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Non-invasive Assessment for Endothelial CD81 Expression via Targeted Microbubbles

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Abstract—The aim of our study was to investigate the use of targeted ultrasound microbubbles (MBs) for molecular imaging of murine endothelial CD81 expression. In the study, the anti-CD81-coated MBs was successfully prepared and characterizated. Murine bEnd.3 cells were stimulated with phenazine methosulfate (PMS) to induce the up-regulation of CD81 expression. Changes in CD81 expression after stimulation were tracked with anti-CD81-coated MBs and imaged by using SONIX RP ultrasound imaging system. Our results showed that endothelial CD81 expression was gradually up-regulated with the increase of PMS concentration. Correspondingly, the accumulation of targeted MBs was also gradually improved and could be inhibited competitively. The mean video intensity of stimulated cells from backscatter of the CD81-targeted MBs was significantly higher than that of the non-stimulated control (mean ± SD: 17.5 ± 3.6 versus 12.1 ± 2.9 pixel intensity; $P < 0.01$). In conclusion, CD81-targeted MBs allows non-invasive assessment of the expression levels of CD81 on the bEnd.3 cells and may provide potential insights into early atherosclerotic plaque detection and treatment monitoring using molecular ultrasound imaging.

Ⅰ. INTRODUCTION

CLUSTER designation 81 (CD81) is a member of the tetraspanin superfamily of cell-surface proteins, which tend to associate with integrins, with other tetraspanins, and with lineage-specific molecules in the immune system and participate in diverse biological activities [1]. Recently, through genetic screens, the tetraspannin CD81 was demonstrated to be a marker of early human atherosclerotic plaques [2]. In this context, the ability to visualize noninvasively and quantify the regulation of the marker molecules would be extremely valuable in preclinical research. Various modalities, such as ultrasound, gamma radiation detector, magnetic resonance, optical light for target-specific imaging have been applied successfully to detection of marker proteins [3-7]. Among these modalities, ultrasound possesses particular attraction for screening large patient groups for a potential disease due to its features, including high sensitivity, availability, rapid execution of imaging protocols, and the relatively low cost. So far, molecular imaging via ultrasound contrast MBs has been applied to characterize arteriosclerosis [8], thrombosis [9], neovasculature [10, 11] lymph nodes [12] as well as inflammations [13, 14]. Moreover, ultrasound molecular imaging has also proven to be highly sensitive for the identification of molecular structures or expression when using targeted contrast agents [15-19], which provides helpful insights into genesis, progress, and prevention of diseases. The purpose of the present study was to investigate the use of targeted contrast agents for molecular imaging of murine endothelial CD81 expression in vitro.

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Ⅱ. EXPERIMENTAL METHOD

A. Preparation and Characterization of targeted MBs

Lipid ultrasound MBs were prepared using mechanical vibration. Anti-CD81 antibodies were coated to the surface of MBs through a biotin-avidin bridge (Fig1A). To confirm the successful conjugation of anti-CD81 antibodies to the surface of MBs, FITC-labeled biotinylated anti-CD81 antibodies were replaced for biotinylated anti-CD81 antibodies. Particle size, size distribution and concentration of MBs were analyzed on an optical particle counter with a 0.5 mm diameter lower detection limit (Accusizer 780; Particle Sizing Systems, Santa Barbara, CA, USA). For each sample, l00 µL of MB suspension was analyzed and repeated three times. One drop of FITC-labeled targeted MB suspension was applied to the microscope slide. A cover slip was used to cover the sample before investigating the sample under × 400 amplification. Morphologic characteristics of MBs were determined under a fluorescent microscope (Olympus, Tokyo, Japan).

B. Examination of targeted MBs binding to bEnd.3 cells

The bEnd.3 cells were seeded in 6-well plates overnight to allow cell adhesion. A given amount of PMS was added into the media and further incubated for 16 h. Static binding of targeted MBs was performed. In brief, a dispersion of 1×10^8 particles /mL targeted MBs or non-targeted MBs were

incubated with the PMS-induced bEnd.3 cells or non-induced cells for 5 min, and free MBs that did not attach to the cells were removed by a PBS rinse. Then, the number of attached MBs was determined using an optical microscope at six random fields of view (Olympus, Tokyo, Japan). As for competitive experiments, 1 μg/mL of anti-CD81 antibodies was used to incubate with the induced cells for 30 min, followed by addition of targeted MBs.

C. In vitro Imaging of Endothelial CD81 Expression

bEnd.3 cells were seeded onto glass cover slips and cultured within a 6 well microplate overnight. 10 μM PMS was added into the media and incubated for 16 h to induce expression of CD81 proteins. 1×10^8 targeted MBs were used to adhesion with PMS-induced cells and non-induced cells. After removing the free MBs with PBS, the cover glass slips attached with cells and MBs were taken out and insert into a 3% agar phantom side by side. The phantom with the cover slips was placed into a water tank. Furthermore, the ultrasound transducer was mechanically positioned at a distance of 2 cm from tissue phantom in the longitudinal direction. For preventing the specular reflection from slips, the position of the slips needed at an angle different from perpendicular to the transducer axis [20]. Adhered MBs were detected by SONIX RP ultrasound imaging system (Ultrasonix, Vancouve, Canada). The B-mode images were performed in harmonics mode at 5.0 MHz transmission center frequency. The quantification and difference in mean video intensity between stimulated cells and non-stimulated cells was calculated using MATLAB (version R2010b, The Mathworks Inc., Natick, MA, USA) and expressed in a box plot.

III. RESULTS

The targeted MBs were successfully prepared and confirmed by fluorescent MBs coated with FITC-labeled anti-CD81 antibodies (Fig.1B). The particle size distribution of targeted MBs showed the mean size of the targeted MBs was 2.61 ± 0.81 μm, with a slightly larger size than that of the non-targeted MBs (Fig.1C). With the increase of PMS concentration, the accumulation of targeted MBs was gradually improved and could be inhibited competitively (Fig. 2). The mean video intensity caused by backscatter of stimulated cells or vessels was significantly higher than that of the non-stimulated cells or vessels (Fig. 3) (mean ± SD: 17.5 ± 3.6 versus 12.1 ± 2.9 pixel intensity; $P < 0.01$).

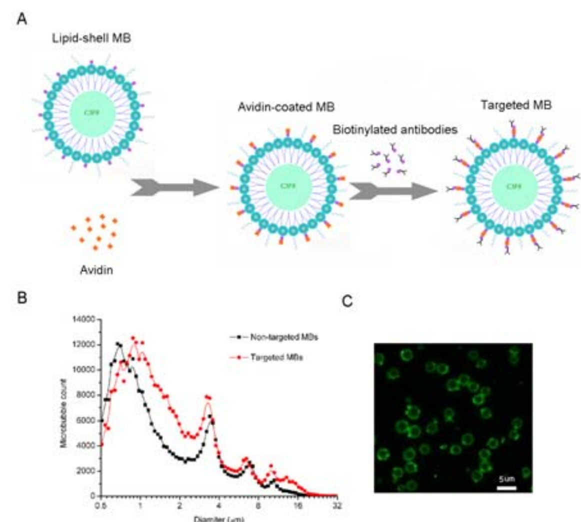

Fig.1 Preparation and characterization of targeted MBs. (A) Schematic diagram of an anti-CD81-coated MB constructed for molecular imaging. (B) Size distribution of the targeted MBs and non-targeted MBs (with 3 replicates). (C) Fluorescent micrograph of FITC-labeled targeted MBs (bar = 5 μm).

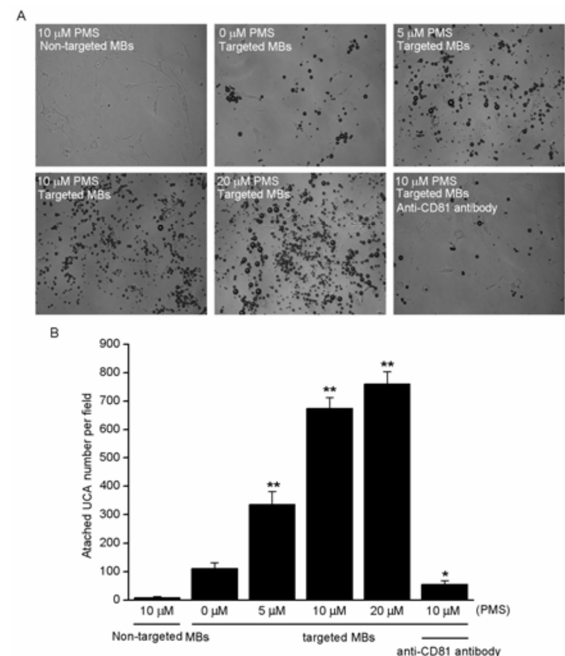

Fig.2: Targeted MBs binding to cultured bEnd.3 cells. (A) Representative micrograph for targeted MBs or non-targeted MBs adhered to cells induced with 0, 5, 10 or 20 μM PMS. (200×). As for competitive experiments, 1 μg/mL of anti-CD81 antibodies was used to incubate with the induced cells for 30 min, followed by addition of targeted MBs. (B) Quantitative assay of the number of MBs adhered onto bEnd.3 cells from six at random view field. * $P < 0.05$ and ** $P < 0.01$ vs non-stimulation control. ($n = 6$).

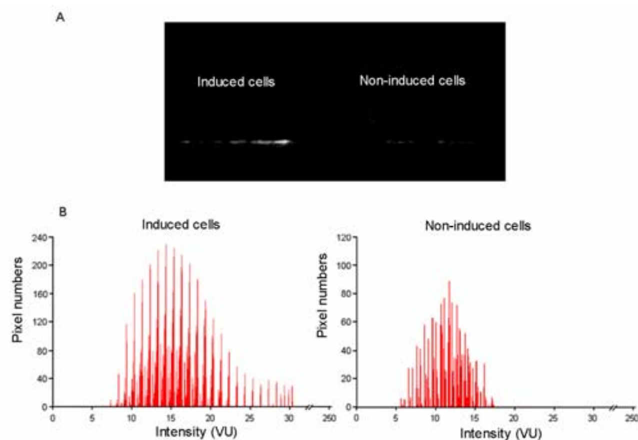

Fig.3: Ultrasound molecular imaging of CD81 expression in vitro. (A) B-mode ultrasound imaging to detect the CD81 expression via CD81-targeted MBs adhered to the induced (left) and non-induced (right) cells which were cultivated on 20 mm glass cover slides inserted into the phantom. Imaging performed with an SONIX RP ultrasound imaging system. (B) Video intensity histograms for the induced (left) and non-induced (right) bEnd.3 cells.

Ⅳ. CONCLUSION

This study reported our initial experience with molecular high-resolution ultrasonography using anti-CD81-coated targeted MBs and showed that it may enable in vitro molecular imaging of CD81 expression on bEnd.3 cells. In the present work, the anti-CD81-coated MBs were successfully prepared and the potential of targeted MBs to detect the increased expression of CD81 proteins induced by PMS stimulation in vitro was demonstrated. It cannot be assumed that the in vitro models we used to test the feasibility of CD81-targeted MBs are identical to disease-related CD81 expression in vivo. In the in vivo setting, more complex factors such as disease-related inducers, individual differences and intricate signal pathway are often involved into the regulation of CD81 expression. Although the cell culture conditions may not be directly comparable to what may be anticipated in vivo, our results indicated that the intensity of the molecular ultrasound signal from the bound CD81-targeted MBs correlates with relative expression of CD81 proteins. These findings are especially valuable since this imaging modality may provide reference values of relative expression of CD81 and information likely to be very useful for detection, prognosis, vulnerable potential of atherosclerosis, or susceptibility to antiatherosclerosis drugs.

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