Journal of Immunological Methods 425 (2015) 97–101

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Determination of lymph flow in murine oral mucosa using depot clearance of near-infrared-labeled albumin

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ARTICLE INFO

Article history:
Received 27 March 2015
Received in revised form 25 June 2015
Accepted 25 June 2015
Available online 2 July 2015

Keywords:
Lymphatic vessel
Gingiva
Inflammation
Optical imaging

ABSTRACT

The lymphatic vessels are playing an important role in inflammation since they return extravasated fluid, proteins, and cells back into the circulation and regulate immune cell trafficking. The oral mucosa, including gingiva, is well supplied with lymphatic vessels and is frequently challenged with inflammatory insults. Lymphatic vessels in gingiva protect against periodontal disease development, but quantification of lymph flow in this area has so far never been performed, due to lack of reliable methods.

Mice of FVB strain (n = 17) were anesthetized with isoflurane and placed on a jaw retraction board allowing the mouth to be kept open and stable. Albumin conjugated with Alexa680-fluorochrome (with or without LPS from *Porphyromonas gingivalis*) was injected superficially in oral mucosa mesio-buccal to the left first molar in each mouse. 60 min post-injection the mouse was transferred to an OptixMX3 optical imager where the total fluorescence was measured in the posterior facial area. The measurements continued further every 60 min for 7 h for each mouse. The mice were awake and active between measurements. The in vivo washout of Alexa680-albumin was calculated using the natural logarithm of the relative values creating a negative slope for each mouse. Statistical analysis of variance was performed. The injection and distribution site for tracer was verified with India ink and shown to be in the interstitium below the oral mucosal epithelium, in an area well supplied with initial lymphatic vessels. Washout of the tracer Alexa680-albumin was log-linear, and the basal lymph flow calculated from depot clearance averaged -0.28 ± 0.08%/min (n = 8). The clearance was significantly faster (-0.30 ± 0.08%/min, n = 9) in acutely inflamed oral mucosa (p = 0.0326).

We developed a method that can successfully quantify the lymph flow in oral mucosa in steady state conditions and under acute perturbation. By use of this method, new information about the lymphatic function in oral mucosa during physiological and pathological conditions can be achieved.

1. Introduction

The lymphatic vessels are playing an important role in tissue fluid balance and immune function. They return extravasated fluid to the circulation (Wiig and Swartz, 2012), are responsible for lipid transport and immune cell trafficking (Weber et al., 2013) and are involved in the development and sustenance of several diseases (Alitalo et al., 2005; Alitalo, 2011; Huggenberger et al., 2011). Moreover, lymphatic vessels contribute to limiting acute inflammation (Huggenberger et al., 2011).

Lymphatic vessels are shown to exist in the gingiva and oral mucosa (Ushijima et al., 2008; Berggreen et al., 2009; Mkonyi et al., 2010; Ando et al., 2011), and seem to have a protective role against periodontal disease development (Mkonyi et al., 2010, 2012b; Berggreen and Wiig, 2013). Moreover, growth of lymphatics from pre-existing vessels (lymphangiogenesis) is shown to take place when periodontal disease is induced (Mkonyi et al., 2012a). Lymphatic vessels are also important in tumor development and metastasis in oral cancer (Yuasa-Nakagawa et al., 2013; Donaduzzi et al., 2014), and lymphangiogenesis has been shown to occur in the cervical lymph nodes during cancer metastasis (Ozasa et al., 2012) and during periapical disease development (Virtej et al., 2015).

Evidently, since lymphatics are involved in several oral diseases, it is of importance to develop tools to test their function in normal as well as pathological conditions. When searching for available methods, near infrared (NIR) imaging has emerged as a novel modality for assessing lymphatic function in vivo (Hofmann et al., 2009; Karlsen et al., 2012; Weiler and Dixon, 2013; Sevick-Muraca et al., 2014). Recently, we developed a minimally invasive method for quantification of lymphatic function in the skin and muscle using Optical imaging of Alexa680-albumin clearance (Karlsen et al., 2012).

To our knowledge, there is no available method for lymph flow measurement in the oral cavity. Here we adapted our previously described NIR imaging method for quantification of lymphatic function

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http://dx.doi.org/10.1016/j.jim.2015.06.014
0022-1759/

and show that this approach can be used to measure lymph flow in healthy and acutely inflamed oral mucosa in mice.

2. Materials and methods

2.1. Experimental animals

Wild type mice (8–30 weeks) of FVB background were used. The animals received a standard pellet diet and tap water ad libitum. All experiments were approved by the Local Institution Board at the University of Bergen and all procedures were performed in accordance to the regulations of the Norwegian State Commission for Laboratory Animals which conforms to recommendations given by the Directive 2010/63/EU.

2.2. Preparation of the fluorescent probe

BSA was conjugated with NIR Alexa Fluor 680 NHS ester by using a SAIVI rapid antibody labeling kit (Invitrogen, Vika, Oslo) according the manufacturer's specifications and as described previously (Karlsen et al., 2012). Aliquots were made and the Alexa680-albumin (near-infrared-albumin) was stored at −20 °C until the experiments were performed. Purity and stability of the conjugated Alexa680-albumin were confirmed as described by Karlsen et al. (2012).

2.3. Time domain optical imaging

In vivo fluorescence was detected with an ART OptixMX3 system with a fixed pulsed laser diode as illumination source with an excitation wavelength of 670 nm with a repetition rate of 80 MHz. In this system, a photomultiplier tube detector with a 700 nm long-pass filter collects the fluorescence signal from the specimen. Images were analyzed using ART Optiview software (version 3.02.00; ART Advanced Research Technologies, Montreal, Quebec), and photon counts per second were calculated for each ROI (Hofmann et al., 2009).

2.4. In vivo lymph flow measurements in mice by NIR optical imaging

The mice were anesthetized with isoflurane that after induction was delivered through a nose cone and placed on a jaw retraction board. The retraction board facilitated visibility and secured free access to the injection site by keeping the mouth open and the head stabilized. One or two microliter Alexa680-albumin solution was injected with a graded Hamilton syringe (34-G needle) superficially in the oral mucosa buccal to the first molar. The injection site was close to the gingiva and the needle was placed in contact with the alveolar bone close to the 1st upper molar and as superficially as possible, in order to avoid uptake from deeper lymphatics and blood vessels of the area (Fig. 1). The mice then were placed in the cages to wake up and move freely for 1 h, shown to be necessary for albumin distribution in the tissue before start of washout (Karlsen et al., 2012). Then the mice were re-anesthetized and immediately after placed on a temperature controlled heating pad in the Optix MX3 scanner to maintain the body temperature at 37 °C. The posterior facial area was scanned as described by Karlsen et al., 2012. The scanning procedure was repeated 6 times. All mice were conscious and allowed to move freely between measurements, such that the measured washout rates are representative for that of awake mice. The ROI was defined as the entire posterior facial area and was superimposed for all subsequent scans, so the possibility for positional differences between scans to influence the fluorescence quantification was minimized as described previously (Karlsen et al., 2012).

2.5. Basal lymph flow measurements

Mice (n = 8) received 1 μl Alexa680-albumin in the oral mucosa as described above and were left for 1 h before scanning. Mice were scanned every 60 min for a total period of 7 h.

2.6. Lymph flow measurements after LPS induced acute inflammation

Mice (n = 9) were injected in oral mucosa as described above with 2 μl, 1:1 solution, of Alexa680-albumin and Ultrapure LPS from *Porphyromonas gingivalis* (*P. gingivalis*) in endotoxin free water (InvivoGen, Toulouse, France) at a final concentration of 5 mg/ml. The mice were scanned every 90 min for a total period of 6 h. The same mice had been injected in the same location with 1:1 solution of Alexa680-albumin and 0.9% NaCl (total volume 2 μl) one week prior to challenge with LPS, as a control. Following Alexa680-albumin/ LPS injection, the mice were scanned every 90 min as described above.

2.7. Statistics

Mean and SD of the remaining albumin dose ($\ln(\frac{m}{m_0})$) were determined for each time point in both experimental groups and were used for calculation of the rate constant *k*. These data were applied for linear regression analysis comparing the slopes. In addition, *k* was calculated as the slope of the curve for each animal. The rate constants before and after LPS-treatment were compared using paired t-tests. A p value ≤0.05 was considered statistically significant. The values on the plots are expressed as mean ± SD. All statistical analysis was performed with Graph pad Prism5 software (California, USA).

2.8. Immunohistochemistry

In order to visualize the distribution of the lymphatic vessels in the area of tracer injection, mouse upper jaws were harvested and fixed with 4% paraformaldehyde overnight and then washed thoroughly with PBS. Thereafter they were decalcified with 10% EDTA solution for 7 days, immersed in 30% sucrose solution overnight, frozen and

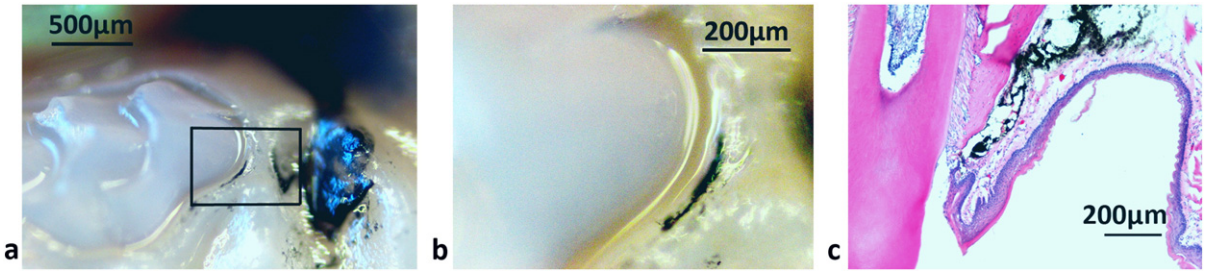

Fig. 1. Injection site buccally of the first molar illustrated with deposition of India ink in the oral mucosa (a) and gingiva (a, b), b represents boxed area shown in a. Section stained with H&E showing distribution of India ink (black label) in the interstitium above alveolar bone (c).