Ultrasonics 62 (2015) 97–102

Contents lists available at ScienceDirect

Ultrasonics

journal homepage: www.elsevier.com/locate/ultras

Combined effect of ultrasound/SonoVue microbubble on $CD4^+CD25^+$ regulatory T cells viability and optimized parameters for its transfection

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ARTICLE INFO

Article history:
Received 19 November 2014
Received in revised form 15 April 2015
Accepted 14 May 2015
Available online 21 May 2015

Keywords:
Ultrasound
Microbubble
$CD4^+CD25^+$ regulatory T cells
Gene therapy
Hepatocellular carcinoma

ABSTRACT

The purpose of this study was to investigate the combined effect of ultrasound and SonoVue microbubble on $CD4^+CD25^+$ regulatory T cells (Tregs) viability and to explore the appropriate parameters for Tregs transfection. Tregs were separated from peripheral venous blood of patients with hepatocellular carcinoma and seeded in 96-well plates. The optimal ultrasound exposure time and optimal SonoVue microbubble concentration for Tregs were measured by mechanical index (MI) of 1.2 or 1.4, exposure time of 0, 30, 60, 90, 120, 150, 180 s, and 0, 10, 20, 30, 40, 50 μL/100 μL microbubble per well, respectively. In addition, the combined effect of ultrasound and microbubble on Tregs viability was evaluated according to the following parameters: MI 1.2/1.4 + exposure time of 120, 150, 180 s + 0, 10, 20, 30, 40, 50 μL/100 μL microbubble per well. Tregs viability investigations were performed in order to explore the optimal transfection condition. The efficiency of plasmid transfer was determined by detection of luciferase activity on the microscopic examinations. The proliferation of Tregs could be promoted by ultrasound exposures, while being decreased with the increasing concentration of microbubbles. Under the current experimental conditions, the optimal ultrasound parameters were MI = 1.4 and exposure time = 150/180 s. The optimal microbubble concentration was 10 μL/100 μL. Compared with treatment with ultrasound or microbubbles alone, the transfection efficiency of Tregs improved 50% by combining ultrasound and microbubble. The results indicate that both ultrasound and microbubble could affect the Tregs proliferation and the optimal Treg transfection rate was obtained by treating with 10% microbubbles and ultrasound exposure for 150/180 s under ultrasound MI of 1.4.

1. Introduction

Since 1996 [1], the combined use of ultrasound and microbubble contrast agents has been proposed as a method of non-viral vectors for drug delivery in these years [2–4]. Compared with the virus, gene transfection reagents and chemical drug delivery system, ultrasound-targeted microbubble destruction (UTMD) has numerous advantages, such as being safe, effective, noninvasive, target-specific and relatively low cost [4–9]. Progresses have been obtained in the application of ultrasound targeted microbubble destruction for drug delivery to solid tumors [10], such as hepatocellular carcinoma (HCC) [11,12]. It is reported that ultrasound-targeted herpes simplex virus thymidine kinase (*HSVtk*) and tissue inhibitor of metalloproteinase 3 (*Timp3*) genes delivery enhanced antitumor effects in hepatoma [13]. The *HSVtk* and *Timp3* genes were trapped into vectors, which were protected from degradation in the circulation by electrostatically bounding to microbubbles. With plasmids releasing by UTMD, *HSVtk* and *Timp3* genes could be expressed in human HCC HepG2 cells. However, the optimal concentration was not discussed in previous studies. Therefore, it is vital to determine the appropriate concentration or dosage for the better use of UTMD.

On the other hand, it is important to choose a suitable target for antitumor treatment using UTMD. $CD4^+CD25^+$ T-regulatory cells (Tregs) play an important role in antitumor immune responses [14]. Treg mediates immune tolerance to self-antigens by suppressing auto reactive immune cells [15]. It may also suppress the immune response against cancer [16,17]. In addition, the excessive presence of Tregs can explain the poor clinical efficacy of immunotherapeutic effects in human tumors [18]. Therefore, targeting Tregs provides an attractive therapeutic strategy to support anti-tumor therapy [19].

Forkhead box P3 (Foxp3) as a member of the transcription factor family controls the development and function of Tregs [20]. siRNA is one of the good options to knock down the expression of target genes [21]. We speculated that using short interfering

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http://dx.doi.org/10.1016/j.ultras.2015.05.006
0041-624X/

RNA (siRNA) technology to silence Foxp3 of Tregs may have a positive effect on HCC treatment. In the present work, we evaluated the optimal condition for Foxp3 siRNA transfection in Tregs by using ultrasound combined with microbubbles. We expected that our findings could provide an objective basis for future gene therapy in HCC treatment by using microbubble ultrasound-mediated Foxp3siRNA delivery.

2. Materials and methods

2.1. Patients

Patients with pathology and cytology confirmed HCC were recruited from Harbin Medical University First Affiliated Hospital (Harbin, China) between October 2013 and March 2014. Patients who were newly diagnosed with HCC and did not receive any treatment were eligible. Other inclusion criterion included patients without other lesions and presented increased serum alpha-fetoprotein (AFP) and transaminase level. Approval was obtained from the ethics committee at our institution and all patients provided written informed consent before study.

2.2. Cells isolation and culture

Peripheral venous blood (40–60 mL) was collected from the eligible patients with HCC and the Tregs were separated from the peripheral blood mononuclear cells (PBMC) by density gradient centrifugation. Then, Tregs were purified by a magnetic cell sorting $CD4^+CD25^+$ Tregs isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Tregs with cell purity > 90% were maintained in RPMI1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) at 37 °C with 5% CO_2.

2.3. Exploration of optimal ultrasound exposure time

In order to analyze the impact of different ultrasound intensity and exposure time on cell proliferation, the isolated Tregs (5.0×10^5/well) were incubated in 96-well plates at 37 °C in 5% CO_2. Then, Tregs were subjected into ultrasound treatment (mechanical index (MI) = 1.2 and MI = 1.4) for different exposure time (0, 30, 60, 90, 120, 150 and 180 s, respectively), using Philips S5-1 transducer (Philips iU22, Philips Medical Systems, Bothell, WA) at the centre frequency of 2.5 MHz (10 μs pulse duration, 100-Hz pulse repetition frequency). The centre 6 wells of the 96-well plate were filled with cell suspension and same volume of PBS was added in the surrounding wells. The ultrasound probe with a few ultrasonic coupling agents was positioned under the centre 6 cell wells at a distance of 3–5 mm. The probe area was corresponding to the area of 6 cell wells, so the cells could reviewed the ultrasound evenly. After scanner machine was warmed up for 30 min, ultrasound was applied and each condition was run in 6 wells with three repeats. Then, the experimental Tregs were maintained at 37 °C in 5% CO_2 for 24 h. Cells without any treatment were considered as controls.

2.4. Exploration of optimal microbubble concentration

In order to evaluate the effect of different concentrations of microbubble on cell viability, freshly isolated Tregs (5.0×10^5-/well) were first incubated in 96-well plates at 37 °C in 5% CO_2. Then, the cells were assigned into 6 groups and each group was added with various concentrations of microbubble (such as 0, 10, 20, 30, 40 and 50 μL/100 μL). SonoVue microbubbles of 2.5–6.0 μm in diameter (Bracco Co. Geneva, Switzerland), were dissolved in 5 mL normal saline (NS), and suspended by inversion or oscillation before used. The density was kept within 2–5×10^8/mL, and the concentration was set as 5 mg/mL. The same experiment was repeated for 3 times. Then, experimental Tregs were cultured at 37 °C in 5% CO_2 for 24 h.

2.5. Detection of the optimal condition for ultrasound with microbubble

In order to explore the optimal effect of ultrasound with microbubble, Tregs were assigned into 36 groups that treated with ultrasound at different exposure time (MI 1.2/1.4; 120, 150, 180 s) combined with microbubbles at different concentrations (0, 10, 20, 30, 40, 50 μL/100 μL per well), respectively (Supplement table). Then, experimental Tregs were cultured at 37 °C in 5% CO_2 for 24 h.

After 24 h of culture under each condition, total 10 μL of cell counting kit-8 (CCK-8) solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well and incubated at 37 °C for 4 h according to the work instruction. Then, the absorbance of each well was measured at 460 nm with Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The cell proliferation rate was calculated with the formula: (OD of experimental group/OD of control) × 100%. The effect of microbubble on cell viability could be represented as the inhibition ratio = (OD of control − OD of the experimental group)/(OD of control) × 100%.

2.6. Plasmid-microbubble mixture preparation

The FOXP3 siRNA fragments were synthesized by Invitrogen (Shanghai, China) and the detailed sequences were listed in Table 1. The plasmid containing Foxp3 siRNA was constructed and amplified in *E. coli* DH5α. Then plasmids were collected and purified by using the plasmid extraction kit (Invitrogen Co., LTD, Shanghai, China) according to the manufactor's instruction. Plasmid concentration (0.5 mg/mL) and purity (the ratio of optical densities at 260 nm and 280 nm ⩾ 1.8) were determined using spectrophotometry. Then, the solution (1:1, v/v) of Foxp3 siRNA plasmid with SonoVue microbubbles was prepared and kept at 4 °C for 2–3 h.

2.7. Transfection and viability measurement

Before transfection, freshly isolated Tregs were suspended in serum-free RPMI1640 for 24 h and seeded in 96-well plates at a cell density of 2×10^4/well. The radiation frequency of ultrasound was set as 2.5 MHz and MI was 1.4. The cells were divided into 4 groups, including group A: treated with empty vector; group B: added with 20 μL plasmid/SonoVue microbubble suspension + 1 μL lipofectamin 2000; group C: treated with 20 μL plasmid/SonoVue microbubble suspension + 1 μL lipofectamin 2000 and exposed to ultrasound for 150 s; group D: treated with 20 μL plasmid/SonoVue microbubble suspension + 1 μL lipofectamin 2000 and exposed to ultrasound for180 s. Then, experimental cells were incubated at 37 °C for 24 h in 5% CO_2.

Since Foxp3 siRNA plasmid expressed green fluorescent protein (GFP), GFP-positive cells were counted under Fluorescence microscope. Transfection efficiency was calculated as follows: fluorescence expression rate = GFP-positive cell/all cells in one field. Transfection efficiency was detected in 5 well of each group, and the same experiment was repeated at least 3 times. Tregs were collected and suspended in 1 mL of phosphate-buffered saline (PBS) buffer for flow cytometry (FCM, Beckman Coulter, Fullerton, CA, USA) 24 h after transfection. The presence of apoptotic cells in each condition was measured by flow cytometric (FCM) analysis.