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● *Original Contribution*

DELIVERY OF MOLECULES TO THE LYMPH NODE VIA LYMPHATIC VESSELS USING ULTRASOUND AND NANO/MICROBUBBLES

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Abstract—Lymph node (LN) dissection is the primary option for head and neck cancer when imaging modalities and biopsy confirm metastasis to the sentinel LN. However, there are no effective alternative treatments to dissection for LN metastasis. Here, we describe a novel drug delivery system combining nano/microbubbles (NMBs) with ultrasound (US) that exhibits considerable potential for the delivery of exogenous molecules into LNs through the lymphatic vessels. A solution containing fluorophores (as a model of a therapeutic molecule) and NMBs was injected into the subiliac LNs of MXH10/Mo-*lpr*/*lpr* mice, which develop systemic swelling of LNs (up to 13 mm in diameter, similar to human LNs). It was found that the NMBs were delivered to the entire area of the proper axillary LN (proper-ALN) via the lymphatic channels and that these were retained there for more than 8 min. Furthermore, exposure to US in the presence of NMBs enhanced the delivery of fluorophores into the lymphocytes near the lymphatic channels, compared with exposure to US in the absence of NMBs. It is proposed that a system using US and NMBs to deliver therapeutic drugs via lymphatic vessels can serve as a new treatment method for LN metastasis. (E-mail: Kodama@bme.tohoku.ac.jp) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Nano/microbubbles, Cavitation, Lymph node metastasis, Drug delivery system.**

INTRODUCTION

The sentinel lymph node (LN) is defined as the first LN or group of LNs to which tumor cells are likely to spread from the primary lesion, and its status is regarded as an independent prognostic factor (Hirakawa et al. 2009; Tobler and Detmar 2006). Metastasis to the sentinel LN is typically diagnosed with imaging modalities such as ultrasound (US), computed tomography (CT), magnetic resonance imaging and positron emission tomography, and the metastasis is histologically confirmed by biopsy (Black et al. 2007; Shigekawa et al. 2012; Yamamoto et al. 2010). In cases of head and neck cancer, when the sentinel LN is positive, neck dissection is performed to improve the survival rate (Liauw et al. 2006). However, because neck dissection can induce severe side effects, such as scar fibrosis and nerve injuries, alternative minimally invasive treatment modalities are desirable for LN metastasis. In a previous study, Curry et al. (2007) reported that contrast-enhanced ultrasound-guided sentinel lymph node detection of the head and neck in a porcine model was feasible. One of the reasons why innovative treatment methods for LN metastasis have not been fully developed is the lack of appropriate animal models of LN metastasis. In our previous work (Li et al. 2013a; Shao et al. 2013), we developed a model of LN metastasis using MXH10/Mo-*lpr*/*lpr* (MXH10/Mo/lpr) mice, in which LNs are similar in size to those found in humans. Using this mouse model, metastasis to the proper axillary LN (proper-ALN) through the lymphatic vessels (LVs) can be induced by injecting tumor cells into the subiliac LN (SiLN) (Shao et al. 2013).

The lymphatic system has a crucial role in antigen collection and the responses of the immune system, and for most solid tumors, the tumor cells eventually migrate into the lymphatics that surround the primary tumor. Therefore, the lymphatic system is regarded as an

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important delivery route for the development of vaccines and the delivery of diagnostic reagents and therapeutic molecules. Subcutaneous, interstitial and pulmonary routes to the lymphatic system have been identified, thereby improving the penetration and retention of a drug and reducing local and systemic cytotoxicity (Cai et al. 2011). However, these systems cannot deliver target molecules into a specific LN with metastasis. Therefore, in the present study we have used MXH10/Mo/lpr mice to develop a novel lymphatic delivery method that uses nano/microbubbles (NMBs) and US to deliver an agent to a specific LN through the LVs. We injected a solution containing fluorophores and NMBs into the SiLN of MXH10/Mo/lpr mice, which resulted in delivery of the solution to the proper-ALN via the LVs. When arrival of the solution to the proper-ALN had been confirmed using a contrast-enhanced high-frequency US (CE-HFUS) imaging system, the proper-ALN was then exposed to destructive US, which resulted in the uptake of fluorophores into lymphocytes. We propose that the present method could potentially be used for the clinical treatment of LN metastasis.

METHODS

In vivo studies were approved by the Institutional Animal Care and Use Committee of Tohoku University, Sendai, Miyagi, Japan (Permit Number: 2010 BeLMO-76-20-255, 2009 BeA-6, 2010 BeA-7). All surgery was performed under isoflurane anesthesia, and great efforts were made to minimize animal suffering.

Mice

Twenty-four MXH10/Mo/lpr mice (16–21 weeks of age) (Shao et al. 2013) were used in the present experiments. The MXH10/Mo/lpr mouse is a substrain of the MXH/lpr recombinant inbred strain of mouse (Tanaka et al. 2010). MXH10/Mo/lpr mice are unique in that most of the peripheral LNs are as large as 10 mm in diameter at 2.5–3 months of age, and both the size and the onset time of LN swelling are stable and predictable (Fig. 1a). The MXH10/Mo/lpr mice were bred under specific pathogen-free conditions in the Animal Research Institute, Graduate School of Medicine, Tohoku University, Sendai, Miyagi, Japan.

Preparation of NMBs

Nano/microbubbles were composed of 1,2-distearoyl-*sn*-glycero-phosphatidylcholine (DSPC; NOF, Tokyo, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-methoxy-polyethylene glycol (DSPE-PEG2000-OMe; NOF) at a ratio of 94:6 mol/mol, containing C_3F_8 gas (lipid concentration, 1 mg/mL). Liposomes were prepared by the reverse-phase evaporation method. NMBs were created from the liposomes by sonication in the presence of C_3F_8 gas for 1 min with a 20-kHz stick sonicator (130 W, Vibra Cell, Sonics & Materials, Danbury, CT, USA). The mean diameter of the NMBs was 199 ± 84.4 nm; <0.01% had diameters exceeding a few micrometers. The size distribution of NMBs was assessed by dynamic light scattering (particle analyzer, ELSZ-2, Otsuka Electronics, Osaka, Japan). Approximately 20% of the NMBs contained both liquid and gas, whereas approximately 80% contained liquid alone (Kodama et al. 2010b).

Detection of LVs and proper-ALN using the CE-HFUS system and pathologic analysis

Seven male mice (19–21 wk) were used for detection of the LVs and proper-ALN, six for ultrasound imaging and one for detection using Indian ink. A high-frequency US (HFUS) imaging system (VEVO 770, VisualSonics, Toronto, ON, Canada) was used to detect the LVs entered by the NMBs (Li et al. 2013a, 2013b). HFUS B-mode images were acquired at a room temperature between 22°C and 26°C. The scanner was equipped with a mechanical single-element transducer (RMV-708, VisualSonics; central frequency: 55 MHz, axial resolution: 30 μm, lateral resolution: 70 μm, focal length: 4.5 mm, depth of field: 1.4 mm). The transducer was fixed to a 3-D stage control system (Mark-204-MS, Sigma Koki, Tokyo, Japan). Mice were anesthetized with 5% isoflurane (Abbott Japan, Tokyo, Japan) and placed on a stage maintained at 38°C (TM150, VisualSonics). To maintain US transmission, US gel was placed on the flank of the mouse (Fig. 1b). The RMV-708 transducer was placed on the animal's flank after detection of the superficial epigastric vein that runs adjacent to the LVs connecting the SiLN to the proper-ALN (n = 1). A 200-μL volume of NMBs was injected into the SiLN from a 1-mL syringe (Terumo, Tokyo, Japan), through a butterfly needle (27 G, Terumo), at the rate of 50 μL/min.

For experiments in which US was applied directly to the proper-ALN, the scanner was equipped with an alternative transducer (RMV-704, VisualSonics; central frequency: 40 MHz, axial resolution: 40 μm, lateral resolution: 80 μm, focal length: 6.0 mm, depth of field: 1.5 mm) to capture the entire picture of a lymph node; this was fixed onto the proper-ALN under anesthesia, using US gel. Reference B-mode images (300 consecutive frames) were acquired before injection of the NMBs. A 200-μL volume of NMBs was injected into the SiLN at the rate of 50 μL/min. Immediately after the injection of NMBs into the SiLN, 300 consecutive frames were acquired (n = 3). The B-mode images were acquired for 8 min. To facilitate a comparison between intralymphatic and intravenous injection, B-mode images were acquired in a similar manner after the intravenous injection of 200