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Effect of ultrasound irradiation on bacterial internalization and bacteria-mediated gene transfer to cancer cells

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ABSTRACT

The present study demonstrates that ultrasound irradiation can facilitate bacteria-mediated gene delivery (bactofection). *Escherichia coli* modified with avidin were employed as a vehicle for delivery of the green fluorescent protein (GFP) gene, a model heterologous gene, into the breast cancer cell line MCF-7. Avidin-mediated binding of *E. coli* to MCF-7 cells enhanced the internalization of *E. coli* by approximately 17%, irrespective of the use of ultrasound irradiation. Furthermore, the use of ultrasound irradiation increased the internalization by approximately 5%, irrespective of the presence of avidin on the *E. coli* cell surface. The percentages of GFP-expressing MCF-7 cells at 24 h after bactofection were below 0.5% and 2% for the case with only avidin-modification of *E. coli* cell surface and only ultrasound irradiation, respectively. However, combining avidin modification with the ultrasound treatment increased this value to 8%. Thus, the use of avidin-modified bacteria in conjunction with ultrasound irradiation has potential as an effective strategy for tumor-targeted bactofection.

1. Introduction

Gene therapy is a promising method for treatment of diseases, and it relies on the delivery of DNA as a pharmaceutical agent to the desired location within the patient's body [1]. To achieve this goal, vehicles for effective gene transfer are needed. Such delivery vehicles are generally categorized into viral (e.g., retroviruses and adenoviruses [1,2]) and nonviral (e.g., cationic liposomes [3]). Another approach is bacteria-mediated gene delivery, termed "bactofection" [4,5]. In this strategy, transformed bacterial strains are used as a vehicle to deliver plasmid-encoded genes of therapeutic proteins (protein antigens, prodrug-converting enzymes, toxins, etc.) into the mammalian cells [4–6]. The bacteria-mediated gene delivery system is superior to viral and nonviral gene delivery systems in terms of cost, the simplicity of carrier production, and the higher amount of DNA that can be delivered [5].

The process of bactofection is reported to be as follows [6]; (i) bacteria enter the mammalian cell via receptor binding and phagocytosis, (ii) plasmid DNA is released into the cytosol of the mammalian cells, and (iii) the plasmid DNA enters the nucleus, where the transgene is then expressed. Many pathogenic bacteria such as *Listeria*, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia* have been used for gene therapy after being genetically attenuated [4–6] because they are capable of penetrating into even nonphagocytic mammalian cells. However, they generally lack the selectivity for targeting a specific diseased site.

To achieve bacteria-mediated gene delivery to a target tumor, the bacteria must selectively accumulate in the tumor region and be internalized by the cancer cells. It has been reported that the certain types of anaerobic bacteria preferentially proliferate and accumulate in tumors, e.g., facultative anaerobes, such as *Salmonella*, *Escherichia coli*, and *Listeria*, and obligate anaerobes, such as *Clostridia* and *Bifidobacteria* [7,8]. Moreover, some researchers have genetically modified the surface of bacteria with cancer-targeting ligands so as to provide a binding affinity for cancer cells. This has resulted in enhanced internalization of the microbes by the cancer cells, enhancing the efficiency of the transgenes [9].

Ultrasound has been widely utilized for targeted and noninvasive medical therapeutics because of its ability to focus and penetrate tissue with less attenuation of energy [10]. In the case of gene therapy, ultrasound has been used for enhancing the entry of naked DNA into mammalian cells, termed sonoporation [11,12]. In this technique, it is considered that ultrasound perturbs the cell membrane, resulting in the formation of transient pores, and thus facilitating gene entry into the cell [11,12]. To date, the sonoporation effect of ultrasound has been used for improving the internalization of membrane-impermeable chemicals and biomacromolecules such as plasmid DNA, proteins, and polysaccharides by mammalian cells [11,12]. However, to the best of our knowledge, there has been no report on ultrasound-facilitated bacterial internalization by mammalian cells.

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The present study demonstrates the use of ultrasound irradiation to facilitate the internalization of transformed *E. coli* by cancer cells, and the subsequent expression of the green fluorescent protein (GFP) gene in the delivered plasmid DNA. Moreover, the surface of the *E. coli* was modified with avidin, which cancer cells have an affinity for, and the effect of this modification on the ultrasound-facilitated bacterial internalization and bacteria-mediated gene delivery was investigated.

2. Materials and methods

2.1. Bacteria and chemicals

E. coli BL21(DE3) and DH5α strains were purchased from Merck Millipore (Darmstadt, Germany) and Takara Bio Inc. (Otsu, Japan), respectively. A plasmid for bacterial GFP expression (pQBI T7-GFP) was purchased from qBiogene Inc. (Irvine, CA, USA), and a plasmid for mammalian GFP expression (pEGFP-C3) was obtained from Addgene (Cambridge, MA, USA). *E. coli* BL21(DE3) harboring a plasmid pQBI T7-GFP (denoted as *E. coli* BL21(DE3)/pQBI T7-GFP) was prepared as GFP-expressing bacteria to allow monitoring of the bacterial internalization by the cancer cells. *E. coli* DH5α, harboring a plasmid pEGFP-C3 (denoted as *E. coli* DH5α/pEGFP-C3), was also prepared to deliver the GFP gene into cancer cells. The *E. coli* cells were cultured in Luria Bertani (LB) medium containing 100-μg/mL ampicillin or 20-μg/mL kanamycin for *E. coli* BL21/pQBI T7-GFP and *E. coli* DH5α/pEGFP-C3, respectively.

2.2. Cell cultures

Human breast cancer cells MCF-7 were purchased from RIKEN cell bank (Tsukuba, Japan). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nakarai Tesque, Kyoto, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies Corporation, Carlsbad, CA, USA). For regular maintenance, culture medium was supplemented with 100-U/mL penicillin, 100-μg/mL streptomycin, and 0.25-μg/mL amphotericin B (Nakarai Tesque). Cells were maintained at 37 °C in a 5% CO_2 atmosphere.

2.3. Preparation of avidin-modified E. coli

The surface of the *E. coli* was modified with avidin so as to enhance its affinity with the cancer cells [13]. A single colony of *E. coli* was isolated from culture on a LB agar plate supplemented with the appropriate antibiotic (see Section 2.1), and inoculated into a test tube containing 5 mL of LB liquid medium with the same antibiotic. The test tube was incubated for 12 h at 37 °C on an orbital shaker at 140 rpm, until the optical density of the broth at a wavelength of 600 nm (OD_{600}) reached unity. GFP expression in the *E. coli* BL21/pQBI T7-GFP strain was induced by adding isopropyl-thio-β-galactoside according to the standard protocol. After incubation, the culture broth containing 2×10^8 cells of *E. coli* BL21/pQBI T7-GFP or 1×10^9 cells of *E. coli* DH5α/pEGFP-C3 was centrifuged at 3000 rpm for 10 min. Then, it was washed thrice with 1 mL of Dulbecco's phosphate buffered saline without calcium and magnesium [D-PBS(−)].

The bacterial sample was then suspended in 500 μL of 20 μg/mL biotin-conjugated rabbit polyclonal anti-*E. coli* antibody (Abcam, Cambridge, UK) in D-PBS(−) containing 0.1% bovine serum albumin (BSA), and incubated for 1 h at room temperature (RT). After washing thrice with 1 mL of D-PBS(−) containing 0.1% BSA, the bacterial sample was suspended in 1 mL of 50 μg/mL avidin solution (Nakarai Tesque) in D-PBS(−) containing 0.1% BSA, followed by incubation for 1 h at RT. After washing thrice with 1 mL of D-PBS(−) containing 0.1% BSA, the bacterial sample was suspended in 2 mL of fresh DMEM containing 10% (v/v) FBS and 100-μg/mL ampicillin, giving the avidin-modified *E. coli* samples. As a control, biotin-modified *E. coli* were prepared using the same protocol, but without the avidin step. As another control, intact *E. coli* were prepared in the same manner, but without the biotin and avidin steps.

2.4. Invasion and bactofection assays employing ultrasound irradiation

Prior to the invasion and bactofection assays, 4×10^5 MCF-7 cells suspended in 2 mL of culture medium were seeded in 35-mm culture dishes and incubated for 48 h to achieve semi-confluence (approximately 1×10^6 cells). After washing the culture dishes with D-PBS(−), 2 mL of the prepared *E. coli* cell suspension was added to the cell monolayer, where the multiplicity of infection (MOI = number of *E. coli* per MCF-7 cell) was 200 for the invasion assay using *E. coli* BL21/pQBI T7-GFP, and 1000 for the bactofection assay using *E. coli* DH5α/pEGFP-C3. After incubation for 2 h, the 35-mm culture dish was placed on the transducer (50 mm in diameter) of the ultrasonic apparatus (Sonic Master ES-2, OG Giken Co., Ltd., Okayama, Japan), with the surface of the transducer covered with 3 mL of water. Ultrasound (frequency, 1 MHz; duty ratio, 50%) was then irradiated from the bottom of the dishes for 30 s at a predefined intensity (0, 0.1, 0.3, or 0.5 W/cm^2 as the reading output of the device). The culture medium was then replaced with 2 mL of fresh medium containing 75-μg/mL gentamicin so as to inactivate the *E. coli* bacterial cells that had not invaded the MCF-7 cells [9,14]. After incubation for a further 2 h, the culture dish was washed 10 times with 1 mL of D-PBS(−) to remove the *E. coli* cells that had not invaded, but had attached to the MCF-7 cells. The resultant cell monolayer was subjected to analysis to evaluate the invasion of GFP-expressing bacteria into the cancer cells, and the subsequent GFP expression.

2.5. Analysis of bacterial invasion into cancer cells, and bactofection

Microscopy and flow cytometry were employed to evaluate the invasion of GFP-expressing bacteria into cancer cells, and the subsequent GFP expression. For microscopy, the cell monolayer was observed using a fluorescence microscope (BZ-8000, KEYENCE, Osaka, Japan). When necessary, the cytoskeleton and cellular nuclei of the MCF-7 cells were fluorescently stained. To achieve this, the cell monolayer was fixed with 500 μL of a 4% solution of paraformaldehyde in PBS for 30 min at RT. After washing twice with 1 mL of D-PBS(−), the cells were stained with 500 μL of phalloidin conjugated to Alexa Fluor 546 (6 μg/mL) and 4′,6-diamidino-2-phenylindole (DAPI, 5 μg/mL) solution in D-PBS(−) for 30 min at RT. After washing twice with 1 mL of D-PBS(−), fluorescence images for blue, green, and red channels were captured at multiple z-positions, and then merged. Three-dimensional images were reconstructed using the software (KEYENCE).

For flow cytometry, the cell monolayer was detached using a solution containing 2.5-g/L trypsin and 1-mM EDTA (Nakarai Tesque). The cells were then washed twice with D-PBS(−), and suspended in 500 μL of D-PBS(−). The fluorescence signal from the cells was detected using a flow cytometer (EPICS XL-MCL ADC, Beckman Coulter Inc., Brea, CA, USA) by counting 20,000 events.

2.6. Statistics

All data are presented as the means ± standard errors of mean. Statistical analysis was performed using unpaired *t*-tests. The difference between the groups was considered significant when the *p*-value was <0.05.