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Localization and proliferation of lymphatic vessels in the tympanic membrane in normal state and regeneration

Takenori Miyashita [a,*], James L. Burford [b], Young-Kwon Hong [c], Haykanush Gevorgyan [b], Lisa Lam [b], Nozomu Mori [a], Janos Peti-Peterdi [b]

[a] Department of Otolaryngology, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan
[b] Department of Physiology and Biophysics and Department of Medicine, Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA
[c] Department of Surgery and Department of Biochemistry and Molecular Biology, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA

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ABSTRACT

We clarified the localization of lymphatic vessels in the tympanic membrane and proliferation of lymphatic vessels during regeneration after perforation of the tympanic membrane by using whole-mount imaging of the tympanic membrane of Prox1 GFP mice. In the pars tensa, lymphatic vessel loops surrounded the malleus handle and annulus tympanicus. Apart from these locations, lymphatic vessel loops were not observed in the pars tensa in the normal tympanic membrane. Lymphatic vessel loops surrounding the malleus handle were connected to the lymphatic vessel loops in the pars flaccida and around the tensor tympani muscle. Many lymphatic vessel loops were detected in the pars flaccida. After perforation of the tympanic membrane, abundant lymphatic regeneration was observed in the pars tensa, and these regenerated lymphatic vessels extended from the lymphatic vessels surrounding the malleus at day 7. These results suggest that site-specific lymphatic vessels play an important role in the tympanic membrane.

1. Introduction

Lymphatic vessels are an important part of the immune system, and the presence of abundant lymphatic vessels in the auricle and external auditory canal has been reported [1]. The auricle and external auditory canal are covered by skin, which contains abundant lymphatic vessel networks [1]. Lymphatic vessels drain interstitial fluid and thereby guide interstitial flow, which has been identified as an important organizing factor in lymphangiogenesis [2], and lymphatic circulation is important for wound healing and prevention of edema and infection [3]. Although lymphatic vessels have been suggested as an important organizing factor in pathological conditions of the tympanic membrane, localization in the tympanic membrane has not been established.

The tympanic membrane is composed of 2 parts: the pars flaccida and the pars tensa. The handle of the malleus is located at the center of the pars tensa and is connected to the tympanic ring via the lateral malleolar ligaments [4]. All of these structures are important for transmitting sound from the air to the ossicles, and dysfunction of the tympanic membrane, commonly due to perforation, causes conductive hearing loss. In this study, we focused on localization of the lymphatic vessels in the tympanic membrane, and the regenerative response after perforation of the tympanic membrane, by using whole-mount imaging of the tympanic membrane of Prox1 GFP mice.

2. Materials and methods

2.1. Prox1-GFP BAC transgenic mouse

Prox1-GFP BAC transgenic mice (Tg[Prox1-EGFP]221Gsat/Mmcd, cryoarchived) were purchased from the Mutant Mouse Regional Resource Centers. The founder mouse (FVB/N) is mated to Crl:CD1 (ICR) mice before cryopreservation, and recovered litters have an outbred background (FVB/N-Crl:CD1(ICR)), as described previously [1]. Overall morphology of the Prox1-GFP BAC transgenic mice appeared normal, and lymphatic vessels could be specifically identified by strong GFP signals.

2.2. Whole-mount imaging of the tympanic membrane

All experiments were performed using protocols approved by the Institutional Animal Care and Use Committee of the University

* Corresponding author. Address: Department of Otolaryngology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. Fax: +81 878 91 2215.
E-mail address: takenori@med.kagawa-u.ac.jp (T. Miyashita).

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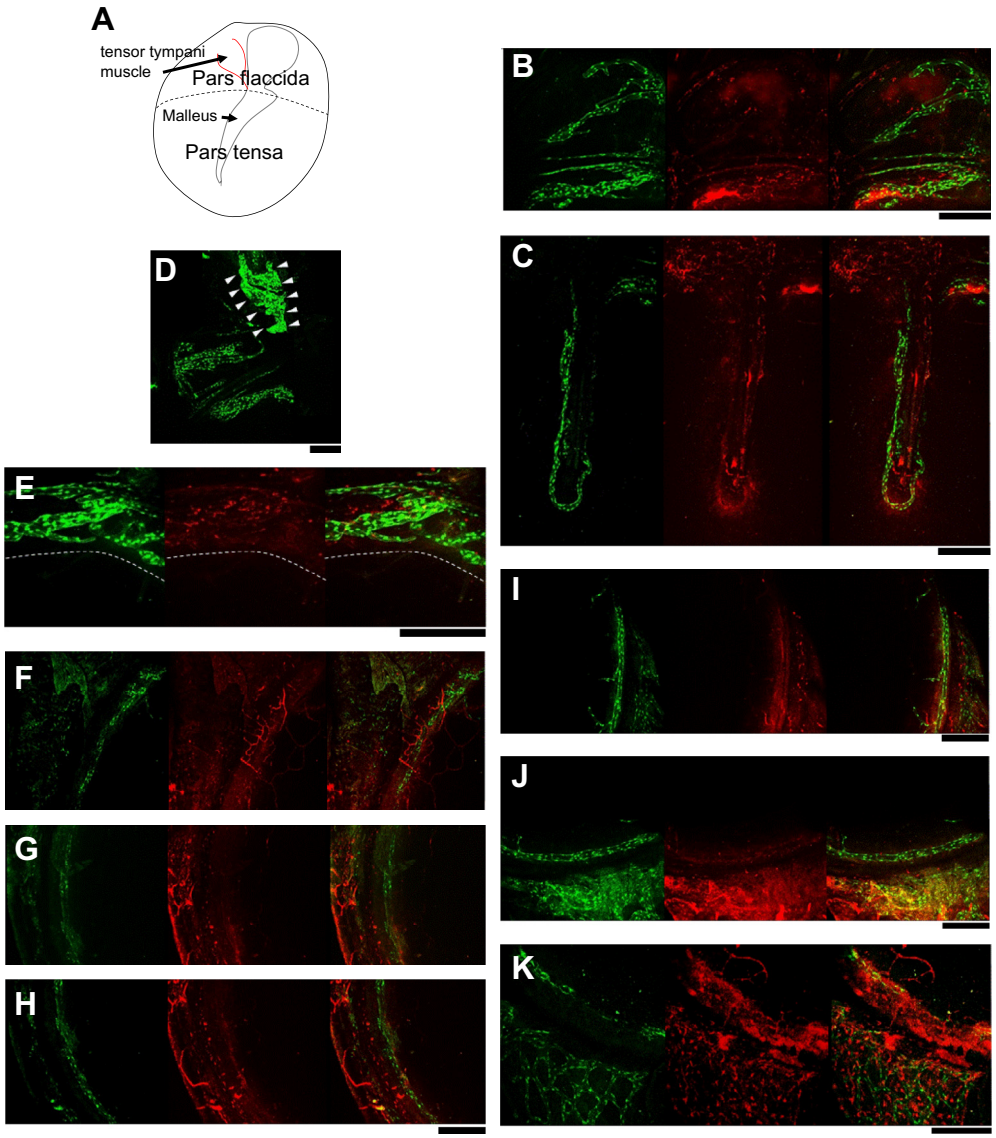

Fig. 1. Localization of the lymphatic vessels in the normal tympanic membrane. Whole-mount projection images of lymphatic vessels (EGFP: green), blood vessels (70 kD dextran-rhodamine: red), and merged images of lymphatic vessels and blood vessels in the tympanic membrane. (B–J) shows whole-mount projection images of the tympanic membrane from the mucosal side, and (K) shows whole-mount projection images of the tympanic membrane from the epithelial side. (A) Schematic structure of the rat tympanic membrane. (B) The pars flaccida of the tympanic membrane. (C) The malleus handle with the pars tensa of the tympanic membrane. (D) Montage image of the tensor tympani muscle and the pars flaccida of the tympanic membrane. Arrowheads shows the lymphatic vessel loops of the tensor tympani muscle. (E) Border (broken line) between the pars flaccida and pars tensa of the tympanic membrane. (F) Anterosuperior area, (G) anterocentral area, (H) anteroinferior area, (I) posterior area, (J) inferior area of the pars tensa of the tympanic membrane and (K) external auditory canal epithelial with annulus tympanicus. Abundant lymphatic loops were observed in the pars flaccida and surrounding the malleus handle, tensor tympani muscle, and annulus tympanicus. Scale bar = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of Southern California (USC). Prox1-GFP BAC transgenic mice (weight, 10–20 g; both sexes) were deeply anesthetized using ketamine (80–100 mg/kg) and xylazine (10 mg/kg) and perfused via the left ventricle with a fixative solution (4% paraformaldehyde in phosphate-buffered saline [PBS]) for approximately 5 min. The temporal bones were removed and the tympanic membrane on both sides, including the surrounding bone tissues, was dissected carefully under a stereomicroscope. The brain and cochlea were also collected. The samples were fixed in 4% paraformaldehyde in PBS for 6 h at 4 °C. The tympanic membrane and the cochlea were then decalcified in 0.12 M ethylene-diaminetetraacetic acid (EDTA; pH 6.5) at 4 °C for 7 days. The tympanic membrane and the cochlea were examined using a 2-photon laser scanning fluorescence microscope (TCS SP5 AOBS MP confocal microscope system; Leica-Microsystems). Images were collected as a z-series file and analyzed with Leica LCS imaging software [5].

3. Results

3.1. Lymphatic vessels in the normal tympanic membrane

Many lymphatic vessel loops were detected in the pars flaccida, whereas no lymphatic vessel loops were observed in the greater part of the pars tensa in the normal tympanic membrane (Fig. 1). In the pars tensa, lymphatic vessel loops were located around the malleus handle and annulus tympanicus (Fig. 1C, F–K). Lymphatic vessel loops surrounding the malleus handle were connected to the

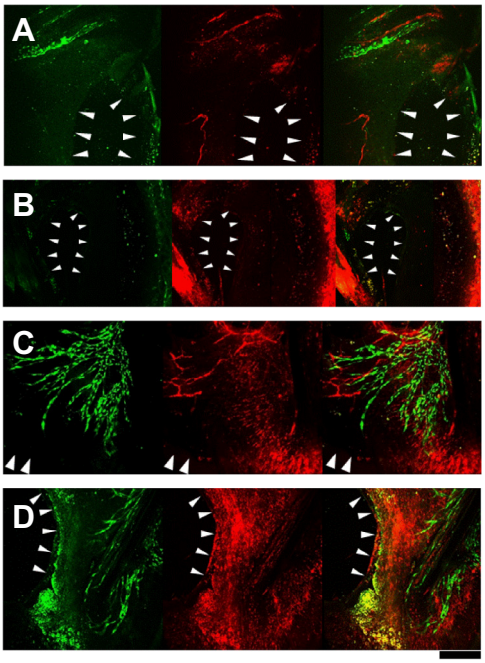

Fig. 2. Lymphatic regeneration of the healing tympanic membrane. Whole-mount projection images of lymphatic vessels (EGFP: green), blood vessels (70 kD dextran-rhodamine: red), and merged images of the tympanic membrane. (A) and (B) show 1-day and 4-day time points. (C) and (D) show the pars tensa of the healing tympanic membrane at 7 days after perforation. Arrowheads indicate perforation area of the tympanic membrane. Scale bar = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lymphatic vessel loops in the pars flaccida (Fig. 1C). Lymphatic vessel loops in the pars flaccida were connected to the lymphatic vessel loops surrounding the tensor tympani muscle or annulus tympanicus (Fig. 1D).

3.2. Lymphatic regeneration in the healing tympanic membrane

To observe lymphatic regeneration in the healing tympanic membrane, we used an animal model of acute tympanic membrane perforation. Almost no lymphatic regeneration was observed at day 1 and day 4 (Fig. 2A, B). At day 7, abundant lymphatic regeneration was observed in the pars tensa, where almost no lymphatic vessels were observed in the normal tympanic membrane (Fig. 2C, D). Many regenerating lymphatic vessels were extended from the lymphatic vessels surrounding the malleus, while comparatively fewer regenerating lymphatic vessels were extended from the annulus tympanicus (Fig. 2C, D).

4. Discussion

The present study clarified the localization of lymphatic vessels in the tympanic membrane. The pars flaccida and the tendon of the tensor tympani muscle are frequent sites of cholesteatoma—a destructive growth of keratinizing squamous epithelium in the middle ear—and the cholesteatoma matrix is fundamentally identical to the epidermis of the tympanic membrane. These sites had abundant lymphatic vessels. Dysfunction of lymphatic vessels could cause edema of the perimatrix and inflammation, and electron microscopical study of human cholesteatoma suggested that dysfunction of the lymphatic vessels might cause cholesteatoma in the middle ear [6]. Localization of the lymphatic vessels might, therefore, be associated with pathological states of the tympanic membrane.

In the pars tensa, which vibrates in response to sounds and is important for hearing, lymphatic vessels were localized around the malleus handle and were connected to lymphatic vessels surrounding the tensor tympani and in the pars flaccida. No lymphatic loop was observed in the pars tensa, except around the malleus handle and the annulus tympanicus. This localization of lymphatic vessels suggests that the normal pars tensa does not require lymphatic vessels, and that the lymphatic vessels surrounding the malleus handle may be the center of the lymphatic system of the pars tensa.

In this study, we also observed lymphatic vessel regeneration during regeneration of the tympanic membrane. In the pars tensa, many regenerating lymphatic vessels were extended from the lymphatic vessels surrounding the malleus at day 7. In the healing tympanic membrane, the malleus plays a crucial role and is the site of significant mitotic activity during the healing process [7]. Therefore, the malleus is the proliferation and budding center of lymphatic vessels for the lymphatic system in the tympanic membrane. The tympanic membrane reaches maximum thickness at 7 days after rupture [7], and the lymphatic circulation is crucial for wound healing and prevention of edema and infection [3]. Therefore, lymphatic vessels in the tympanic membrane may play an important role in the healing state. Further studies are needed to reveal the function and pathological state of lymphatic vessels in the tympanic membrane.

In conclusion, we demonstrated localization of the lymphatic vessels of the tympanic membrane, and lymphatic vessel response during regeneration of the tympanic membrane. These results suggest that site-specific lymphatic vessels play an important role in the integrity of the tympanic membrane.

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