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Original research

Influence of high mobility group box 1 (HMGB1) derived from SCC7 cells on mouse normal tongue muscle fibers

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ABSTRACT

Objective: High mobility group box 1 (HMGB1) is present in the nucleus of all normal cells and known for its participation in the maintenance of homeostasis. But recent studies suggested an association with the invasion and metastasis of cancer. Therefore, we aimed to clarify how HMGB1 released from cancer cells affects mice tongue muscle fibers.

Methods: In this study, we conducted immunohistochemical investigation of the localization of HMGB1 and the receptor for advanced glycation endproducts (RAGE) in the carcinoma and adjacent muscle tissues of the consecutive serial frontal section of mice tongues, into which SCC7 cells (isolated from a murine squamous cell carcinoma) had been implanted. The amounts of HMGB1 and RAGE mRNAs expressed were also examined.

Results: At first, observation of hematoxylin/eosin-stained sections showed muscle fibers that had become reduced in size in the anterior and middle tongue from the SCC7 group. Immunohistochemical staining revealed that HMGB1 and RAGE were expressed not only in the carcinoma but also in the muscle fibers peripheral of the carcinoma and distal to the carcinoma. As with the immunohistochemical staining, mRNA expression levels of HMGB1 and RAGE were also expressed in the carcinoma and peripheral/distal muscle fibers.

Conclusion: These results indicated the HMGB1 protein released from the carcinoma had reduced the size of the muscle fibers, through the RAGE. Furthermore, the possibility that this promotes cancer invasion through the formation of a clearance gap in high density skeletal muscle tissue was suggested.

1. Introduction

Oral cancers develop frequently in the tongue, with the most common histologic type being squamous cell carcinoma [1–6]. Although the extent of surgical resection varies, the procedure induces eating, articulation, and swallowing disorders, causing a marked influence on the body [7–12]. Therefore, studies on the development, invasion, and metastasis of oral cancers are being actively performed, prompting the analysis of related genes and various factors [13–16]. Recent studies on oral cancers have strongly suggested the involvement of a protein called high mobility group box 1 (HMGB1) in the invasion and metastasis of cancers. HMGB1 is present in the nucleus of all normal cells and involved in the maintenance of homeostasis [17]. However, when it is released from necrotic cells it functions as a late-stage mediator of various serious pathological conditions, such as sepsis, promoting inflammatory reactions and chemotaxis [18–21]. HMGB1 has been reported to be expressed not only as an inflammatory mediator, but also as being overexpressed in cancer cells present in the boundary region between tumors and normal cells in gastric and colon cancers [22,23]. In addition, HMGB1 is also used as a tumor marker for squamous cell carcinoma [24,25]. The objective of this study was to clarify the influence of HMGB1 released from cancers on the surrounding muscle tissue. Squamous cell carcinoma (SCC7 cells [26,27]) isolated from the abdominal walls of the mice were implanted into the experimental mouse's tongues. HMGB1 and its receptor, the receptor for advanced glycation end products (RAGE) [28], were observed using immunohistochemical staining. In addition, HMGB1 and RAGE mRNA expression levels were investigated and compared.

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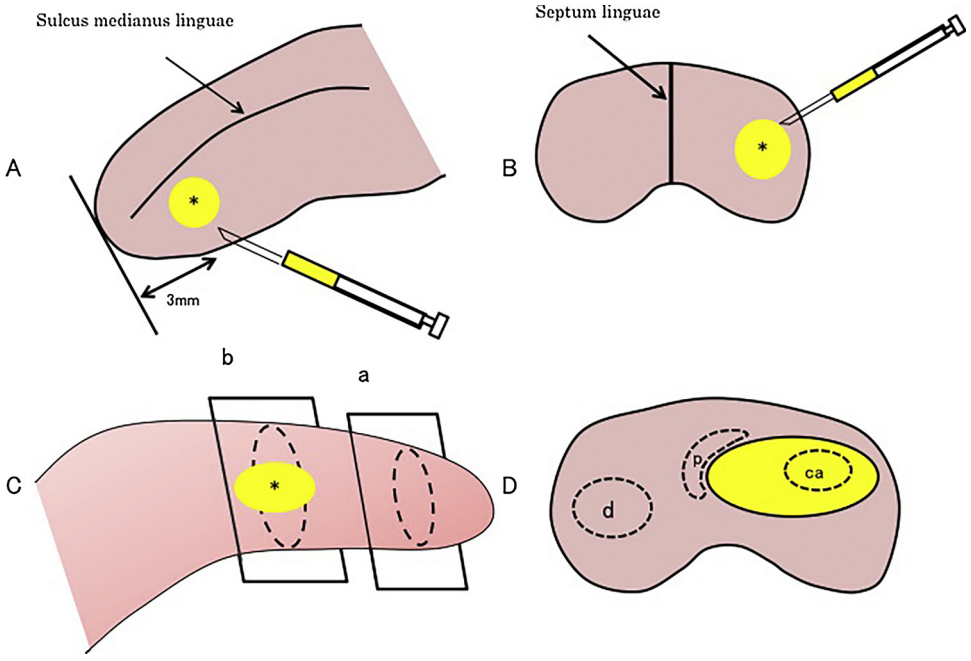

Fig. 1. Schema showing the site in the mouse tongue for injection of the SCC7 cells and the location of tissue samples of mouse tongue for H-E and immunohistochemical staining.
*: Carcinoma.
SCC7 cells were injected at a distance of 3 mm from the apex on the left lateral aspect of the tongue (A), and into only the left half of the tongue (B).
C: Serial frontal section of tongue.
a: Sections of anterior tongue.
b: Sections of middle tongue.
*: Carcinoma.
D: Observation area in a section of middle tongue of the SCC7 group.
ca: Carcinoma.
p: Muscle fibers in the periphery of the carcinoma.
d: Muscle fibers distal to the carcinoma.

2. Method

2.1. Animals

Twenty-four male BALB/cAJcl-nu/nu nude mice aged 4 weeks (Clea Japan, Tokyo, Japan) were used. Errors are generated among samples when cancer develops spontaneously in the lingual muscle. For this reason, squamous cell carcinoma (SCC7 cells: Riken, Saitama, Japan) isolated from the abdominal walls of mice were implanted. The SCC7 cell concentration was adjusted to 1×10^6 cells/mL with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA). Under ether anesthesia, 100 µL of the cell suspension was injected into the left side of the tongue 3 mm posterior to the tongue apex, so as to avoid crossing the lingual septum. A 26G injection needle (Terumo, Tokyo, Japan) was used, injecting the SCC7 cells as slowly as possible (Fig. 1A and B). Three groups were prepared: one receiving the injection of SCC7 cells, one receiving an injection of DMEM as a negative control, and another conventional non-stimulated group. They were designated SCC7, DMEM, and non-stimulated, respectively. SCC7 cells and DMEM were injected once a week, for four weeks. The animals were normally maintained, and samples were collected one week after the final injection.

2.2. Histochemical staining

2.2.1. Preparation of sections

Animals in each group were euthanized by ether anesthesia. The tongues were resected at the root and immediately frozen in isopentane cooled with liquid nitrogen. The specimens from all three groups were then cut into 10 µm serial frontal cross sections of the anterior and middle tongue using a cryostat (Fig. 1C). Some sections were stained with hematoxylin and eosin (H-E) in accordance with the standard method for morphological observation.

2.2.2. Immunohistochemical staining

After drying at room temperature, sections were placed in 0.01% H_2O_2/methanol solution for 10 min to remove endogenous peroxidase, followed by washing with 0.1% Tween 20/0.05 M Tris-buffered saline (TBST, pH 7.6) for 10 min. The sections were reacted with 10% normal goat serum (Histofine SAB-PO (R) kit, Nichirei Corp., Tokyo, Japan) at 25 °C for 30 min. Next they were reacted overnight with rabbit anti-human HMGB1 monoclonal antibody diluted with 10% normal goat serum (dilution rate: 1:100, Abcam, Cambridge, UK), and rabbit anti-human RAGE monoclonal antibody diluted with 10% normal goat serum (dilution rate: 1:100, Abcam) as the primary antibodies.

After washing with TBST, the sections were reacted for one hour at room temperature with biotinylated goat anti-rabbit IgG antibody (Histofine SAB-PO (R) kit, Nichirei Corp.) as the secondary antibody. After washing with TBST, the sections were reacted with peroxidase-labeled streptoavidin solution (Histofine SAB-PO (M) kit, Nichirei Corp.) for one hour at room temperature, followed by washing again with TBST and colored with 0.02% H_2O_2/0.01% 3,3′-diaminobenzidene tetrahydrochloride (DAB, Dojindo, Kumamoto, Japan) solution. The sections were washed with water, dehydrated, penetrated, and mounted. The preparations were observed and photographed under a light microscope. Negative control sections were reacted with 10% normal goat serum instead of the primary antibody and stained similarly, and the absence of nonspecific immune reactions was confirmed. As positive controls, specific staining of HMGB1 in the nucleus of skeletal muscle fiber cells within the same section, and of RAGE in vascular endothelial cells, was confirmed.

2.2.3. Observated regions

a) SCC7 group

Four regions were observed: the cancer tissue in the anterior region and middle region of the tongue, the muscle fiber region in the periphery of the cancer tissue (peripheral region), and the distal muscle region not in contact with the cancer tissue (distal region) (Fig. 1D).

b) DMEM group

Two regions were observed: the anterior and middle tongue.

c) Non-stimulated group

Two regions were observed: the anterior and middle tongue.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

2.3.1. Preparation of sections for laser microdissection (LMD)

Specimens prepared for histochemical staining were cut into 10 µm frontal cross sections using a cryostat, and affixed to LMD slide glass Membrane Slides (Leica, Wetzlar, Germany). The slides were placed in 70% ethanol on the surface of ice for 3 min and then in ice-chilled water