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Blocking the Na+/H+ exchanger 1 with cariporide (HOE642) reduces the hypoxia-induced invasion of human tongue squamous cell carcinoma

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Abstract. The Na+/H+ exchanger 1 (NHE1) plays a significant role in acidifying the tumour microenvironment, potentially contributing to the tumour's malignant potential. The mechanism by which NHE1 mediates cell invasion and migration, especially in human tongue squamous cell carcinoma (hTSCC), is unknown. In this study, the authors demonstrated that inhibition of NHE1 by cariporide (HOE642) suppressed the invasion and migration of Tca8113 cells under hypoxic conditions. Hypoxia also promoted the production of MMP-9, and the suppressive role of cariporide was enacted through the downregulation of MMP-9. The data demonstrated that NHE1 plays a role in hTSCC invasion and that NHE1 might be a new target for hTSCC treatment.

Keywords: human tongue squamous cell carcinoma; Na+/H+ exchanger 1; cariporide; cell invasion; tumour microenvironment.

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Human tongue squamous cell carcinoma (hTSCC) is the most common type of oral cavity neoplasm. The 5-year survival rate for patients with hTSCC is 63%,[1] with the poor prognosis of TSCC being attributed to the high rate of local invasion and distant metastasis.[2] For this reason, the development of novel therapeutic agents targeting the malignant behaviour of TSCC cells, especially their invasiveness, is important in improving the prognosis of patients.

In 1889, Paget described his 'seed and soil' hypothesis. Since then, numerous studies have described the importance of the tumour microenvironment in promoting an invasive and metastatic phenotype.[3] Among these studies, several highlighted the importance of an acidic environment in driving the invasive capacity and subsequent malignant progression.[4] An acidic extracellular pH (pHe) is thought to be critical in the activation of extracellular proteases and the degradation of the extracellular matrix (ECM) within tumours.[5] In the early stages of cell invasion, ECM breakdown is primarily mediated by matrix metalloproteinases (MMPs), which are a family of zinc metallo-endopeptidases.[6] MMP-9, which is secreted by invasive cancer cells, is thought to be a crucial factor in facilitating

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tumour invasion and metastasis,[7,8] but the precise role of pH regulation in TSCC has not been fully elucidated.

The Na+/H+ exchanger 1 (NHE1) is an ubiquitously expressed member of the Na+/H+ exchanger family that catalyses the extrusion of intracellular proton (H+) ions in exchange for extracellular sodium (Na+) ions, thereby regulating both intracellular pH and extracellular pH (pHi and pHe, respectively).[9] Furthermore, NHE1 plays a role in acidifying the tumour microenvironment.[10] The authors hypothesized that NHE1 activation may induce extracellular acidification and subsequent MMP-9 expression, resulting in motility and invasion of tumour cells. Therefore, inhibition of NHE1 may be a novel anti-tumour therapy. Previous research demonstrated that NHE1 activation enhanced tumour invasion and that the inhibition of NHE1 reduced tumour cell invasion and motility.[11–13] The role of NHE1 in TSCC and the mechanism behind it have not been established.

A hypoxic environment is a common component of the tumour microenvironment and closely linked to tumour cell invasion and motility potency.[14–17] A previous study showed that exposure to hypoxia led to increased NHE1 expression and activity.[18] In the present study, cariporide (HOE642), a selective inhibitor of NHE1, was chosen to inhibit NHE1 activity. The authors evaluated the role of NHE1 in TSCC cells cultured in hypoxic medium, which mimics *in vivo* tumour conditions, and investigated the potential mechanisms of NHE1.

Materials and methods

Tca8113 cells (human tongue SCC cells) were obtained from the China Center for Type Culture Collection (Wuhan University) and cultivated in RPMI-1640 medium supplemented with 10% foetal calf serum (Sigma Chemical Co, St Louis, MO). According to the presence or absence of hypoxic media, cells were divided into two groups; a basal group (without hypoxic media) and a hypoxic group. The hypoxic group was separated into three sub-groups based on different doses of cariporide (Hoechst Frankfurt, Germany), including a control group (no drug), a 5 μM cariporide group and a 10 μM cariporide group. Each group was seeded at 1.5×10^6 cells/100 mm diameter dish. All experiments were performed with confluent cultures. To attain the normoxic condition, cultures were maintained at 37 °C in a humidified incubator containing 20% O_2, 5% CO_2 and 75% N_2. To attain the hypoxic condition, cells were cultured and handled at 37 °C in a compact gas oxygen controller Proox model 110 (Reming Bioinstruments, Redfield, NY, USA) that was flushed with a gas mixture of 2% O_2, 5% CO_2, and 93% N_2. The presence of pO_2 in the culture medium was measured using a trace oxygen analyzer (Oxi 315i/set, WTW, Germany). Cariporide (HOE642) was obtained from Aventis Pharma (Frankfurt, Germany).

Measurement of pHi levels

Fluorescence spectroscopy was used to determine the pHi value in the cells. Briefly, cells were incubated at 37 °C with gentle shaking in sodium HEPES buffer containing 1 μg/ml of BCECF-AM (2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (Calbiochem, San Diego, CA, USA). The data were obtained as the ratio of the pH-sensitive excitation wavelength (490 nm) to the pH-insensitive excitation signal wavelength (450 nm), with the emission wavelength being set at 530 nm. For every experiment, a calibration curve was constructed by incubating BCECF-AM-labelled cells in a high potassium HEPES buffer at a specific pH level (6.2, 6.6, 7.0, 7.2, 7.4, 7.6, and 7.8) and by the addition of the K+/H+ ionosphere, nigericin (1 μg/ml). Under these conditions, nigericin equilibrated intra- and extracellular pH and calibration curves were constructed by plotting pHe against the corresponding background-corrected fluorescence ratios.

Measurement of pHe levels

The pHe was measured using phenolsulfonephthalein (phenol red) absorbance. Specifically, cells were washed twice with Hanks' balanced salt solution (HBSS; 138 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.35 mM Na_2HPO_4) containing 0.03 mM phenol red. Cells were pre-warmed to 37 °C, equilibrated with CO_2, and were incubated with 1.5 ml of the above unbuffered solution without bicarbonate at 37 °C in a water-saturated atmosphere devoid of CO_2. The ratio of the 450/490 nm absorbance of phenol red was monitored using a spectrophotometer and converted to pH using the following equation: $pH = \log((R - R_{min})/(R_{max} - R)) + pK'a$, where R is the experimentally derived ratio of absorbance, R_{max} and R_{min} indicate the limiting values of R, and $pK'a$ indicates the negative log of the dissociation constant (pKa = 7.5 for phenol red). R_{max} and R_{min} values were calculated from a standard curve for each experiment.

NHE1 activity

The NHE1 activity was measured via Na+-dependent pHi recovery after an NH_4Cl-induced acid load. Specifically, BCECF-labelled cells were incubated with the HEPES buffer (bicarbonate-free Na+ medium) containing 20 mM NH_4Cl followed by replacement of the medium with Na+-free, N-methylglucamine chloride solution, which resulted in acidification of the cytoplasm. The addition of 135 mM Na+ produced a rapid rise in pHi, which allowed the initial rate of cellular pHi recovery (dpHi/dt) to be determined. This recovery rate represented a direct measure of the acid-extruding capability of the cell membrane, primarily the activity of the Na+/H+ exchanger. The rate of pHi recovery (dpHi/dt) from an acid load that was induced by the rapid removal of NH_4Cl was calculated at pHi intervals of 0.05 units and used as an index of NHE1 activity.

Protein extraction and Western blot analysis

The total cellular protein from the cultured tumour cells was extracted using a lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, and 0.5% NP40. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples containing 50 mg of protein were separated on polyacrylamide SDS–PAGE gels and were transferred electrophoretically to a Hybond-C nitrocellulose membrane (GE-Healthcare, Arlington Heights, IL, USA). The membranes were incubated overnight. The next day, the membranes were incubated with a monoclonal antibody or anti-β-actin antibody. After washing three times with tris buffered saline, the membrane was incubated with a goat anti-mouse IgG antibody. Next, the membranes were incubated with an enhanced chemiluminescence solution (GE-Healthcare), and the protein levels quantified using an autoradiogram densitometry.

Migration and invasion assays

Cell migration and invasion were determined using transwell cell culture chambers, with or without matrigel coating (8 μm pore size) (Millipore, Billerica, MA, USA), as described previously.[19,20] Cells were seeded (5×10^4 cells/well) into the upper chamber with serum-free medium and were incubated in the bottom chamber with 0.5% dimethyl sulphoxide