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Note

Gene delivery system involving Bubble liposomes and ultrasound for the efficient *in vivo* delivery of genes into mouse tongue tissue

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ABSTRACT

Oral squamous cell carcinoma is the most common type of head and neck cancer. Recently, efficient, easy, and minimally invasive gene delivery methods are expected to be developed as cancer gene therapies. However, the optimal method for delivering therapeutic genes into oral tissue for cancer treatment has not been elucidated. Therefore, we hypothesized that the tongue is a good target tissue for gene delivery with Bubble liposomes and ultrasound. To assess this, we attempted to deliver a mixture of plasmid DNA encoding a luciferase or enhanced green fluorescent protein, and Bubble liposomes into murine tongue with or without ultrasound exposure. The ultrasound conditions were 1 MHz, 2 W/cm^2, 60 s, and duty cycle: 50%. The time-course of gene expression in the tongue was investigated with a luciferase assay and fluorescent microscopy. Luciferase expression was significantly increased in tongue transfected using Bubble liposomes and ultrasound compared with that of the tongue untreated with ultrasound, and this high level of luciferase activity was maintained for 2 weeks. From these results, Bubble liposomes can be used in combination with ultrasound to efficiently deliver plasmid DNA into the tongue *in vivo*. This technique is a highly promising approach for gene delivery into oral tissue.

1. Introduction

Although the most common types of oral disease are dental caries and periodontal disease, oral cancer such as squamous cell carcinoma (SCC) is associated with an unfavorable prognosis. Tongue SCC is the most common type of oral SCC, and metastasis to the lymph nodes and/or proximal tissues often occurs (Ohba et al., 2010; Shiga et al., 2007). The current treatments for tongue SCC include surgery, radiation therapy, and chemotherapy, all of which have severe side effects. Therefore, cancer cell-specific treatment that does not damage normal cells is desired. Recently, gene delivery to tumor cells such as using adenovirus-based p53 gene therapies has gained attention (Edelman and Nemunaitis, 2003; Huang et al., 2009). The two main gene carrier systems for gene therapy are viral vectors and non-viral delivery systems. Viral vectors are efficient carriers for gene transfection (Lundstrom, 2003), but some serious problems such as immunogenicity and toxicity have been reported (Check, 2002, 2003; Marshall, 1999). On the other hand, the transfection efficiency of non-viral methods remains a problem. Therefore, it is necessary to develop a safe and highly efficient gene transfer method.

Recently, it has been reported that the use of microbubbles in combination with low energy ultrasound (US) enhances transfection efficiency (Greenleaf et al., 1998; Shohet et al., 2000; Sonoda et al., 2006; Taniyama et al., 2002a,b). Regarding the orofacial area, there have been a few reports about gene delivery using microbubbles and US, for example, Sakai et al. (2009) and Chen et al. (2009) reported transient gene transfection in the target tissue using different microbubbles. However, prolonged gene expression is necessary in the clinical setting, and the size and stability of the microbubbles employed also needs to be improved. Previously, we developed "Bubble liposomes (BL)" as a novel gene delivery carrier system and reported that gene delivery using a combination of BL and US is safer and more efficient in both *in vitro* and *in vivo* compared to other non-viral methods (Negishi et al., 2008, 2011; Suzuki et al., 2007). However, there are no reports about gene delivery to

Abbreviations: SCC, squamous cell carcinoma; US, ultrasound; BL, Bubble liposome; PEG, polyethylene glycol; EBD, Evans blue dye; QOL, quality of life.

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oral tissue using this technique. Therefore, in the present study, we assessed whether efficient gene delivery into mouse tongue tissue could be achieved using BL and US.

2. Materials and methods

2.1. Animals

Five-week-old male ICR mice were used for all animal experiments (Tokyo Laboratory Animals Science, Tokyo, Japan). All studies were approved by the Animal Experiment Committee of Tokyo University of Pharmacy and Life Sciences. The mice were given feed and tap water *ad libitum* throughout the experimental period.

2.2. Preparation of Bubble liposomes

The BL were prepared using the previously described method (Negishi et al., 2008; Suzuki et al., 2007). In brief, PEG liposomes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (NOF Corporation, Tokyo, Japan) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-polyethyleneglycol (DSPE-PEG_{2000}-OMe) (NOF Corporation) in a molar ratio of 94:6 were prepared using a reverse phase evaporation method. In brief, all reagents were dissolved in 1:1 (v/v) chloroform/diisopropyl ether. Phosphate-buffered saline was added to the lipid solution, and the mixture was sonicated and then evaporated at 47 °C. Then, the organic solvent was completely removed, and the size of the liposomes was adjusted to less than 200 nm using extruding equipment and a sizing filter (pore size: 200 nm) (Nuclepore Track-Etch Membrane, Whatman plc, UK). The lipid concentration was measured using a Phospholipid C test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan), and BL were prepared from liposomes and perfluoropropane gas (Takachio Chemical Ind. Co. Ltd., Tokyo, Japan). First, 2-mL sterilized vials containing 0.8 mL of a liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressurized with a further 3 mL of perfluoropropane gas. The vial was placed in a bath-type sonicator (42 kHz, 100 W) (Bransonic 2510j-DTH, Branson Ultrasonics Co., Danbury, CT, USA) for 5 min to form BL.

2.3. Plasmid DNA

Two reporter plasmids were used. The pcDNA3-Luc plasmid, which is derived from pGL3-basic (Promega, Madison, WI), is an expression vector encoding the firefly luciferase gene under the control of the cytomegalovirus promoter. The pEGFP-N3 plasmid (Clontech Laboratories, Inc., Mountain View, CA) is an expression vector encoding enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus promoter.

2.4. In vivo gene delivery using BL and US

ICR mice were anesthetized with 10 mg/mL pentobarbital throughout each procedure. A 20 μL mixture of pDNA (20 μg) and BL (10 μg) was injected into the tongue tissue of the mice using a 33-gauge syringe (Hamilton Company, USA), and US exposure (frequency: 1 MHz; duty: 50%; intensity: 2 W/cm^2; time: 60 s) was immediately applied to the injection site. A Sonitron 2000 (Nepa Gene Co., Ltd.) was used as an ultrasound generator. Several days after the injection, the mice were sacrificed, and the tongue tissue in the US-exposed area was collected and homogenized with Polytron (Kinematica, Inc., New York, USA). The cell lysate and tissue homogenates were prepared with a lysis buffer (0.1 M Tris–HCl (pH 7.8), 0.1% Triton X-100, and 2 mM EDTA). Luciferase activity was then measured using a luciferase assay system (Promega, Madison, WI) and a luminometer (LB96V, Belthold Japan Co. Ltd., Tokyo, Japan). Activity is indicated as relative light units (RLU) per mg of protein. To analyze EGFP expression, the collected tongue was fixed with paraformaldehyde and dehydrated in sucrose solution. The specimens were then embedded in OCT compound and immediately frozen at −80 °C. Serial 10 μm thick sections were then cut using a cryostat and observed with a fluorescence microscope (Axiovert 200 M, Carl Zeiss).

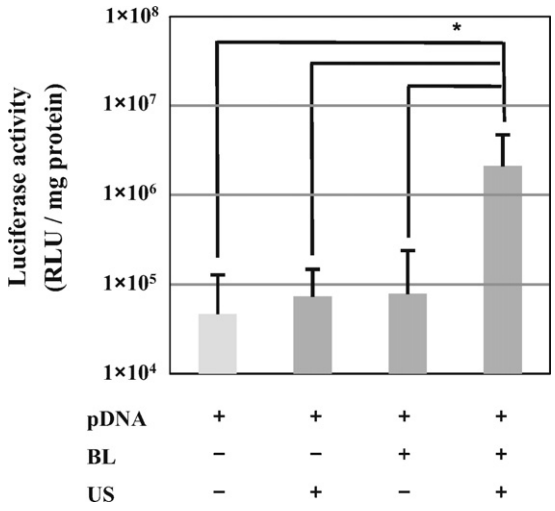

Fig. 1. Luciferase activity in tongue tissue transfected with a reporter gene using BL and US. Mice were subjected to BL and US-mediated luciferase gene transfer. Relative luciferase activity was determined on day 5 after transfection. The data are shown as the mean ± S.D. *$P < 0.05$, Mann–Whitney's U test ($n = 5$), compared to other groups. pDNA (pCMV-luciferase): 20 μg; BL: 10 μg; US conditions: frequency: 1 MHz, duty: 50%, and intensity: 2 W/cm^2, time: 60 s. BL, Bubble liposomes; US, ultrasound.

2.5. Tissue damage testing using Evans blue dye (EBD)

Tissue-damage testing using EBD was performed as reported previously (Liu and Huang, 2002). Briefly, EBD was dissolved in PBS (10 mg/mL) and sterilized using 0.2 μm membrane filters. The mice treated with pDNA, BL, and US were administered EBD (0.5 mg dye/10 g body weight) by tail vein injection and then sacrificed 1 day after the EBD injection. Their tongue tissues were collected, fixed with paraformaldehyde, embedded in OCT compound, and immediately frozen at −80 °C. Serial 10 μm thick sections were cut using a cryostat and observed with a fluorescence microscope (Axiovert 200 M, Carl Zeiss).

2.6. Statistical analysis

All data are shown as the mean ± S.D. ($n = 5$ or 6). Mann–Whitney's U test was used to determine the statistical significance of any differences. The differences detected in multiple comparison tests were assessed by two-way repeated-measures analysis of variance (ANOVA). Differences associated with a $P < 0.05$ were considered significant.

3. Results

We first tried to deliver naked pDNA into tongue tissue using BL and US under the conditions used in a previous study, in which naked pDNA was delivered into skeletal muscles (Negishi et al., 2011). Significantly increased gene expression was detected in the group treated with BL and US exposure (Fig. 1.); i.e., it was 12-fold higher than that of the group treated with pDNA alone. In the groups

treated with pDNA + BL and pDNA + US, the relative luciferase activity remained as low as that of the pDNA alone group.

Then, to optimize the conditions for *in vivo* gene delivery into tongue tissue, we examined three transfection condition parameters, the total pDNA, US intensity, and US exposure time. First, to assess whether the pDNA injection volume affected transfection efficiency, we adjusted it from 0.2 μg to 20 μg. As a result, the increase in luciferase activity was found to be dependent on the amount of pDNA, and the most significant increase in relative luciferase activity was detected at 20 μg pDNA (Fig. 2a.). Next, we investigated the relationship between US intensity and the transfection efficiency of gene delivery into tongue tissue. The US intensity was varied within the 0–4 W/cm^2 range. The relative luciferase activity was significantly higher in the groups treated with US intensities of 2.0 W/cm^2 and 4.0 W/cm^2 (Fig. 2b.). Moreover, we also examined the effect of the US exposure time and found that luciferase activity was highest when US was delivered for 60 s (Fig. 2c.). In contrast, when US was delivered for a longer period, the transfection efficiency tended to decrease. We further examined the duration of gene expression induced after treatment with BL and US exposure. As a result, we found that high luciferase activity was maintained for about 2 weeks (Fig. 3).

Next, the localization of EGFP-expressing cells and tissue damage after gene delivery with BL and US was observed with fluorescence microscopy. In histological observations, distinct EGFP expression was observed in the tongue tissue treated with BL and US (Fig. 4.). In the group treated with BL and US, there were many EGFP expressing cells throughout the muscle layer. In the other groups, only a few sporadically distributed cells were found to express EGFP. However, using a high US intensity to achieve efficient gene transfection leads to tissue damage (Duvshani-Eshet and Machluf, 2005; Kim et al., 1996). Therefore, to investigate the tissue damage caused by gene transfection, mice had EBD injected into their tail veins one day before they were euthanized, as enhanced EBD uptake indicates increased cell damage. As a result, we found that severe tissue damage was observed after the application of high intensity US (4.0 W/cm^2) or a US exposure time of 120 s or more (Fig. 5).

From these results, we suggest that the optimal conditions for gene delivery into the murine tongue using BL and US are as follows: total pDNA (2 μg/μL): 20 μg, US intensity: 2 W/cm^2, and US exposure time: 60 s.

In addition, *Sonazoid*™, a commercially available microbubble, has been used as an echo-contrast gas in clinical. We therefore also test the transfection efficacy of *Sonazoid*™ in the same experiment. However, the luciferase activity was moderate increase even in the combination of US exposure (data not shown).

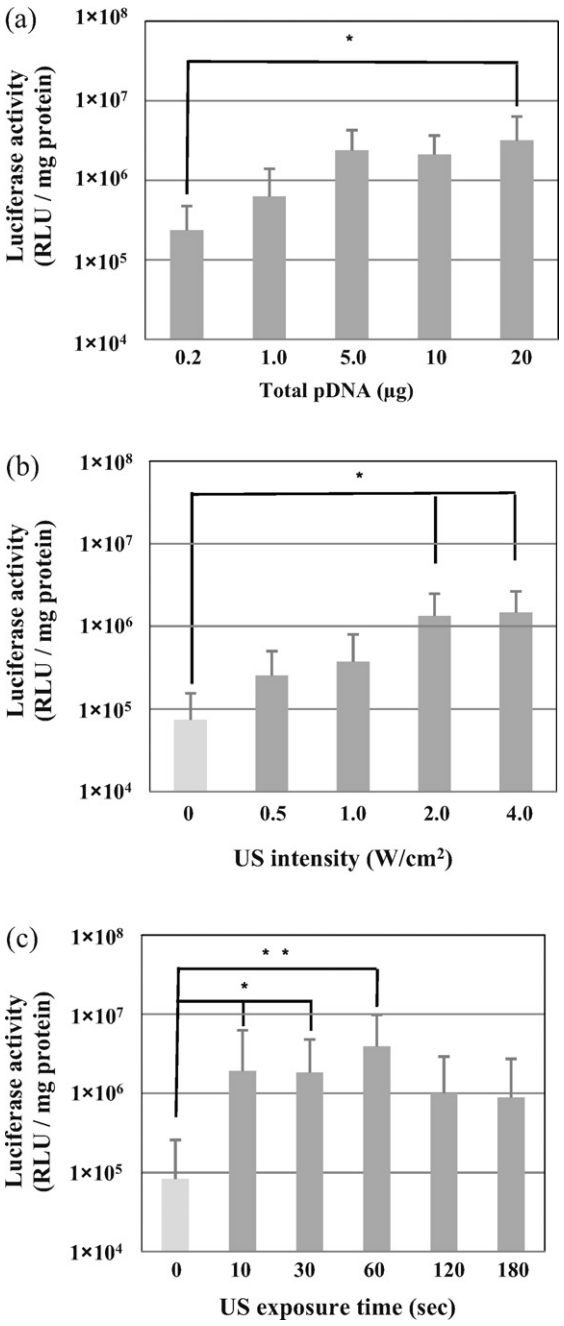

Fig. 2. Characteristics of ultrasound gene delivery systems using BL. To examine the optimal parameters for BL and US-mediated gene transfer into tongue tissue, the mice were subjected to various transfection conditions; i.e., by altering the amount of pDNA, US intensity, and US exposure time. The other conditions were as follows: US frequency: 1 MHz, duty: 50%. The data are shown as the mean ± S.D. (a) The variation in the gene expression level induced by changing the amount of pDNA. The amount of pDNA was changed from 0.2 μg to 20 μg. The total injection volume remained constant at 20 μL. $^*P < 0.05$, Mann–Whitney's U test ($n = 5$), compared with 0.2 μg of pDNA. (b) The variation in the gene expression level induced by changes in the US intensity. The US intensity was set at 0, 0.5, 1.0, 2.0, or 4.0 W/cm^2. $^*P < 0.05$, Mann–Whitney's U test ($n = 5$), compared with 0 W/cm^2 (no US exposure). (c) The variation in the gene expression level induced by changes in the US exposure time. The US duration was set at 0, 10, 30, 60, 120, or 180 s. $^*P < 0.05$, $^{**}P < 0.01$, Mann–Whitney's U test ($n = 6$), compared with 0 s (no US exposure).

4. Discussion

Gene therapy is expected to be clinically useful for treating genetic diseases, cancer, and/or infectious diseases. These diseases are also found in the orofacial area, and a number of studies have recently examined the usefulness of gene therapy for a variety of oral diseases. In those studies, gene delivery into the orofacial area was performed with viral vectors due to their high transfection efficiency. For example, it has been reported that reporter genes were transfected into rat salivary glands using several kinds of viral vector (Shai et al., 2002; Zheng and Baum, 2005). In addition, viruses are the most common transfer system used to deliver gene therapy to oral SCC (Ladeinde et al., 2005). However, viral systems are not perfect because of their safety and immunogenicity (Check, 2002, 2003; Marshall, 1999).

Therefore, many researchers have tried to establish non-viral gene delivery systems that combine high transfection efficiency with reduced invasiveness. Among these non-viral gene delivery methods, Fechheimer et al. (1987) first reported the US-mediated gene delivery technique, and since then gene transfer using ultrasonic waves has developed into a safe and non-viral gene transfection technology. A physical phenomenon known as

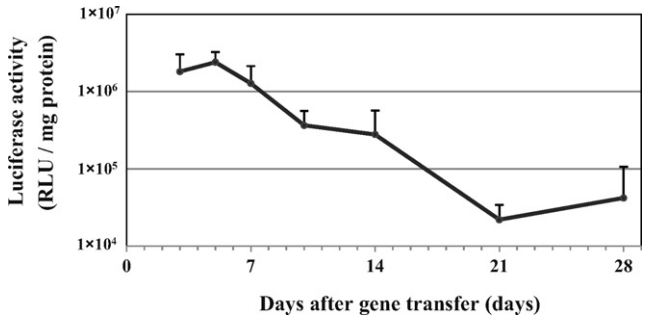

Fig. 3. Time-dependent changes in luciferase activity in tongue tissue transfected using BL and US. Relative luciferase activity was examined at 3, 5, 7, 10, 14, 21, and 28 days after the gene transfection. The transfection conditions were as follows: pDNA (pCMV-luciferase): 20 μg; BL: 10 μg; US conditions: frequency: 1 MHz, duty: 50%, intensity: 2 W/cm^2, and time: 60 s. The data are shown as the mean ± S.D.

cavitation is assumed to be the mechanism responsible for US-mediated gene delivery. When the "cavitation bubble" generated by US energy is destroyed, it produces a jet stream, which in turn produces transient pores in cell membranes, allowing extracellular plasmid DNA to enter the cytosol. In addition, it has been shown that transfection efficiency often improves in the presence of microbubbles, and the utility of their application has been demonstrated both *in vitro* and *in vivo* (Greenleaf et al., 1998; Shohet et al., 2000; Sonoda et al., 2006; Taniyama et al., 2002a,b).

Microbubbles were originally used as an ultrasonic contrast agent for clinical ultrasound diagnosis. A variety of microbubbles with different encapsulated gas types, shell materials, and diluents have been designed. The mean diameter of microbubbles that are marketed as echo-enhanced contrast agents is several micrometers. Several reports have compared the transfection efficiencies of these microbubbles during their use in combination with US (Alter et al., 2009; Hassan et al., 2009; Li et al., 2003; Wang et al., 2005).

However, the intravascular application of microbubbles is hindered by problems with their stability, targeting ability, and particle size. Thus, we developed and applied a new liposome composed of hydrophilic polyethylene glycol (PEG) as a drug delivery system. PEG-liposomes containing perfluoropropane gas are known as "Bubble liposomes (BL)". We have reported that this BL-mediated US gene transfer system enhances transfection efficiency both *in vitro* and *in vivo* (Negishi et al., 2008, 2010; Suzuki et al., 2007, 2008a,b, 2010). Negishi et al. (2011) used gene transfer methods involving BL and US to transfect genes into murine skeletal muscle and discussed their reasons for selecting skeletal muscle as a target tissue for gene therapy. They stated that as skeletal muscle cells are large and display stability and longevity, they are an attractive target tissue for gene therapy.

The generation and oscillation of "cavitation bubbles" after ultrasound exposure is influenced by the composition and pressure of the organs surrounding the transfection site. Therefore, it is important to optimize each transfection condition in the targeted tissue. The majority of tongue tissue is composed of skeletal muscle and is covered with a keratinized oral mucosa. In our histological observations, distinct EGFP expression was observed in the tongue muscle, as shown in Fig. 4. On the other hand, scattered EGFP expression was observed on the surface area of the mucoepithelial layer (data not shown). Moreover, a previous report described that repeated US exposure enhances transfection efficiency and prolonged gene expression compared with single US exposure (Bekeredjian et al., 2003). In this study, we showed that high luciferase activity was maintained for 2 weeks in murine tongue tissue treated with BL and single US exposure. Such persistent expression is thought to be suitable for therapy against tongue cancer. Whether the effect of the therapeutic gene needs to be continuous depends on the disease being targeted; for example, whether it is an infectious or genetic disease. Therefore, if the gene delivery method involving BL and US is to be applied to other oral tissues, it is very important to optimize the transfection conditions for long-term gene expression in the target tissue. Further

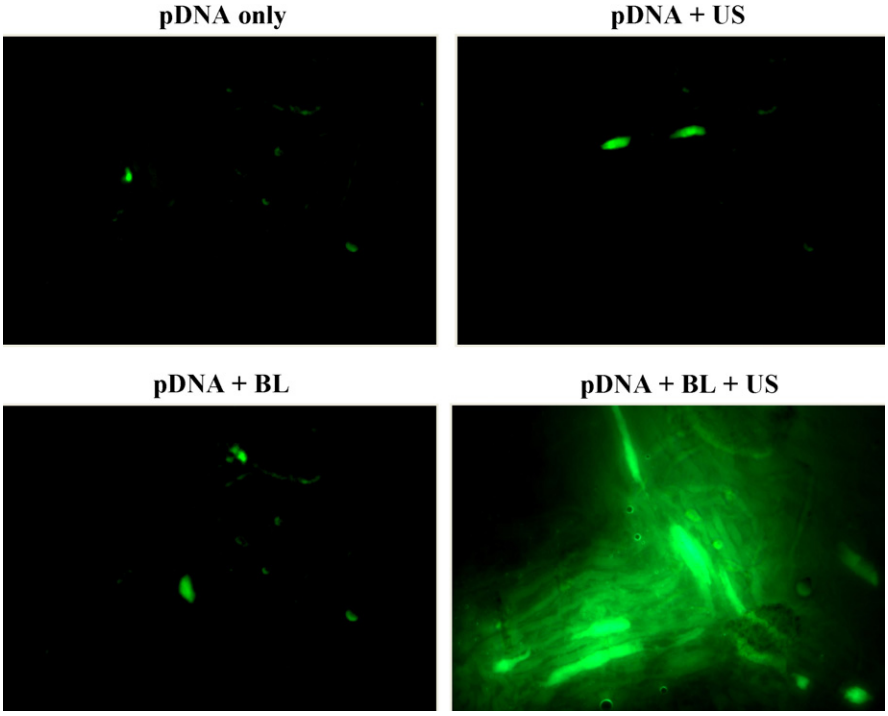

Fig. 4. EGFP expression in tongue tissue transfected with a reporter gene using BL and US. Mice were treated with BL and then subjected to US-mediated EGFP transfer into the tongue. On day 5 after the transfection, the tongue was sectioned into 10 μm thick slices using a cryostat, and EGFP expression was analyzed by fluorescent microscopy. Each of the gene transfer conditions is indicated above the pictures. Magnification: 200×. BL, Bubble liposomes; US, ultrasound.

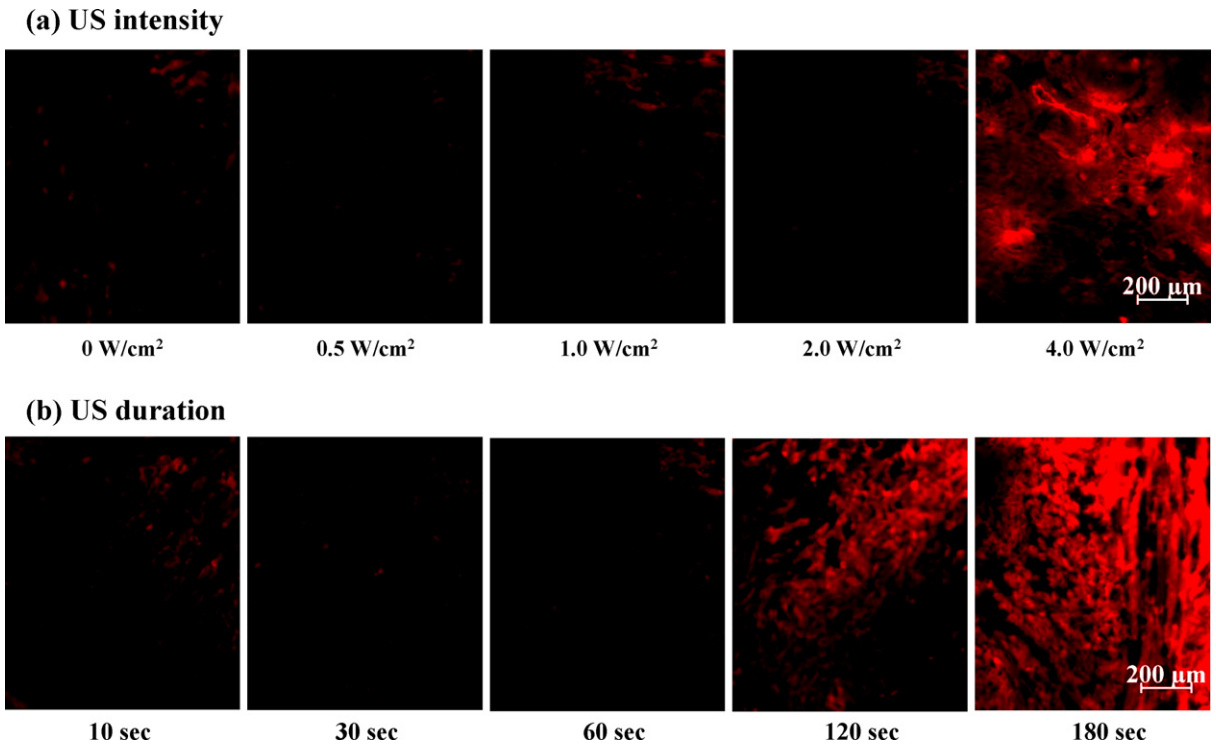

Fig. 5. Tissue-damage testing using EBD. To assess the tissue-damage by BL and US-mediated gene transfer into tongue tissue, the mice were treated pDNA with BL and various US exposure conditions. (a) At a frequency of 1 MHz with an intensity of 0, 0.5, 1.0, 2.0, or 4.0 W/cm^2 for 60 s (upper section). (b) At a frequency of 1 MHz with an intensity of 2.0 W/cm^2 for 10, 30, 60, 120, or 180 s (lower). Evans-blue fluorescence of 10 μm cryosections from the tongue was examined with fluorescence microscopy. Scale bar: 200 μm.

experiments using genes that encode therapeutic proteins are required to assess the clinical application of this US-mediated BL method.

This is the first report regarding gene transfer to tongue tissue using BL and US as a therapeutic method for diseases of the oral cavity. As mentioned above, tongue SCC is one of the most common forms of head and neck cancer; nevertheless, no standardized treatment strategy for this condition has been established (Shiga et al., 2007). Oral dysfunction and decreased quality of life (QOL) are often seen after surgical treatment in tongue SCC patients. Therefore, gene delivery systems involving BL and US exposure that enable cancer cell-specific treatment may improve the QOL of tongue SCC patients.

5. Conclusion

In conclusion, the results of this study suggest that our gene delivery method involving BL and US could be a useful treatment for patients with tongue SCC. This US-mediated BL technique is a highly promising approach for gene delivery into oral tissue.

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