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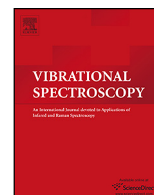

Towards the development of a non-bioptic diagnostic technique for eosinophilic esophagitis using Raman spectroscopy☆

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ABSTRACT

Eosinophilic esophagitis (EoE) is believed to be a rare pathological condition that is characterized by dense infiltration of eosinophils in esophageal epithelial layer. Occurrence of this disease worldwide has started increasing rapidly in the last decade. Routine endoscopic observations can help in diagnosis only if clear longitudinal furrows or multiple concentric rings are observed but does not give any definitive conclusion in the early stages. Hence esophageal tissue samples are collected from multiple sites by biopsy and the number of eosinophils is counted after staining. Such a procedure is time consuming and has an inherent risk of bleeding, eventually damaging esophagus. Hence we developed a resonance Raman spectroscopy based approach to detect eosinophils in esophagus using mouse models. Our results show the presence of eosinophils in mice esophageal tissues suffering from inflammation by administering interleukin-33 while there are none in control mice. We believe this method can be used in clinical application for diagnosing EoE in the near future.

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1. Introduction

Eosinophils, one of the three types of granulocytes, are polymorphonuclear leukocytes that make up about 2–6% of white blood cells. They play key roles in regulating inflammation in addition to fighting parasites. Though inflammation is indispensible with a defensive role in isolating and controlling a disease site, unnecessary or over inflammation leads to a variety of inappropriate immunological responses resulting in allergic diseases eventually causing tissue damage. Recently, cases of eosinophil related inflammation of airway mucosa including sinuses, esophagus and lungs are reported to be rapidly increasing. Eosinophilic gastrointestinal disorders, characterized by dense infiltration of eosinophils in esophago-gastro-intestinal mucosa, can be categorized into eosinophilic esophagitis (EoE) and eosinophilic gastroenteritis. Ever since Landres et al. first reported EoE over 30 years ago, it was found to be prevalent only among pediatric population [1–3]. However, a growing trend of EoE in adults worldwide has been observed in the last decade, especially more so in western countries. Also, it develops more frequently in males than females (76% of the patients are male in Europe or North America and 80% in Japan) with the average age of these patients being 51 years [4].

Predominant symptoms of patients with EoE are dysphagia, heartburn, chest discomfort, throat discomfort and food impaction which is primarily triggered by dense eosinophil infiltration into the upper mucosal layer of esophagus resulting in chronic inflammation [4–6]. This leads to increase in the permeability and growth of esophageal squamous layer while also promoting edema or fibrotic response in submucosal layer, which are difficult to observe in patients suffereing from EoE at the early stage. Effective remedy for this disease is already established with local action steroid or with anti-interleukin-5 or -13 antibody. However, the differentiating from reflux esophagitis, which shows similar symptoms to EoE is difficult by simple diagnosis using endoscope [7]. But definite diagnosis, which is done by counting number of eosinophils in mucosal epithelial tissue by staining, forces the

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patients to undergo biopsy of the mucosal tissue of esophagus. Since there is no guide for determining the appropriate position, multiple biopsies are considered superior to a single biopsy (sometimes more than five biopsies are done) in diagnosing EoE. This increases the risk of bleeding, especially when the biopsy sampling are done in multiple areas in esophagus [5,8]. Such diagnosis gets even more complicated in patients undergoing treatment with antithrombotic drugs. Adding to the complexity is important fact that many of these patients also simultaneously suffer from other difficulties such as allergies or asthma. Hence alternative technologies that do not depend on biopsy are need of the hour.

We propose to develop Raman spectroscopy based diagnostic method for EoE, for the advantages are manifold. First, Raman spectrum, which can be thought of as a chemical fingerprint, gives rich chemical information. Secondly, it does not require any external dye probes. Thirdly, it is a non-invasive technique that is suitable for *in vivo* applications [9–11]. It is well known that granulocytes are different in their chemical make up, especially in the heme peroxidases, and that they can be distinguished by Raman spectroscopy [12–14]. In fact, we have successfully automated distinguishing each white blood cell type using Raman spectroscopy and multivariate curve resolution analysis recently [15]. Particularly in the case of eosinophil peroxidase, there is an absorption band at 640 nm and the nearby ± 20 nm region can be used for resonance enhancements of this heme proteins [14,16]. With this background, we set out to explore the possibility of applying such a method to detect eosinophil infiltration in esophageal mucous membrane in model mice using eosinophil peroxidase as a marker. We prepared model disease mouse with EoE by inducing inflammation in esophagus using interleukin-33 (IL-33), an IL-1 family of proteins that produce T helper-2 associated cytokines [17–19]. Pathologically, in the case of EoE, eosinophils are observed only in the esophageal epithelial layer but not in gastric or intestinal mucosa. Our results on successful detection of eosinophils during such inflammation in esophageal tissues of IL-33 administered model mouse by resonance Raman spectroscopy is reported in this article.

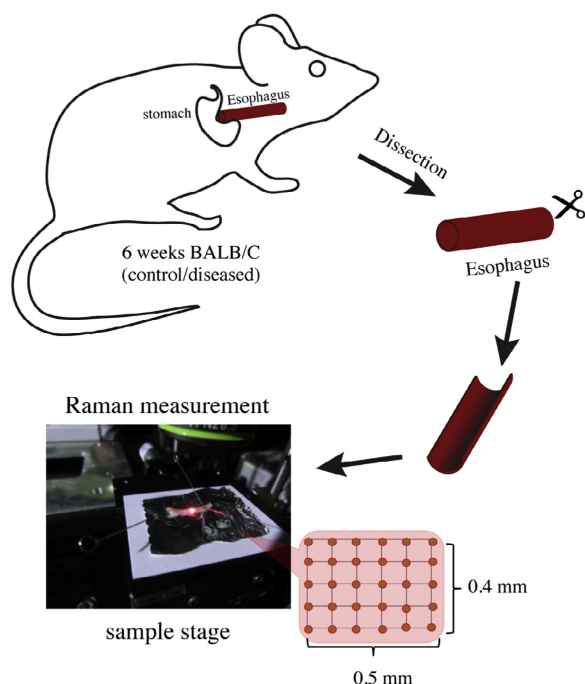

Fig. 1. Steps involved in preparing esophageal tissue for Raman spectroscopic measurements.

2. Materials and methods

2.1. Preparation of esophageal tissue

Six week BALB/c female mice (Charles River) were used to prepare two kinds of model mice, control and diseased (IL-33 administered). One control mouse (wildtype) was prepared by administering 25 μl of PBS while three EoE model mice (diseased) was prepared by injecting IL-33 (2 μg in 150 μl PBS) intra-peritonealy for seven consecutive days from day 7–13. On day 14, mice were euthanized and esophagus dissected in order to carry out Raman measurements. The mice were anatomized after euthanasia according to the guide for the treatment of laboratory animals of Shimane University. The dissected esophageal tissues were first thoroughly rinsed using PBS. The cylindrical shaped esophageal tissue was then cut lengthwise so as to expose the inner membrane. The cut esophageal tissues of control or IL-33 injected mice were kept in a bath of cooled PBS until further measurements. The desired tissue was then carefully transferred onto a plate with little PBS to avoid sample drying to perform Raman spectroscopic measurements. To check infiltration of eosinophils after Raman experiments, hematoxylin and eosin staining (HE) was done on both control and IL-33 treated mice.

2.2. Preparation of granulocytes obtained from human blood

Granulocytes of human blood were extracted from one of the authors to compare and discuss the Raman spectra for esophageal tissue of mice. Polymorphprep was purchased from COSMO BIO for the purpose of blood separation. Red Blood Cell Lysis Solution was purchased from Miltenyi Biotec K.K. for hemolyzing red blood cells. 5 ml of human blood was collected and treated with heparin to prevent aggregation. It was then slowly added to equal amount of blood separating solution kept prior in a centrifuge tube, to give two layers. The tube was centrifuged at 500g for 30 min at room temperature to get six separated layers. Granulocytes separated in the fourth layer from top. 200 μl from this fourth layer was collected into another centrifuge tube and 5 ml of PBS was added followed by centrifugation at 400g for 10 min at room temperature to give white pellet at the bottom. The upper layer was removed and rinsed with PBS. Then 1 ml of PBS and 9 ml of diluted Red blood Cell Lysis Solution was added to the centrifuge tube and left to stand still for 10 min after ultrasonic agitation. It was again centrifuged at 400g for 10 min at room temperature to give pure

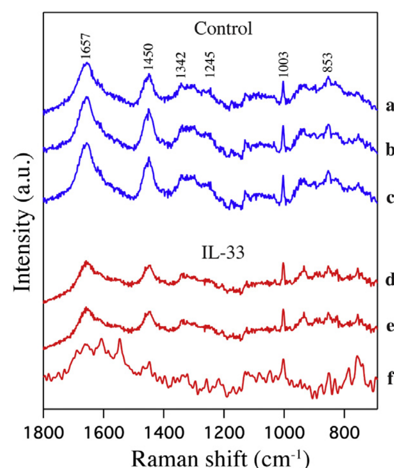

Fig. 2. Space-resolved Raman spectra from esophageal tissue of mouse. Raman spectra obtained from; (a–c) a control mouse and (d–f) IL-33 administered mouse.

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