim is to find out key mechanisms that induce the oral cancer cell invasion significantly worsening the prognosis.

Materials and methods

Cell culture

Human tongue squamous cell carcinoma cells (HSC-3) (JCRB 0623, Osaka National Institute of Health Sciences, Osaka, Japan), primary and recurrent oral squamous cell carcinoma cells (UT-SCC-43A and UT-SCC-43B) were used in the experiments. HSC-3 cell line originates from tongue of a 64-year-old male patient and has a high metastatic potential [22]. The HSC-3 media contained DMEM:F12 supplemented with 0.4 mg/ml hydrocortisone, 250 ng/ml fungizone, 50 g/ml ascorbic acid, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Sigma-Aldrich) and 10% heat-inactivated fetal bovine serum (FBS) (Perbio Science, Erembodegem, Belgium). Oral squamous cell carcinoma cell lines UT-SCC-43A and -43B were isolated from a 75-year-old Caucasian female patient's primary carcinoma of mandibular gingiva (UT-SCC-43A) and the recurrent tumor (UT-SCC-43B) [23]. Media for UT-SCC cells contained DMEM (Sigma-Aldrich) supplemented with 2 mML-glutamine (Finnzymes, Espoo, Finland), 1 × NEAA (Finnzymes), 100 U/ml penicillin (Sigma-Aldrich), 100 μg/ml streptomycin (Sigma-Aldrich) and 10% FBS. All the cell lines were cultured at 37 °C of 5% CO_2 in a humified atmosphere, and passaged routinely using 0.025% Trypsin–0.02% EDTA (Sigma-Aldrich, Ayrshire, UK).

Migration assay

The migratory capabilities of HSC-3, UT-SCC-43A and -43B cells were studied in 24-well-plates with Transwell inserts (diameter 6.5 mm, pore size 8 μm, Corning, NY). The wells were first equilibrated in media at 37 °C for 2 h. Then 30,000 cells suspended in 100 μl were applied evenly on each of the inserts, and the cells were allowed to migrate for 20 h at 37 °C. Subsequently, the cells were fixed with 500 μl of 10% trichloroacetic acid (TCA) for 15 min. The cells were washed three times with distilled water and stained with crystal violet (0.1% in 200 mM boric acid, pH 6.0) for 20 min. The results were quantified with a microscope (Leica, Germany).