Involvement of endothelin in tongue cancer pain

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Table of Contents

The following article and new unpublished data (Fig. 2 and 7) are part of this doctoral thesis: Furukawa A, Shinoda M, Kubo A, Honda K, Akasaka R, Yonehara Y, Iwata K: Endothelin signalling contributes to modulation of nociception in early-stage tongue cancer in rats. Anesthesiology 2018; 128:1207-1219

Abstract

Background: Patients with early stage tongue cancer do not frequently complain of tongue pain. Endothelin-1 signaling is upregulated in the cancerous tongue at the early stage. The hypothesis that endothelin-1 signaling contributes to the modulation of tongue nociception associated with tongue cancer.

Methods: Squamous cell carcinoma cells were inoculated into the tongue under general anesthesia. Lingual mechanical sensitivity under light anesthesia using forceps from days 1 to 21 (n = 8) and the amounts of endothelin-1 and β-endorphin in the tongue on days 6, 14, and 21 (n = 5 to 7) were examined after the inoculation. The effect of endothelin-A or μ-opioid receptor antagonism on the mechanical sensitivity was examined (n = 5 to 7).

Results: Lingual mechanical sensitivity did not change at the early stage (days 5 to 6) but increased at the late stage (days 13 to 14). Contrarily, heat sensitivity did not change throughout the entire study period. The amount of endothelin-1 increased (25.4 ± 4.8 pg/ml *vs*. 15.0 ± 5.2 pg/ml; $p = 0.008$), and endothelin-A receptor antagonism in the tongue induced mechanical hypersensitivity at the early stage (51 ± 9 g *vs*. 81 ± 6 g; $p = 0.0001$). The μ-opioid receptor antagonism further enhanced mechanical sensitivity (39 ± 7 g *vs*. 81 ± 6 g; $p < 0.0001$), and the amount of β-endorphin increased at the early stage.

Conclusions: β-Endorphin released from the cancer cells *via* endothelin-1 signaling is involved in analgesic action in mechanical hypersensitivity at the early stage.

Introduction

It has long been known that pain is a dominant clinical feature of oral cancer, which accounts for 2.1% of all new cancer cases worldwide. The chief complaint of one-third of oral cancer cases is oral pain, which is correlated with tumor node metastasis classification.[1,2] Squamous cell carcinoma, which accounts for more than 90% of all oral cancers, is known to be malignant.[3] Interestingly, some patients do not complain of oral pain until the primary malignant lesions have expanded to an outstanding size, indicating that early oral squamous cell carcinoma regularly proceeds unobtrusively, although severe pain is the main symptom at the later stage of oral squamous cell carcinoma.[4] This pathophysiologic feature of oral squamous cell carcinoma frequently interferes with its early detection. However, it is unknown why oral squamous cell carcinoma at the early stage is asymptomatic, although focal cancer growth apparently resides in the oral cavity.

Endothelins, which are composed of 21 amino acid residues, are peripherally involved in hypersensitivity induced by local inflammation, peripheral nerve injury, or carcinogenesis.[5-8] Endothelin-1 and endothelin-2 have been identified as two main isoforms of endothelins in the peripheral tissue; endothelin-1 and endothelin-2 signaling is transmitted *via* two receptors, the Gq protein–coupled receptors and endothelin-A and endothelin-B, respectively.[9] Recent evidence has shown that intradermal endothelin-1 administration induces hypersensitivity in humans and animals, suggesting that endothelin-A activation peripherally mediates nociceptive neuronal excitability. [10,11] Interestingly, nonpainful oral squamous cell carcinoma is known to significantly up-regulate endothelin-1 in humans.[12,13] In fact, squamous cell carcinoma cells consist of malignant keratinocytes; it was reported that the release of β-endorphins was stimulated by activation of endothelin-1 signaling in normal keratinocytes.[14] The conflicting data suggest that endothelin-1 released from oral squamous cell carcinoma cells may not modulate oral cancer sensitivity, speculating unique mechanisms related to an endogenous opioid-mediated analgesia, which modulates excitability in the primary afferent nociceptors at the early stage of tongue cancer.

On the hypothesis that β-endorphin released from cancer cells *via* endothelin-1 signaling is involved in analgesic action in tongue cancer-related mechanical hypersensitivity at the early stage of tongue cancer, the following experiments were performed in this study. Carcinogenesis was established by craniocervical squamous cell

carcinoma cell inoculation into the tongue. In various stages of tongue cancer, changes in lingual mechanical sensitivity and electrophysiological properties of trigeminal ganglion neurons innervating the tongue after tongue carcinogenesis, levels and functional significance of endothelin-1, and β-endorphin release in tongue cancer were examined to determine the contribution of endothelin signaling to nociceptive modulation in tongue cancer. Though early detection of early-stage squamous cell carcinoma is key to its successful management, it is challenging because of its asymptomatic nature. The elucidation of tongue cancer nociceptive mechanisms may potentially facilitate the accurate diagnosis of oral squamous cell carcinoma at an early stage.

Materials and Methods

Animals

Male 344 Fischer rats (100 to 200 g; Japan SLC, Japan) were housed in a temperature-controlled room (23°C) with a light–dark cycle of 12 h:12 h, with *ad libitum* access to food and water. The Animal Experimentation Committee of Nihon University, Tokyo, Japan, (AP15D024) approved this study. The experiments were conducted in accordance with the guidelines issued by the International Association for the Study of Pain.[15] The number of animals and animal suffering were reduced maximally in all experiments.

Squamous Cell Carcinoma Cell Culture

To develop a rat model of tongue cancer, the craniocervical squamous cell carcinoma cell line (SCC-158; JCRB, Japan) comprising squamous cell carcinoma cells derived from the external acoustic meatus was used in this study. The SCC-158 cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, USA) containing 100 units·ml^{-1} penicillin (Gibco, United Kingdom), 100 µg·ml^{-1} streptomycin (Gibco), and 10% fetal bovine serum (Gibco), and maintained by subculture every 3 to 4 days according to JCRB's guidelines. The cultured cells were incubated at 37°C at 5% CO_2 and 95% minimum relative humidity. At 80% confluency, the cells were detached by 0.1% trypsin/ ethylenediaminetetraacetic acid solution (Worthington Biochemical, USA) and harvested after washing with 1× phosphate-buffered saline.

Lingual Inoculation of Squamous Cell Carcinoma Cells

Rats were randomly assigned to each experimental condition by means of simple randomization; anesthetized with intraperitoneal administration of a mixture consisting of 2.5 mg·kg^{-1} butorphanol (Meiji Seika Pharma, Japan), 0.375 mg·kg^{-1} medetomidine (Zenoaq, Japan), and 2.0 mg·kg^{-1} midazolam (Sandoz, Japan); and placed on a warm mat (37°C). The mouth was gently opened, and the tongue was pulled out, and a 30 µl suspension of the cultured SCC-158 cells was inoculated into the right edge of the tongue with a 26-gauge needle. The suspension of the cultured SCC-158 cells consisted of SCC-158 cells (5×10^6 cells) with 30 µl of phosphate-buffered saline. As a control, 30 µl of phosphate-buffered saline, instead of the suspension of the cultured SCC-158 cells, was inoculated into the tongue.

Behavioral Analysis

The threshold intensities for evoking the head-withdrawal reflex elicited by mechanical stimulation of the left tongue edge were measured under light anesthesia, as previously described.[16,17] Briefly, the rats were anesthetized using 2% isoflurane (Mylan, USA). After stopping the isoflurane supply, it was confirmed that the depth of anesthesia was adequate, and the level was appropriately adjusted, under which a complete identical hindlimb-withdrawal reflex was induced through identical noxious pinch stimulation (150 g) to the hind paw. The breathing pattern, cardiac rhythm, and body temperature were also identical. Under the adjusted depth of anesthesia, mechanical stimulation (0 to 150 g, 10 $g \cdot s^{-1}$, cutoff: 150 g) using forceps equipped with flat tips (6-mm^2 area; Panlab, Spain) and heat stimulation (35°C-60°C, 1°C/sec, cut off: 60°C) using a contact heat probe (9 mm^2 square; Intercross, Tokyo, Japan) were applied to the left tongue edge. The mechanical stimulus intensity was slowly increased to elicit head withdrawal; the lowest intensity required for eliciting head withdrawal was defined as the mechanical head-withdrawal reflex threshold. The sustained heat stimulation elicits head withdrawal; the duration required for eliciting head withdrawal was defined as the heat head-withdrawal reflex latency.

Mechanical stimulation (three times at 5-min intervals) was delivered to the tongue, and the mean intensity was defined as the mechanical head-withdrawal reflex threshold. Heat stimulation (three times at 5-min intervals) was delivered to the tongue, and the mean latency was defined as the heat head-withdrawal reflex latency. The mechanical head-withdrawal reflex threshold and the heat head-withdrawal reflex latency were determined daily both before and after inoculating the tongue with squamous cell carcinoma cells or vehicle. All behavioral analyses were performed under blinded conditions.

Whole Cell Patch-Clamp Recording

Precise tongue injection of 10% 1,1′-dioctadecyl-3,3,3′,3tetramethylindocarbocyanine methanesulfonate (Molecular Probes, USA) dissolved in ethanol into the squamous cell carcinoma cell- or vehicle-inoculated site was performed 3 h before squamous cell carcinoma-cell or vehicle inoculation to identify the neurons innervating the tongue using a 26-gauge needle. At the early stage (days 5 to 6) and late stage (days 13 to 14) after the inoculation, the rats were decapitated and the trigeminal ganglia rapidly isolated under

deep anesthesia. They were digested in serum-free Dulbecco's modified Eagle's medium supplemented with 1% penicillin/streptomycin with collagenase type IV (1.0 $mg \cdot ml^{-1}$; Worthington Biochemical, USA) and neutral protease (1.0 $mg \cdot ml^{-1}$; Worthington Biochemical) at 37°C in 5% CO_2 for 40 min. After washing with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, USA) and 1% penicillin/streptomycin (Life Technologies), 4.5 $g \cdot l^{-1}$ glucose, the trigeminal ganglia were gently triturated to facilitate the dissociation of trigeminal ganglion neurons. Dissociated trigeminal ganglion neurons were plated on glass coverslips precoated with poly-D-lysine (Becton Dickinson Labware, USA). Only 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine methanesulfonate-labeled trigeminal ganglion neurons were used for the ensuing whole cell patch-clamp recording, within 6 h after plating at 25°C. The whole cell configuration was established on 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine methanesulfonate-labeled trigeminal ganglion neurons by fire-polished patch-pipettes (3 to 6 MΩ), which were filled with a solution consisting of 130 mM KCl0, 4 mM NaCl, 0.2 mM $CaCl_2$, 10 mM HEPES, 10 mM ethylene glycol-bis-β-aminoethyl ether N,N,N′,N′-tetraacetic acid, 2 mM adenosine triphosphate, and 0.5 mM guanosine triphosphate, adjusted pH 7.25. A standard external solution of pH 7.4, consisting of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose were used. All recordings were performed using an Axopatch 200B amplifier (Molecular Devices, USA), digitized at 1 kHz (Digidata 1440A; Molecular Devices), and controlled by Clampex software (pClamp 10.5; Molecular Devices). Cell capacitance and resting membrane potential were obtained from the Axopatch 200B amplifier. The recorded data from the neurons were used and analyzed which showed their resting membrane potentials more negative than −40 mV and generated action potentials with a distinct overshoot of more than 0 mV in response to depolarizing current injections. To determine the action potential threshold (*i.e.*, the greatest membrane potential without evoking an action potential), a series of 5-ms current pulses incremented by 50-pA were administered (1s apart). The minimum current amplitude to evoke an action potential was regarded as rheobase. To examine firing patterns in the trigeminal ganglion neurons, suprathreshold current (2× rheobase) was injected for 500 ms, and the number of action potentials was counted in voltageclamp mode. Access resistance did not show any significant changes during the experiments.

Tongue Histologic Examination

On days 6, 14, and 21 after the squamous cell carcinoma cell or vehicle inoculation in the tongue, the tongue was removed under deep anesthesia with intraperitoneal sodium pentobarbital (50 $mg \cdot kg^{-1}$; Schering Plough, USA) after perfusion with saline and embedded in tissue Tek (Sakura Finetek, Japan). The tongue sections were obtained using a cryostat (at −20°C) and placed on MAS-coated Superfrost plus microscope slides (Matsunami, Japan). Sections were incubated at 25°C and fixed in 4% paraformaldehyde for 10 min. The sections were stained with hematoxylin and eosin to assess any pathologic changes in the tongue.

Western Blot Analysis

The SCC-158 cells (5×10^5 cells) were cultured in Dulbecco's modified Eagle's medium more than 36 h, after which the culture medium was refreshed. The trigeminal ganglion was enucleated from the rats anesthetized with intraperitoneal sodium pentobarbital (50 $mg \cdot kg^{-1}$; Schering Plough) and perfused with saline on days 6, 14, and 21 after the intervention. The trigeminal ganglion was homogenized using a tube pestle (Thermo Fisher Scientific) in lysis buffer (4°C, 137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu g \cdot ml^{-1}$ aprotinin, 1 $g \cdot ml^{-1}$ leupeptin, and 0.5 mM sodium vanadate) and centrifuged at 15,000 rpm at 4°C for 10 min. Its supernatant was collected, and the protein concentrations of the culture medium or the supernatant from the trigeminal ganglion were measured with a protein assay kit (Bio-Rad, USA). After heat denaturation in Laemmli sample buffer solution (Bio-Rad), the protein samples (15 μg in the culture medium and 30 μg in the trigeminal ganglion) were subjected to electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel followed by electroblotting onto polyvinylidene difluoride membrane (Trans-Blot Turbo Transfer Pack; Bio-Rad). After rinsing with Tris-buffered saline with 0.1% Tween 20, the membrane was immersed in 3% bovine serum albumin (Bovogen, Australia) for 30 min and incubated with rabbit anti-endothelin-1 polyclonal antibody (1:5,000, Abcam, United Kingdom) or rabbit anti-μ-opioid receptor polyclonal antibody (1:500, Abcam) diluted in Tris-buffered saline with 0.1% Tween 20 with 5% bovine serum albumin for 12 h at 4°C. A horseradish peroxidase-conjugated donkey anti-rabbit antibody (Cell Signaling, USA) and Western Lightning ELC Pro (PerkinElmer, USA) visualized each protein binding. ChemiDoc MP system (BioRad) quantified the signal

intensity, which was normalized to β-actin on blots reprobed with the anti-β-actin antibody (1:200; Santa Cruz Biotechnology, USA), after removal of protein binding with a stripping reagent (Thermo Fisher Scientific).

Measurement of Endothelin-1 and β-Endorphin Released from the Squamous Cell Carcinoma Cell-inoculated Tongue

The amounts of endothelin-1 and β-endorphin in the squamous cell carcinoma cell- and vehicle-inoculated tongue were determined by enzyme-linked immunosorbent assay. On days 6, 14, and 21 after the intervention, the tongues were removed from the rats anesthetized with intraperitoneal sodium pentobarbital (50 $mg \cdot kg^{-1}$) and homogenized in lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, 10 $\mu g \cdot ml^{-1}$ aprotinin, 1 $g \cdot ml^{-1}$ leupeptin, and 0.5 mm sodium vanadate) at 4°C. The tongue homogenate was centrifuged at 15,000 rpm at 4°C for 10 min, and the supernatant was collected. The protein concentrations of the supernatant were calculated with a protein assay kit (BioRad) and stored at −80°C until analysis. After adjusting the protein level of the samples, the amount of endothelin-1 and β-endorphin in the sample was measured with a rat β-endorphin enzyme-linked immunosorbent assay kit (MyBioSource, USA) and endothelin-1 enzyme-linked immunosorbent assay kit (Abcam), respectively. Additionally, the fluid medium in which SCC-158 cells (5×10^5 cells) were cultured more than 36 h was collected. After adjusting the protein level of the fluid medium, the amount of β-endorphin in the fluid medium was also determined by enzyme-linked immunosorbent assay as mentioned previously.

Effect of Endothelin-1 and μ-Opioid Signaling on Mechanical Sensitivity in Tongue Cancer

To assess the involvement of endothelin-1 and μ-opioid signaling in the cancerous tongue in mechanical allodynia, 20 μl of the selective endothelin-A receptor antagonist BQ123 (Abcam; 20 ng dissolved in phosphate-buffered saline) or 20 μl of the selective μ-opioid receptor antagonist CTAP (Abcam; 5 μg dissolved in phosphate-buffered saline) was administered into the squamous cell carcinoma cell- and vehicle-inoculated tongue on days 6, 14, and 21 after the inoculation with a 26-gauge needle under anesthesia using the aforementioned mixture anesthetics. After confirmation of complete recovery from anesthesia at 2 h after BQ123 or CTAP administration, the mechanical head-withdrawal

reflex threshold of the left edge of the tongue was measured under light anesthesia, as described above.

To assess the involvement of endothelin-1 signaling in the tongue in mechanical allodynia, 5 μl of endothelin-1 (1 mg/ml dissolved in dimethyl sulfoxide; Enzo Biochem, USA) was administered into the tongue under light anesthesia using 2% isoflurane (Mylan). The mechanical head-withdrawal reflex threshold of the left edge of the tongue was measured under light anesthesia at 30 min, 120 min, 24 h, 48 h, 72 h, and 1 week after the endothelin-1 administration, as described above.

Effect of Endothelin-A Antagonist on β-Endorphin Release from the SCC-158 Cells

To assess the effect of endothelin-1 signaling on β-endorphin release from the squamous cell carcinoma cell-inoculated tongue, the SCC-158 cells were cultured in Dulbecco's modified Eagle's medium in which BQ123 (10 ng, 100 ng, and 1,000 ng·5×10^5 cells^{-1}) was added in variable concentrations. To measure the potential induction of β-endorphin after the addition of endothelin-1 *in vitro* in squamous cell carcinoma cells, the SCC-158 cells were cultured in Dulbecco's modified Eagle's medium containing endothelin-1 (2 μg; Enzo Biochem) or vehicle. To assess the involvement of nuclear factor-κB signaling in β-endorphin release from the squamous cell carcinoma cells *via* endothelin-1 signaling, the SCC-158 cells were cultured in Dulbecco's modified Eagle's medium containing endothelin-1 (2 μg; Enzo Biochem), in which NF-κB inhibitor (SM7368; 6.5 μg / 5×10^5 cells dissolved in dimethyl sulfoxide; Abcam) or vehicle was added. Thirty-six hours after the incubation, the fluid medium was collected and centrifuged for 5 min at 15,000 rpm at 4°C. Protein concentration of the collected supernatant was determined with a protein assay kit (Bio-Rad). The amounts of β-endorphin in the sample were measured using a rat β-endorphin enzyme-linked immunosorbent assay kit (MyBiosource), as described above.

Immunocytochemistry for Cultured SCC-158 Cells

SCC-158 cells were incubated in Dulbecco's modified Eagle's medium mixing 100 units·ml^{-1} penicillin (Gibco), 100 μg·ml^{-1} streptomycin (Gibco), and 10% fetal bovine serum. Twelve hours after incubation, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 10 min. After washing with phosphate-buffered saline, the cells were incubated in 1% bovine serum albumin in phosphate-

buffered saline for 1 h at 25°C. Thereafter, the cells were incubated with the sheep anti-endothelin-A receptor polyclonal antibody (1:100; Abcam)[18] or the rabbit anti-μ-opioid receptor polyclonal antiserum (1:100; Abcam) diluted with 0.01 M phosphate-buffered saline containing 1% bovine serum albumin. The cells were reacted with Alexa Fluor 488-conjugated donkey anti-sheep IgG (1:200 diluted in 0.01 M phosphate-buffered saline; Thermo Fisher Scientific) or Alexa Fluor 488-conjugated goat antirabbit IgG (1:200 diluted in 0.01 M phosphate-buffered saline; Thermo Fisher Scientific) and 4′,6-diamidino-2phenylindole (1:1,000 diluted in 0.01 M phosphate-buffered saline; Sigma Aldrich) for nuclear staining. As a negative control, no specific labeling was observed in the absence of a primary antibody. 4′,6diamidino-2-phenylindole-labeled endothelin-A receptor immunoreactive cells were analyzed (BZ-9000 system; Keyence, Japan).

Immunohistochemistry for Trigeminal Ganglion Neurons Innervating the Tongue

Further, trigeminal ganglion neurons innervating the tongue were identified *via* the retrograde labeling technique using 4% hydroxystilbamidine (FluoroGold; Fluorochrome, USA) dissolved in distilled saline. To label trigeminal ganglion neurons innervating the tongue, a FluoroGold (4 μl) single submucosal injection into the left side of the tongue was administered with a 27-gauge needle before the squamous cell carcinoma cell inoculation. On day 14 after the inoculation, the rats were transcardially perfused with saline and a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia (intraperitoneal sodium pentobarbital, 50 $mg \cdot kg^{-1}$; Schering Plough). The ipsilateral trigeminal ganglia were extracted and immersed in phosphate-buffered saline containing 20% sucrose for 12 h. The trigeminal ganglia, which were embedded in tissue Tek, were cut in the horizontal plane along the long axis at 15 μm. The trigeminal ganglion sections, which were thaw-mounted onto MAS-coated Superfrost plus microscope slides (Matsunami), were incubated with rabbit anti-μ-opioid receptor polyclonal antiserum (1:100; Abcam)[19,20] or sheep anti-endothelin-A receptor polyclonal antibody (1:100; Abcam) and then they were reacted with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:200 diluted in 0.01 M phosphate-buffered saline; Thermo Fisher Scientific) or Alexa Fluor 488-conjugated donkey anti-sheep IgG (1:200 diluted in 0.01 M phosphate-buffered saline; Thermo Fisher Scientific). The sections were coverslipped, and FluoroGold-labeled μ-opioid receptor or endothelin-A receptor immunoreactive cells were identified. As a negative control, no specific labeling was

observed in the absence of a primary antibody.

Statistical Analysis

Data for all experiments were sampled from 141 male rats of the 344 Fischer strain. The estimation of sample sizes was based on previous experience.[21-23] Individual sample sizes for behavioral, enzyme-linked immunosorbent assay, behavioral pharmacologic, and Western blot analysis are separately described, and the number of neurons is reported for electrophysiologic experiments in each experiment in the Results section. There were no excluded or missing data of any kind in any experiment. The sample sizes were increased during the review process, because it was determined that the number of samples was originally inadequate based on the estimation of sample sizes for the enzyme-linked immunosorbent assay analysis (fig. 5C). After confirmation that the raw data for all experiments fitted a normal distribution, two-way ANOVA followed by Bonferroni's multiple-comparison tests or two-way ANOVA with repeated measures followed by Bonferroni's multiple-comparison tests with/without a combination of one-way ANOVA were used for the analysis of the head-withdrawal reflex elicited by mechanical stimulation, as appropriate. Student's t-test was used for the analysis of changes in trigeminal ganglion neuronal excitability after tongue squamous cell carcinoma inoculation using electrophysiologic techniques and the analysis of the amount of endothelin-1 and β-endorphin released from the squamous cell carcinoma cell-inoculated tongue using enzyme-linked immunosorbent assay or Western blot experiments. One-way ANOVA was used for the analysis of the amount of β-endorphin in the squamous cell carcinoma-cultured fluid medium using enzyme-linked immunosorbent assay. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Prism Software Inc., USA). The data are expressed as means ± SD. A value of $p < 0.05$ was considered to be statistically significant.

Results

Mechanical and Heat Sensitivity in Cancerous Tongue

A small tumor was observed at the squamous cell carcinoma cell-inoculated site on day 6 after the inoculation; the tumor grew as far as the contralateral side on day 14 and entirely infiltrated the tongue on day 21 (fig. 1A). There were no histologic changes in the vehicle-inoculated tongue. The mechanical head-withdrawal reflex threshold was significantly lower in the squamous cell carcinoma cell-inoculated tongue than in the vehicle-inoculated tongue on day 8 (squamous cell carcinoma: 67 ± 9 g, vehicle: 97 ± 14 g) after the inoculation (fig. 1B). There was no significant change in the heat head withdrawal reflex latency between SCC group and vehicle group during the experimental period (fig. 2).

Trigeminal Ganglion Neuronal Excitability

The mean diameter of trigeminal ganglion neurons innervating the tongue did not significantly differ between squamous cell carcinoma cell- and vehicle-inoculated rats at the early stage (squamous cell carcinoma: 27.7 ± 2.3 μm, n = 5 to 18 neurons; vehicle: 27.5 ± 2.7 μm, n = 5 to 18 neurons) and at the late stage (squamous cell carcinoma: 27.9 ± 1.6 μm, n = 5 to 16 neurons; vehicle: 27.6 ± 2.3 μm, n = 5 to 15 neurons) after inoculation. The passive and active properties of trigeminal ganglion neurons innervating the squamous cell carcinoma cell- or vehicle-inoculated tongue were examined in current-clamp mode. In 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine methanesulfonate-labeled trigeminal ganglion neurons, the tongue squamous cell carcinoma cell inoculation did not affect resting membrane potentials at the early stage (squamous cell carcinoma: −67.1 ± 6.8 mV, n = 5 to 18 neurons; vehicle: −66.5 ± 7.1 mV, n = 5 to 18 neurons) and at the late stage (squamous cell carcinoma: −66.9 ± 5.5 mV, n = 5 to 16 neurons; vehicle: −68.9 ± 6.1 mV, n = 5 to 15 neurons), whereas rheobase significantly decreased at the late stage after squamous cell carcinoma cell inoculation (squamous cell carcinoma: 250 ± 100 pA, n = 5 to 16 neurons; vehicle: 360 ± 120 pA, n = 5 to 15 neurons; fig. 3, A and B). The action potential threshold of trigeminal ganglion neurons innervating the squamous cell carcinoma cell-inoculated tongue was significantly decreased at the late stage after squamous cell carcinoma cell inoculation (squamous cell carcinoma: −43.1 ± 9.6 mV, n = 5 to 16 neurons; vehicle: −34.9 ± 7.7 mV,

n = 5 to 15 neurons; fig. 3C). The rheobase and suprathreshold current injections (twofold rheobase) did not change spike frequency in 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine methanesulfonate-labeled trigeminal ganglion neurons at the early stage (spike number at rheobase; squamous cell carcinoma: 4 ± 7, n = 5 to 18 neurons; vehicle: 3 ± 4, n = 5 to 18 neurons; spike number at 2× rheobase; squamous cell carcinoma: 8 ± 9, n = 5 to 18 neurons; vehicle: 7 ± 7, n = 5 to 18 neurons) and at the late stage after squamous cell carcinoma cell inoculation (spike number at rheobase; squamous cell carcinoma: 4 ± 5, n = 5 to 16 neurons; vehicle: 2 ± 2, n = 5 to 15 neurons; spike number at 2× rheobase; squamous cell carcinoma: 10 ± 10, n = 5 to 16 neurons; vehicle: 7 ± 7, n = 5 to 15 neurons; fig. 3, D and E).

Endothelin-1 Release from Squamous Cell Carcinoma Cells

After 36-h incubation of squamous cell carcinoma cells, endothelin-1 was detected in the culture medium in which the SCC-158 cells were cultured (fig. 4A). The amount of endothelin-1 in the squamous cell carcinoma cell-inoculated tongue on days 6 (25.4 ± 4.8 $pg \cdot ml^{-1}$) and 14 (17.5 ± 4.4 $pg \cdot ml^{-1}$) after squamous cell carcinoma cell inoculation was significantly more abundant than that in the vehicle-inoculated (day 6: 15.0 ± 5.2 $pg \cdot ml^{-1}$; day 14: 12.4 ± 2.6 $pg \cdot ml^{-1}$) or naive (13.4 ± 4.8 $pg \cdot ml^{-1}$) tongues (fig. 4B). The endothelin-A receptor, which is a high-affinity receptor for endothelin-1, was expressed in most of the cultured SCC-158 cells (fig. 4Ca). In addition, the endothelin-A receptor was expressed in trigeminal ganglion neurons innervating the vehicle- or squamous cell carcinoma cell-inoculated tongue (fig. 4Cb). To assess the effect of endothelin-1 signaling on mechanical sensitivity in the cancerous tongue, the tongue mechanical head-withdrawal reflex threshold after 2 h of BQ123 tongue administration was measured on days 6, 14, and 21 after squamous cell carcinoma cell inoculation of the tongue. The mechanical head-withdrawal reflex threshold in the BQ123-administered tongue was significantly lower than that in the vehicle-administered tongue on day 6 (squamous cell carcinoma + vehicle: 81 ± 6 g, squamous cell carcinoma + BQ123: 51 ± 9 g) and day 14 (squamous cell carcinoma + vehicle: 60 ± 6 g, squamous cell carcinoma + BQ123: 44 ± 6 g), and no significant difference was observed between these groups on day 21 (fig. 4D). BQ123 administration to the vehicle-inoculated tongue did not change the tongue's mechanical sensitivity. To assess the effect of endothelin-1 signaling on mechanical sensitivity in the tongue, the tongue mechanical head-withdrawal reflex threshold after

endothelin-1 tongue administration was measured. The mechanical head-withdrawal reflex threshold in the endothelin-1-administered tongue was significantly lower than that in the vehicle-administered tongue from 30 min to 48 h after the endothelin-1 administration (30 min, vehicle: 110 ± 10 g; endothelin-1: 72 ± 10 g; fig. 4E). No significant difference was observed from 72 h onward.

Involvement of Endothelin-1 Signaling in β-Endorphin Release from Squamous Cell Carcinoma Cells

β-endorphin protein was significantly increased in the culture medium after SCC-158 cell incubation with vehicle for 36 h (6.5 ± 3.5 $ng \cdot ml^{-1}$). The increase of β-endorphin protein was depressed by BQ123 addition in the culture medium (10 μg: 1.7 ± 0.6 $ng \cdot ml^{-1}$, 100 μg: 1.3 ± 0.5 $ng \cdot ml^{-1}$, 1,000 μg: 1.1 ± 0.3 $ng \cdot ml^{-1}$; fig. 5A). Additionally, the amount of β-endorphin protein in the endothelin-1-containing culture medium after SCC-158 cell incubation was depressed by SM7368 addition in the culture medium (SM7368: 8.0 ± 5.6 $ng \cdot ml^{-1}$; vehicle: 1.3 ± 0.5 $ng \cdot ml^{-1}$; fig. 5B). The amount of β-endorphin was significantly increased in the squamous cell carcinoma cell-inoculated tongue on days 6 and 14 after squamous cell carcinoma cell inoculation; the increase of β-endorphin was significantly suppressed by successive BQ123 administration to the tongue (fig. 5C).

Peripheral β-Endorphin Signaling in Mechanical Sensitivity in the Cancerous Tongue

FluoroGold-labeled trigeminal ganglion neurons expressed the μ-opioid receptor on day 14 after the squamous cell carcinoma cell- or vehicle-inoculation, signifying that the μ-opioid receptor was expressed in trigeminal ganglion neurons innervating the tongue (fig. 6Aa). Furthermore, the μ-opioid receptor was expressed in the cultured SCC-158 cells (fig. 6Ab). There were no changes in the total amount of μ-opioid receptor protein in the squamous cell carcinoma cell-inoculated tongue on days 6, 14, and 21 after the inoculation compared to vehicle-inoculated and naive tongues (fig. 6B). The decrement of mechanical head-withdrawal reflex threshold on day 6 (squamous cell carcinoma + vehicle: 81 ± 6 g, squamous cell carcinoma + CTAP: 39 ± 7 g) and day 14 (squamous cell carcinoma + vehicle: 60 ± 6 g, squamous cell carcinoma + CTAP: 39 ± 6 g) after squamous cell carcinoma cell-inoculation was significantly augmented by CTAP administration to the tongue (fig. 6C).

Discussion

Currently, various models of orofacial cancer have been developed with cancer cell inoculation. Human oral squamous cell carcinoma cell or melanoma inoculation into the hind paw produced significant spontaneous nocifensive behaviors, as well as thermal and mechanical hypersensitivity shortly thereafter.[12,24] A rat model of facial cancer established by carcinosarcoma cell inoculation into the whisker pad skin developed spontaneous facial nocifensive behavior, mechanical allodynia, and thermal hyperalgesia less than 3 days after the inoculation.[25] However, it has been reported that the patients with tongue squamous cell carcinoma are often unaware of early-stage tongue cancer because the tumor growth progresses asymptomatically.[4,26] Therefore, it is difficult to affirm that the aforementioned models of orofacial cancer mirror the actual pathophysiologic features in patients with tongue cancer. In this study, though overt development of a malignancy was already identified at a premature stage of cancerous tongue induction by craniocervical squamous cell carcinoma cell inoculation, there were no changes in tongue mechanical sensitivity. On the one hand, mechanical allodynia was induced in the squamous cell carcinoma cell-inoculated tongue in the later phase of tongue carcinogenesis.

Accordingly, the tongue cancer model established by squamous cell carcinoma cell inoculation partially mimics some aspects of the human condition and must be well suited to study the mechanisms underlying pathologic changes in sensory function in the cancerous tongue. Incidentally, there is a possibility that pain behavioral data collected under shallow general anesthesia in this study may mask some pain behaviors in the awake state. It is reported that there is no significant effect on tail-flick antinociception after a 5-min exposure to 1.2% isoflurane.[27] Because the depth of anesthesia during the behavioral tests in this study was almost certainly shallower than that in 5-min exposure to 1.2% isoflurane, the reflexive hypersensitivity found in our behavioral testing must mirror peripheral nociceptive hypersensitivity. Hence, it is reasonable to conclude that the reflexive hypersensitivity relates to aspects of clinically relevant pain, which is induced by peripheral nociceptive neuronal hypersensitivity. Interestingly, heat sensitivity in the cancerous tongue did not change in common with our previous study[28], though peripheral mechanical nociceptive neuronal sensitivity was enhanced. In contrast, it was reported that inoculation of cancer cells induced marked heat hyperalgesia in the inoculated site.[29,30] This conflicting result may be due to difference in cancer species or

cancerous region.

In trigeminal ganglion neurons innervating the tongue, tongue squamous cell carcinoma cell inoculation did not affect the resting membrane potential and action potential generation by current injection during the experimental period. On the other hand, rheobase and the action potential threshold after squamous cell carcinoma cell inoculation were significantly decreased at the late stage of tongue carcinogenesis. These data show that changes in the excitability of trigeminal ganglion neurons innervating the tongue correlate with the tongue's mechanical sensitivity after tongue carcinogenesis, suggesting that peripheral neuronal regulatory mechanisms may be dominant in mechanical hypersensitivity in the cancerous tongue.

In this study, the amount of endothelin-1 protein that is assumed to be released from squamous cell carcinoma cells was increased in the squamous cell carcinoma cell-inoculated tongue at the early and late stages of tongue carcinogenesis. The endothelin-A receptor was expressed in squamous cell carcinoma cells, and antagonism of the endothelin-A receptor expressed in cultured squamous cell carcinoma cell membranes completely depressed the augmentation of β-endorphin release from cultured squamous cell carcinoma cells; the further addition of endothelin-1 increased β-endorphin release. Moreover, the inhibition of NF-κB signaling in the cultured squamous cell carcinoma cells also depressed the augmentation of β-endorphin release. The EDN1 gene is well known to encode endothelin-1 and thought to be regulated by various signaling and transcriptional pathways.[31] Endothelin-1 signaling reportedly triggers the activation of a diverse intracellular cascade, such as the NF-κB and phosphatidylinositol 3-kinase signaling pathways.[32] In many types of cancer cells derived from oral squamous cell carcinoma, prostate cancer, or colorectal carcinoma, the nuclear factor-κB signaling pathway is activated.[33-36] For instance, tumor necrosis factor α treatment induces a dose-dependent enhancement of nuclear factor-κB signaling *via* phosphatidylinositol 3-kinase activation, resulting in the promotion of transcription of EDN1 in human glioblastoma cells.[37] Administration of interleukin1β, which is known to be markedly upregulated in human non-small cell lung carcinoma cells, leads to nuclear factor-κB activation and the ensuing EDN1 expression in renal inner medullary collecting duct cells.[38,39] Thus, it has been reported that the nuclear factor-κB signaling pathway leads to EDN1 activation. Thus, cancer cells release endothelin-1, which is synthesized through EDN1 activation, and autocrine or paracrine endothelin-1 signaling by endothelin-1 binding to endothelin-

A receptor further enhances EDN1 activation through a similar intracellular signaling cascade.[40] Recent studies have demonstrated a high abundance of the endothelin-A receptor in various carcinoma cells.[41,42] The inhibition of endothelin-1 signaling by endothelin-A receptor antagonism led to depression of β-endorphin production in cultured squamous cell carcinoma cells derived from a human oral squamous cell carcinoma.[43] Endothelin-A receptor antagonism in the squamous cell carcinoma cell- inoculated tongue also completely depressed the increase of β-endorphin in the squamous cell carcinoma cell-inoculated tongue at the early and late stages of tongue carcinogenesis after squamous cell carcinoma cell inoculation in the current study. Taken together, these findings propose the possibility that squamous cell carcinoma cells secrete endothelin-1 and express the endothelin-A receptor, indicating that endothelin-1 signaling in squamous cell carcinoma cells accelerates endothelin-1 secretion from squamous cell carcinoma cells; this autocrine or paracrine pathway stimulates further β-endorphin release from squamous cell carcinoma cells at the early stage of tongue cancer. However, the activation of nuclear factor-κB regulates the functions of miRNAs involved in the synthesis and release of numerous molecules from cancer cells,[44,45] suggesting that other molecules that depend on nuclear factor-κB signaling may be involved in the modulation of tongue nociception. Further studies are thus required to investigate this phenomenon.

The trigeminal ganglion neurons innervating the squamous cell carcinoma cell-inoculated tongue expressed the μ-opioid receptor, and the amount of μ-opioid receptor protein in the cancerous tongue was not altered in this study. The selective endothelin-A antagonist in the squamous cell carcinoma cell-inoculated tongue depressed the enhancement of tongue mechanical sensitivity and the increase of β-endorphin amount in the squamous cell carcinoma cell-inoculated tongue at the early stage of tongue cancer. In addition, the tongue's mechanical hypersensitivity after squamous cell carcinoma cell inoculation was significantly augmented by selective μ-opioid receptor antagonism. Opioids directly act on the central nociceptive pathways and exert powerful action on peripheral nociception. The excitability of sensory neuronal membranes is known to be suppressed by opioid signaling to modulate the functioning of potassium channels. β-endorphin signaling *via* μ-opioid receptor activation on nociceptive afferents induces the activation of the G-protein-coupled inward potassium channel, and the ensuing neuronal membrane hyperpolarization depresses nociceptive neuronal excitability.[14,46] Similarly, peripheral μ-opioid signaling stimulates intracellular signaling involving the L-arginine-

nitric oxide-cyclic guanosine monophosphate cascade, and the subsequent opening of adenosine triphosphate-sensitive potassium channels leads to the depression of nociceptive neuronal hyperexcitability relevant to mechanical hypersensitivity.[47,48] Therefore, it can be hypothesized that β-endorphin released from squamous cell carcinoma cells by endothelin-1 signaling depresses the hyperexcitability of nociceptive neuronal afferents innervating the squamous cell carcinoma cell-inoculated tongue through the activation of potassium channels, resulting in the depression of the tongue's mechanical hypersensitivity at the early stage of tongue carcinogenesis. Furthermore, μ-opioid receptor expression in the cultured SCC-158 cells was confirmed in this study, speculating that β-endorphin signaling plays any role in the functional modulation of squamous cell carcinoma cells. Further studies may be needed.

Alternately, the localization of endothelin-A receptors has been confirmed in small-diameter primary afferent neurons, which are assumed nociceptive neurons in the trigeminal and dorsal root ganglia, by immunocytochemical analysis.[49-51] The endothelin-A receptor was also expressed in trigeminal ganglion neurons innervating the vehicle- or squamous cell carcinoma cell-inoculated tongue, and endothelin-1 administration induced the tongue's mechanical hypersensitivity in this study. Endothelin-1 exposure of the isolated nociceptive sensory neurons enhances their neuronal excitability, which is caused by the depression of the delayed rectifier type of potassium current.[50] In addition, endothelin-1 administration into the hind paw induces a decrease of the mechanical nociceptive threshold dose-dependently,[52] potentially suggesting that the inhibitory effect of trigeminal ganglion neuronal hyperexcitability by β-endorphin signaling is more intensive than the effect of endothelin-1 signaling on trigeminal ganglion neuronal excitability at the early stage of tongue cancer.

Interestingly, the high concentration of β-endorphin in the squamous cell carcinoma cell-inoculated tongue was preserved up to the late stage of tongue carcinogenesis, although trigeminal ganglion neuronal excitability was enhanced at the late stage. Further, selective μ-opioid receptor antagonism in the squamous cell carcinoma cell-inoculated tongue enhanced the tongue's mechanical hypersensitivity at the early stage of tongue cancer. Alternative mechanisms contributing to the cancerous nociceptive neuronal hyperexcitability are expected to be involved in tongue mechanical hypersensitivity associated with tongue cancer; also, exposure of the nociceptive sensory neurons to endothelin-1 is expected to enhance their neuronal excitability. Squamous cell carcinoma

cells produce nerve growth factor in abundance, and cancer-induced mechanical hypersensitivity is depressed by nerve growth factor neutralizing treatment.[53,54] Human squamous cell carcinoma cells produce and secrete serine proteases, resulting in increased proteolytic activity[55]; serine protease signaling *via* protease-activated receptor 2 located on primary nociceptive afferents contributes to cancer-induced mechanical hypersensitivity.[56] The injection of lipids released from oral squamous cell carcinoma cells into the hind paw also produced spontaneous nocifensive behavior and mechanical allodynia.[24]

Taken together, these results show that mechanical sensitivity in the cancerous tongue at the early stage of tongue carcinogenesis is modulated *via* analgesic action on μ-opioid receptor-expressed peripheral afferents attributable to β-endorphin *via* endothelin-1 signaling within the cancer microenvironment, indicating that the cancerous tongue's mechanical sensitivity may depend on the balance of excitatory and inhibitory effects on primary nociceptive afferents at the early and late stages of tongue cancer and requires consideration of a multitude of molecules and pathways.

Acknowledgements

I would like to express my deepest gratitude to Profs. Yoshiyuki Yonehara and Koichi Iwata, and Assoc. Prof. Masamichi Shinoda for their support and guidance throughout the research. I would also like to express my gratitude to member of Departments of Clinical Medicine and Physiology for their cooperation and support.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127:2893-917
2. Cuffari L, Tesseroli de Siqueira JT, Nemr K, Rapaport A: Pain complaint as the first symptom of oral cancer: A descriptive study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006; 102:56-61
3. Chen YK, Huang HC, Lin LM, Lin CC: Primary oral squamous cell carcinoma: An analysis of 703 cases in southern Taiwan. Oral Oncol 1999; 35:173-9
4. Bagan J, Sarrion G, Jimenez Y: Oral cancer: Clinical features. Oral Oncol 2010; 46:414-7
5. Fujita M, Andoh T, Saiki I, Kuraishi Y: Involvement of endothelin and ET(A) endothelin receptor in mechanical allodynia in mice given orthotopic melanoma inoculation. J Pharmacol Sci 2008; 106:257-63
6. Klass M, Hord A, Wilcox M, Denson D, Csete M: A role for endothelin in neuropathic pain after chronic constriction injury of the sciatic nerve. Anesth Analg 2005; 101:1757-62
7. Chichorro JG, Zampronio AR, Souza GE, Rae GA: Orofacial cold hyperalgesia due to infraorbital nerve constriction injury in rats: Reversal by endothelin receptor antagonists but not non-steroidal anti-inflammatory drugs. Pain 2006; 123:64-74
8. Griswold DE, Douglas SA, Martin LD, Davis TG, Davis L, Ao Z, Luttmann MA, Pullen M, Nambi P, Hay DW, Ohlstein EH: Endothelin B receptor modulates inflammatory pain and cutaneous inflammation. Mol Pharmacol 1999; 56:807-12
9. Davenport AP, Hyndman KA, Dhaun N, Southan C, Kohan DE, Pollock JS, Pollock DM, Webb DJ, Maguire JJ: Endothelin. Pharmacol Rev 2016; 68:357-418
10. Hans G, Deseure K, Robert D, De Hert S: Neurosensory changes in a human model of endothelin-1 induced pain: A behavioral study. Neurosci Lett 2007; 418:117-21
11. McKelvy AD, Mark TR, Sweitzer SM: Age- and sex-specific nociceptive response to endothelin-1. J Pain 2007; 8:657-66
12. Pickering V, Jay Gupta R, Quang P, Jordan RC, Schmidt BL: Effect of peripheral endothelin-1 concentration on carcinoma-induced pain in mice. Eur J Pain 2008; 12:293-300

13. Schmidt BL, Pickering V, Liu S, Quang P, Dolan J, Connelly ST, Jordan RC: Peripheral endothelin A receptor antagonism attenuates carcinoma-induced pain. Eur J Pain 2007; 11:406-14
14. Khodorova A, Navarro B, Jouaville LS, Murphy JE, Rice FL, Mazurkiewicz JE, Long-Woodward D, Stoffel M, Strichartz GR, Yukhananov R, Davar G: Endothelin-B receptor activation triggers an endogenous analgesic cascade at sites of peripheral injury. Nat Med 2003; 9:1055-6
15. Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16:109-10
16. Honda K, Noma N, Shinoda M, Miyamoto M, Katagiri A, Kita D, Liu MG, Sessle BJ, Yasuda M, Iwata K: Involvement of peripheral ionotropic glutamate receptors in orofacial thermal hyperalgesia in rats. Mol Pain 2011; 7:75
17. Liu MG, Matsuura S, Shinoda M, Honda K, Suzuki I, Shibuta K, Tamagawa T, Katagiri A, Kiyomoto M, Ohara K, Furukawa A, Urata K, Iwata K: Metabotropic glutamate receptor 5 contributes to inflammatory tongue pain via extracellular signal-regulated kinase signaling in the trigeminal spinal subnucleus caudalis and upper cervical spinal cord. J Neuroinflammation 2012; 9:258
18. Abed HS, Samuel CS, Lau DH, Kelly DJ, Royce SG, Alasady M, Mahajan R, Kuklik P, Zhang Y, Brooks AG, Nelson AJ, Worthley SG, Abhayaratna WP, Kalman JM, Wittert GA, Sanders P: Obesity results in progressive atrial structural and electrical remodeling: Implications for atrial fibrillation. Heart Rhythm 2013; 10:90-10
19. Margas W, Mahmoud S, Ruiz-Velasco V: Muscarinic acetylcholine receptor modulation of mu (μ) opioid receptors in adult rat sphenopalatine ganglion neurons. J Neurophysiol 2010; 103:172-82
20. Jedynak JP, Cameron CM, Robinson TE: Repeated methamphetamine administration differentially alters fos expression in caudate-putamen patch and matrix compartments and nucleus accumbens. PLoS One 2012; 7:e34227
21. Honda K, Shinoda M, Kondo M, Shimizu K, Yonemoto H, Otsuki K, Akasaka R, Furukawa A, Iwata K: Sensitization of TRPV1 and TRPA1 via peripheral mGluR5 signaling contributes to thermal and mechanical hypersensitivity. Pain 2017; 158:1754-64
22. Kubo A, Shinoda M, Katagiri A, Takeda M, Suzuki T, Asaka J, Yeomans DC, Iwata

K: Oxytocin alleviates orofacial mechanical hypersensitivity associated with infraorbital nerve injury through vasopressin-1A receptors of the rat trigeminal ganglia. Pain 2017; 158:649-59

23. Shinoda M, Takeda M, Honda K, Maruno M, Katagiri A, Satoh-Kuriwada S, Shoji N, Tsuchiya M, Iwata K: Involvement of peripheral artemin signaling in tongue pain: Possible mechanism in burning mouth syndrome. Pain 2015; 156:2528-37
24. Ruparel S, Bendele M, Wallace A, Green D: Released lipids regulate transient receptor potential channel (TRP) dependent oral cancer pain. Mol Pain 2015; 11:30
25. Hidaka K, Ono K, Harano N, Sago T, Nunomaki M, Shiiba S, Nakanishi O, Fukushima H, Inenaga K: Central glial activation mediates cancer-induced pain in a rat facial cancer model. Neuroscience 2011; 180:334-43
26. Scully C, Bagan J: Oral squamous cell carcinoma: Overview of current understanding of aetiopathogenesis and clinical implications. Oral Dis 2009; 15:388-99
27. Kingery WS, Agashe GS, Guo TZ, Sawamura S, Davies MF, Clark JD, Kobilka BK, Maze M: Isoflurane and nociception: Spinal α_{2A} adrenoceptors mediate antinociception while supraspinal α_1 adrenoceptors mediate pronociception. Anesthesiology 2002; 96:367-74
28. Tamagawa T, Shinoda M, Honda K, Furukawa A, Kaji K, Nagashima H, Akasaka R, Chen J, Sessle BJ, Yonehara Y, Iwata K: Involvement of microglial P2Y12 signaling in tongue cancer pain. J Dent Res 2016; 95:1176-82
29. Asai H, Ozaki N, Shinoda M, Nagamine K, Tohnai I, Ueda M, Sugiura Y: Heat and mechanical hyperalgesia in mice model of cancer pain. Pain 2005; 117:19-29
30. Nagamine K, Ozaki N, Shinoda M, Asai H, Nishiguchi H, Mitsudo K, Tohnai I, Ueda M, Sugiura Y: Mechanical allodynia and thermal hyperalgesia induced by experimental squamous cell carcinoma of the lower gingiva in rats. J Pain 2006; 7:659-70
31. Stow LR, Jacobs ME, Wingo CS, Cain BD: Endothelin-1 gene regulation. FASEB J 2011; 25:16-28
32. Nelson J, Bagnato A, Battistini B, Nisen P: The endothelin axis: Emerging role in cancer. Nat Rev Cancer 2003; 3:110-6
33. Allen CT, Ricker JL, Chen Z, Van Waes C: Role of activated nuclear factor-κB in the pathogenesis and therapy of squamous cell carcinoma of the head and neck.

Head Neck 2007; 29:959-71
34. Ahn KS, Aggarwal BB: Transcription factor NF-κB: A sensor for smoke and stress signals. Ann N Y Acad Sci 2005; 1056:218-33
35. Garg R, Blando J, Perez CJ, Wang H, Benavides FJ, Kazanietz MG: Activation of nuclear factor κB (NF-κB) in prostate cancer is mediated by protein kinase Cε (PKCε). J Biol Chem 2012; 287:37570-82
36. Sakamoto K, Maeda S, Hikiba Y, Nakagawa H, Hayakawa Y, Shibata W, Yanai A, Ogura K, Omata M: Constitutive NF-κB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. Clin Cancer Res 2009; 15:2248-58
37. Terragni J, Graham JR, Adams KW, Schaffer ME, Tullai JW, Cooper GM: Phosphatidylinositol 3-kinase signaling in proliferating cells maintains an anti-apoptotic transcriptional program mediated by inhibition of FOXO and non-canonical activation of NFκB transcription factors. BMC Cell Biol 2008; 9:6
38. Vikhreva P, Petrova V, Gokbulut T, Pestlikis I, Mancini M, Di Daniele N, Knight RA, Melino G, Amelio I: TAp73 upregulates IL-1β in cancer cells: Potential biomarker in lung and breast cancer? Biochem Biophys Res Commun 2017; 482:498-505
39. Boesen EI, Sasser JM, Saleh MA, Potter WA, Woods M, Warner TD, Pollock JS, Pollock DM: Interleukin-1β, but not interleukin-6, enhances renal and systemic endothelin production *in vivo*. Am J Physiol Renal Physiol 2008; 295:F446-53
40. Rosanò L, Di Castro V, Spinella F, Nicotra MR, Natali PG, Bagnato A: ZD4054, a specific antagonist of the endothelin A receptor, inhibits tumor growth and enhances paclitaxel activity in human ovarian carcinoma *in vitro* and *in vivo*. Mol Cancer Ther 2007; 6:2003-11
41. Ahmed SI, Thompson J, Coulson JM, Woll PJ: Studies on the expression of endothelin, its receptor subtypes, and converting enzymes in lung cancer and in human bronchial epithelium. Am J Respir Cell Mol Biol 2000; 22:422-31
42. Zhang Y, Tang L, Su M, Eisen D, Zloty D, Warshawski L, Zhou Y: Expression of endothelins and their receptors in nonmelanoma skin cancers. J Cutan Med Surg 2006; 10:269-76
43. Quang PN, Schmidt BL: Endothelin-A receptor antagonism attenuates carcinoma-induced pain through opioids in mice. J Pain 2010; 11:663-71

44. Wu WK, Lee CW, Cho CH, Fan D, Wu K, Yu J, Sung JJ: MicroRNA dysregulation in gastric cancer: A new player enters the game. Oncogene 2010; 29:5761-71
45. Chow JY, Ban M, Wu HL, Nguyen F, Huang M, Chung H, Dong H, Carethers JM: TGF-β downregulates PTEN via activation of NF-κB in pancreatic cancer cells. Am J Physiol Gastrointest Liver Physiol 2010; 298:G275-82
46. Ikeda K, Kobayashi T, Kumanishi T, Niki H, Yano R: Involvement of G-protein–activated inwardly rectifying K (GIRK) channels in opioid-induced analgesia. Neurosci Res 2000; 38:113-6
47. Gutierrez VP, Zambelli VO, Picolo G, Chacur M, Sampaio SC, Brigatte P, Konno K, Cury Y: The peripheral L-arginine-nitric oxide-cyclic GMP pathway and ATP-sensitive K^+ channels are involved in the antinociceptive effect of crotalphine on neuropathic pain in rats. Behav Pharmacol 2012; 23:14-24
48. Sachs D, Cunha FQ, Ferreira SH: Peripheral analgesic blockade of hypernociception: Activation of arginine/NO/cGMP/ protein kinase G/ATP-sensitive K^+ channel pathway. Proc Natl Acad Sci U S A 2004; 101:3680-5
49. Pomonis JD, Rogers SD, Peters CM, Ghilardi JR, Mantyh PW: Expression and localization of endothelin receptors: Implications for the involvement of peripheral glia in nociception. J Neurosci 2001; 21:999-1006
50. Feng B, Strichartz G: Endothelin-1 raises excitability and reduces potassium currents in sensory neurons. Brain Res Bull 2009; 79:345-50
51. Yamamoto T, Ono K, Hitomi S, Harano N, Sago T, Yoshida M, Nunomaki M, Shiiba S, Watanabe S, Nakanishi O, Inenaga K: Endothelin receptor-mediated responses in trigeminal ganglion neurons. J Dent Res 2013; 92:335-9
52. Joseph EK, Gear RW, Levine JD: Mechanical stimulation enhances endothelin-1 hyperalgesia. Neuroscience 2011; 178:189-95
53. Halvorson KG, Kubota K, Sevcik MA, Lindsay TH, Sotillo JE, Ghilardi JR, Rosol TJ, Boustany L, Shelton DL, Mantyh PW: A blocking antibody to nerve growth factor attenuates skeletal pain induced by prostate tumor cells growing in bone. Cancer Res 2005; 65:9426-35
54. Mantyh WG, Jimenez-Andrade JM, Stake JI, Bloom AP, Kaczmarska MJ, Taylor RN, Freeman KT, Ghilardi JR, Kuskowski MA, Mantyh PW: Blockade of nerve sprouting and neuroma formation markedly attenuates the development of late stage cancer pain. Neuroscience 2010; 171:588-98

55. Nyberg P, Ylipalosaari M, Sorsa T, Salo T: Trypsins and their role in carcinoma growth. Exp Cell Res 2006; 312:1219-28
56. Lam DK, Schmidt BL: Serine proteases and protease-activated receptor 2-dependent allodynia: A novel cancer pain pathway. Pain 2010; 149:263-72

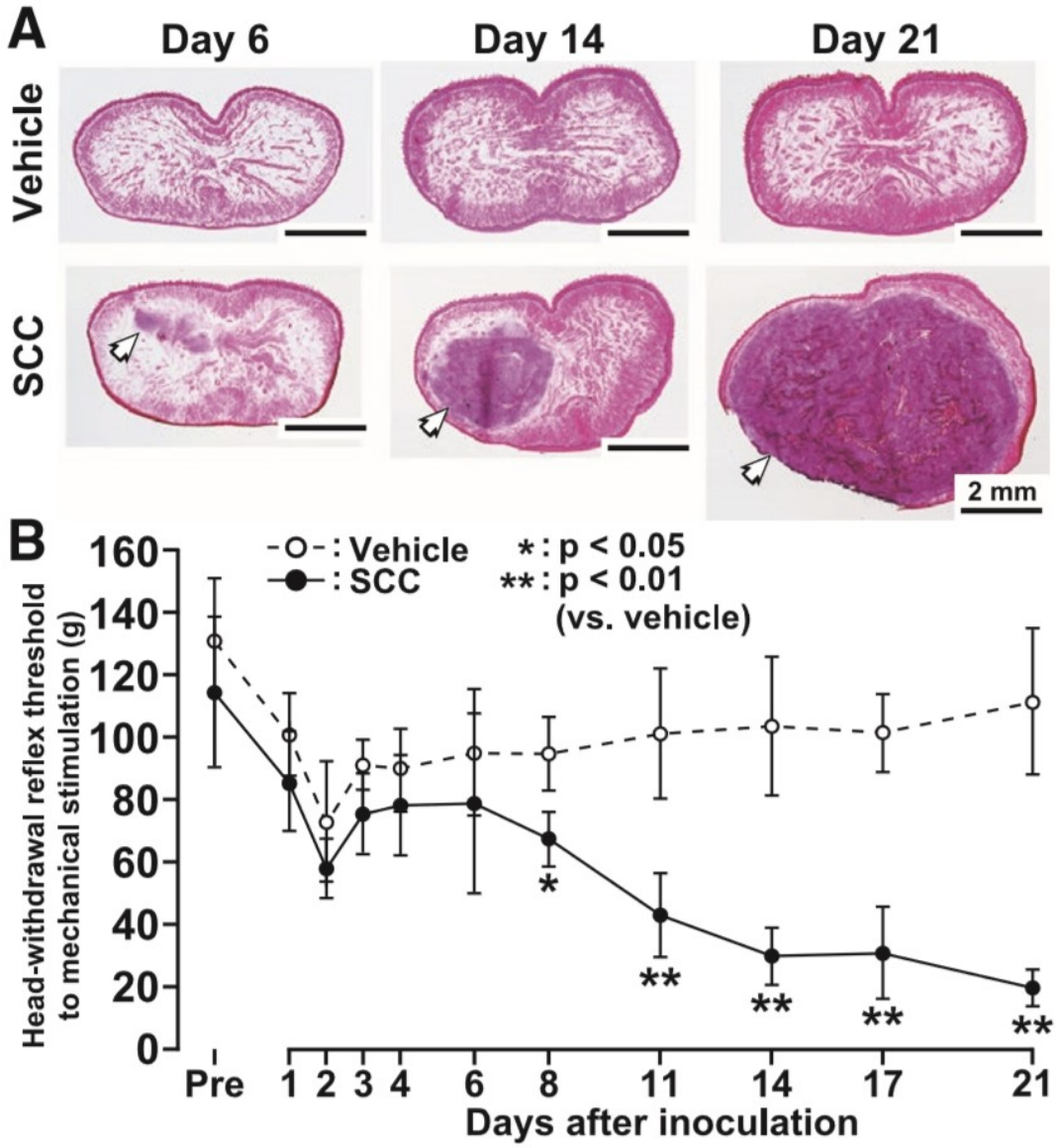

Fig. 1 Cancerous tongue and changes in tongue mechanical sensitivity after squamous cell carcinoma (SCC) cell inoculation. (A) Sequential histologic observation of the vehicle- and squamous cell carcinoma cell-inoculated sites in the tongue. Arrows indicate tumor mass. (B) Mechanical head-withdrawal reflex threshold after vehicle and squamous cell carcinoma cell inoculation into the tongue. The data represent mean ± SD (n = 8 each; two-way ANOVA with repeated measures followed by Bonferroni's multiple-comparison tests). "Pre" indicates before inoculation.

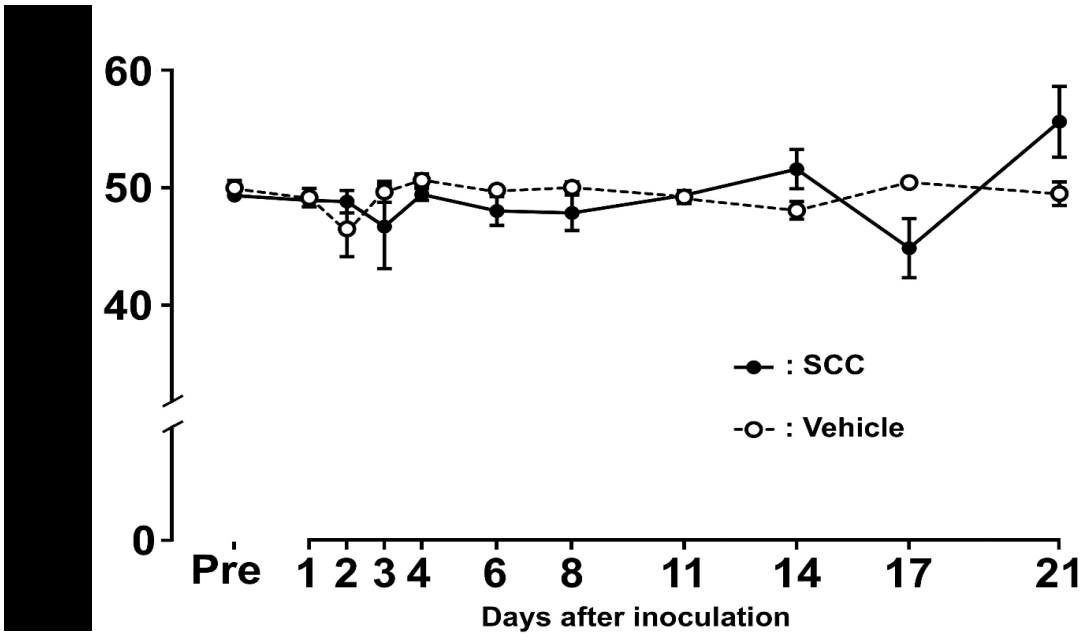

Fig. 2 Heat head-withdrawal reflex latency after vehicle and squamous cell carcinoma cell inoculation into the tongue. The data represent mean ± SD (n = 3 each; two-way ANOVA with repeated measures followed by Bonferroni's multiple-comparison tests). "Pre" indicates before inoculation.

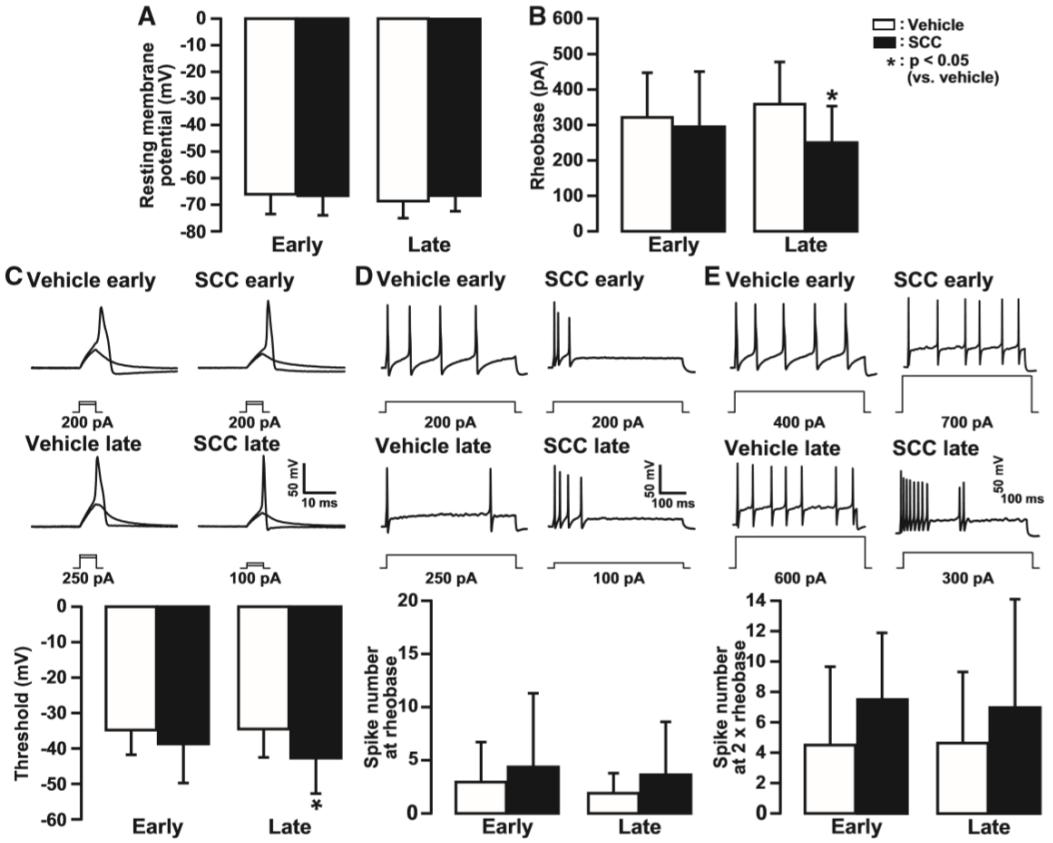

Fig. 3 Electrophysiologic properties of trigeminal ganglion neurons innervating the cancerous tongue. (A, B) Resting membrane potential (A) and rheobase (B) of trigeminal ganglion neurons innervating the tongue at the early and late stages after vehicle and squamous cell carcinoma (SCC) cell inoculation into the tongue. (C) The depolarization wave induced by the threshold current injection and current injection 50 pA smaller than the threshold. (D) The depolarization wave induced by the threshold current injection. (E) The depolarization wave induced by the 2× rheobase injection. (C-E) Passive and active properties (C, threshold; D, spike number at the rheobase; E, spike number of 2× rheobase) of trigeminal ganglion neurons innervating the tongue. The data represent mean ± SD. Student's t-tests were used for statistical analysis.

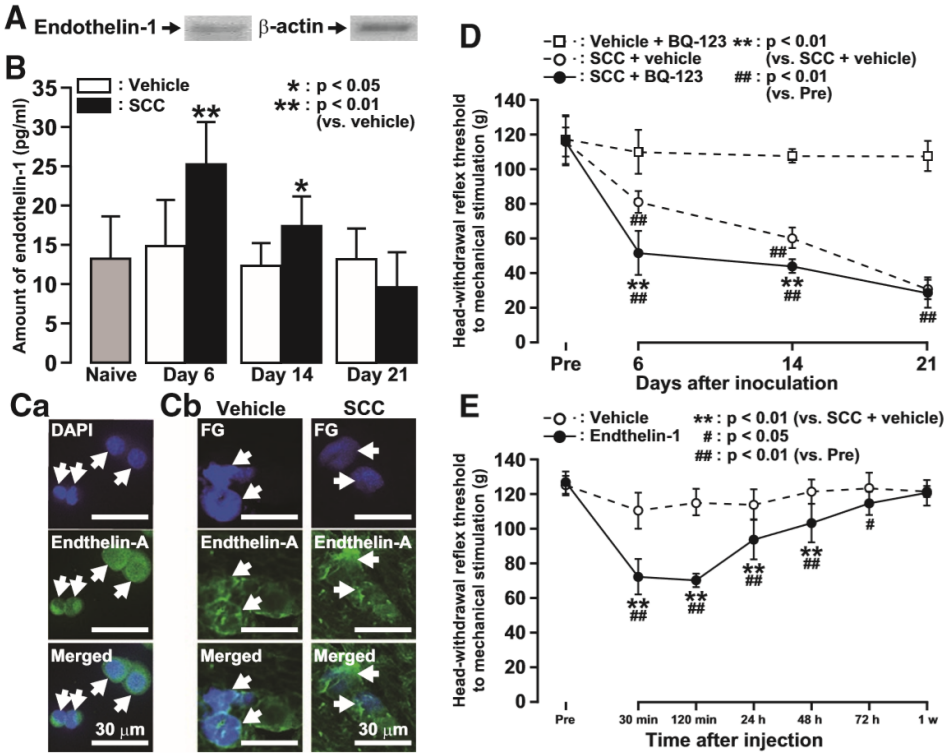

Fig. 4 Involvement of endothelin-1 signaling in tongue mechanical sensitivity after squamous cell carcinoma (SCC) cell inoculation. (A) Blotting example of endothelin-1 protein in the SCC-158 culture medium. (B) Sequential changes in the amount of endothelin-1 protein in the vehicle- and squamous cell carcinoma cell-inoculated tongue. The data represent mean ± SD (n = 6 each; Student's t-tests). (C) Endothelin-A receptor expression in cultured SCC-158 cells (a), and in trigeminal ganglion neurons innervating the vehicle- or squamous cell carcinoma cell-inoculated tongue (b). Arrows indicate 4′,6-diamidino-2-phenylindole (DAPI)- or FluoroGold (FG)-labeled endothelin-A receptor-immunoreactive cells. (D) Changes in the cancerous tongue mechanical head-withdrawal reflex threshold after BQ123 administration in the tongue. The data represent mean ± SD (vehicle + BQ123: n = 5, squamous cell carcinoma + vehicle: n = 5, squamous cell carcinoma + BQ123: n = 7; one-way and two-way ANOVA followed by Bonferroni's multiple-comparison tests). (E) Changes in the tongue mechanical head-withdrawal reflex threshold after endothelin-1 administration in the tongue. The data represent mean ± SD (vehicle: n = 8, endothelin-1: n = 8; one-way and two-way ANOVA with repeated measures followed by Bonferroni's multiple-comparison tests). "Pre" indicates before inoculation.

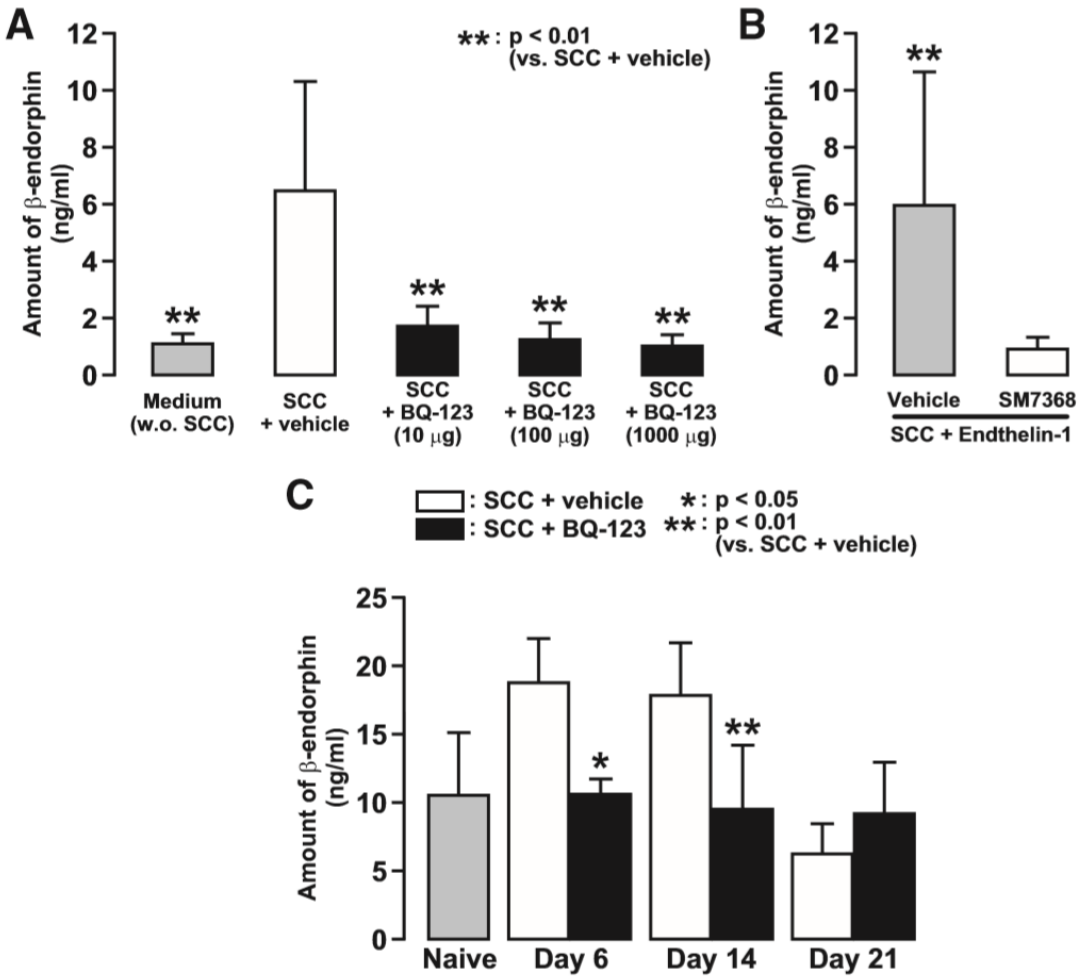

Fig. 5 β-endorphin release from squamous cell carcinoma cells depends on endothelin-1 signaling. (A) The release of β-endorphin protein from the cultured squamous cell carcinoma (SCC) cells under various concentrations of the BQ123-applied condition. Medium indicates only cultivation medium without squamous cell carcinoma cells (n = 6 each, one-way ANOVA followed by Bonferroni's multiple-comparison tests). (B) The release of β-endorphin protein from the cultured squamous cell carcinoma cells in the endothelin-1-containing culture medium under the SM7368-applied condition (n = 6 each, Student's t-tests). (C) Sequential changes in the amount of β-endorphin protein in the cancerous tongue under the BQ123- or vehicle-applied condition. The data represent mean ± SD (naive: n = 5, squamous cell carcinoma + vehicle day 6: n = 5, squamous cell carcinoma + vehicle day 14: n = 5, squamous cell carcinoma + vehicle day 21: n = 5, squamous cell carcinoma + BQ123 day 6: n = 5, squamous cell carcinoma + BQ123 day 14: n = 6, squamous cell carcinoma + BQ123 day 21: n = 7; Student's t-tests).

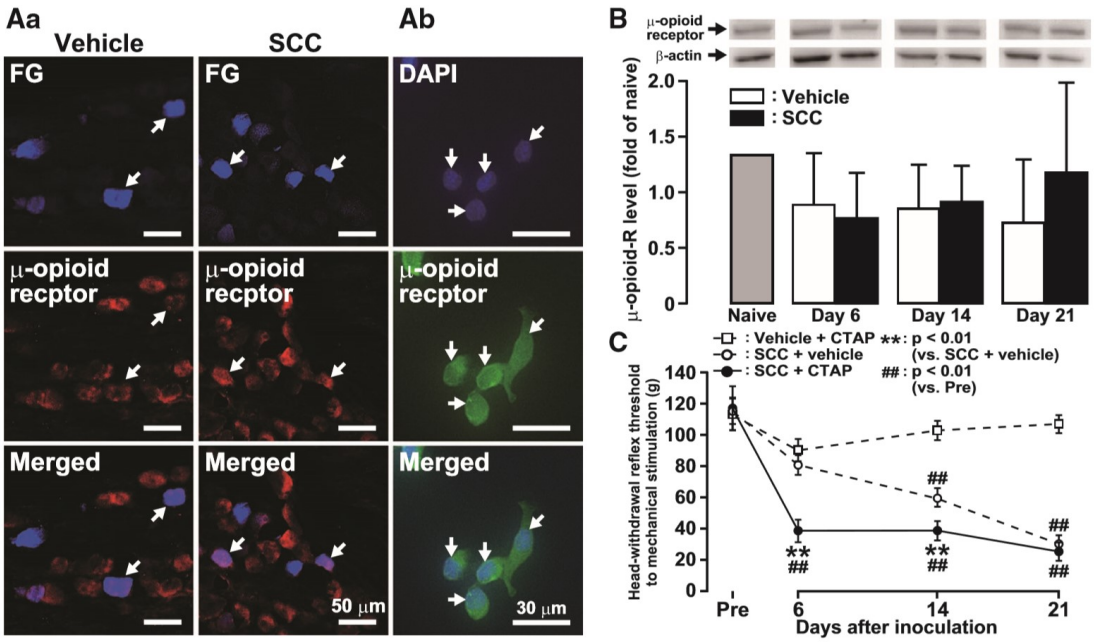

Fig. 6 Involvement of β-endorphin signaling in tongue mechanical sensitivity after squamous cell carcinoma (SCC) cell inoculation. (Aa) μ-opioid receptor expression in trigeminal ganglion neurons innervating the cancerous tongue on day 14 after vehicle or squamous cell carcinoma cell inoculation into the tongue. Arrows indicate FluoroGold-labeled μ-opioid receptor immunoreactive cells. (Ab) μ-opioid receptor expression in the cultured SCC-158 cells. Arrows indicate 4′,6-diamidino-2-phenylindole–labeled μ-opioid receptor immunoreactive cells. (B) Sequential changes in the amount of μ-opioid receptor protein in the squamous cell carcinoma cell- or vehicle-inoculated tongue (n = 5 in naive, n = 7 in vehicle, n = 7 in squamous cell carcinoma). The data represent mean ± SD. (C) Changes in the cancerous tongue mechanical head-withdrawal reflex threshold after CTAP administration in the tongue. The data represent mean ± SD (vehicle + CTAP: n = 6, squamous cell carcinoma + vehicle: n = 5, squamous cell carcinoma + CTAP: n = 7; one-way and two-way ANOVA followed by Bonferroni's multiple-comparison tests).

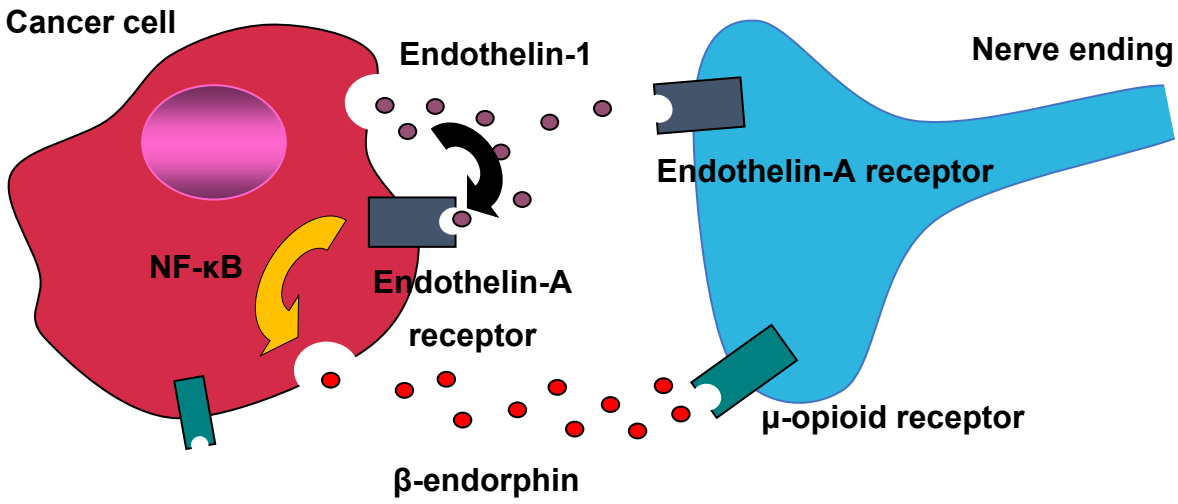

Fig. 7 Summary diagrams of the present results. Endothelin-1 is released from cancer cells, and it binds to endothelin-A receptor in cancer cells, and then β-endorphin is released from cancer cells through endothelin-NF-kB pathway. β-endorphin binds to μ-opioid receptor in nociceptive neurons, and suppresses excitability enhancement of neurons, resulting in the suppression of tongue mechanical allodynia associated with cancer cell inoculation.