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The pathogenesis of chronic eosinophilic esophagitis in SHARPIN-deficient mice

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ABSTRACT

Increased numbers of eosinophils in the esophagus are common in several esophageal and systemic diseases, and a prominent feature of eosinophilic esophagitis. Mouse models can provide insight into the mechanisms of eosinophil infiltration and their pathogenic role. SHARPIN-deficient *cpdm* mice develop a chronic proliferative dermatitis and an esophagitis characterized by epithelial hyperplasia and the accumulation of eosinophils in the serosa, submucosa, lamina propria and epithelium of the esophagus. We conducted a detailed investigation of the pathogenesis of the esophagitis by light microscopy, immunohistochemistry, and gene expression as the mice aged from 4 to 10 weeks. The thickness of the esophageal epithelium and the number of eosinophils in the esophagus both increased with age. There were scattered apoptotic epithelial cells in mice at 6–10 weeks of age that reacted with antibodies to activated caspase 3 and caspase 9. The expression of CCL11 (eotaxin-1), IL4, IL13 and TSLP was increased in *cpdm* mice compared with wild type (WT) mice, and there was no change in the expression of CCL24 (eotaxin-2), IL5 and IL33. The expression of chitinase-like 3 and 4 (YM1 and YM2) proteins, markers of type 2 inflammation, was greatly increased in *cpdm* mice, and this was replicated in vitro by incubation of WT esophagus in the presence of IL4 and IL13. Immunohistochemistry showed that these proteins were localized in esophageal epithelial cells. The severity of the esophagitis was not affected by crossing SHARPIN-deficient mice with lymphocyte-deficient *Rag1* null mice indicating that the inflammation is independent of B and T lymphocytes.

1. Introduction

An increased number of eosinophils in the esophagus is a feature of a wide spectrum of organ-specific and systemic diseases, including eosinophilic esophagitis (EoE), gastroesophageal reflux disease (GERD), Crohn's disease, and hypereosinophilic syndromes (Atkins et al. 2009; Liacouras et al. 2011). EoE is a recently recognized chronic disease in children and adults with a predisposition for males characterized by the presence of many eosinophils throughout the epithelium of the esophagus. It is thought to be caused by an allergic reaction to airborne or food antigens (Blanchard 2015; Rothenberg 2015). The light microscopic features include accumulation of eosinophils in the esophageal epithelium, fibrosis of the subepithelial lamina propria, and epithelial cell hyperplasia. Eosinophils accumulate superficially in the epithelium sometimes forming microabscesses. EoE may be differentiated from GERD by the larger number of eosinophils (>15/HPF), distribution of eosinophils along the length of the esophagus, and lack of response to proton pump inhibitors (PPI) (Liacouras et al. 2011). However, it was recently recognized that a substantial subpopulation of patients with clinical symptoms and histologic features indistinguishable from EoE, responds to PPIs (Dellon et al. 2013; Moawad et al. 2014). This is considered a separate disease entity termed PPI-responsive esophageal eosinophilia. EoE and PPI-responsive esophageal eosinophilia have a very similar gene expression profile except for a small subset of genes that appears to distinguish the two groups of patients (Wen et al. 2015).

The mechanisms by which eosinophils accumulate in the epithelium of the esophagus are not completely understood and likely vary depending on the disease. The epithelium of the esophagus is normally devoid of eosinophils, but eosinophils can accumulate as a non-specific response to various types of injuries (Odze 2009). Gene expression analysis revealed greatly increased expression of *Ccl26* mRNA in the esophageal epithelium of patients with EoE. Furthermore, a single nucleotide polymorphism in the 3′ untranslated region of *Ccl26* correlated with increased susceptibility to the disease supporting a role of this chemokine in the accumulation of eosinophils (Blanchard et al. 2006). Clinical trials with anti-IL5 monoclonal antibodies demonstrated a partial reduction of the number of intraepithelial eosinophils in the esophagus suggesting the involvement of this cytokine in eosinophil accumulation in EoE (Assa'ad et al. 2011; Spergel et al. 2012; Straumann et al. 2010).

Mouse models may provide further insight into the pathogenesis of EoE and related diseases characterized by esophageal eosinophilia.

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Intranasal administration of fungal or house dust mite antigens, ovalbumin, and peanut allergens to mice resulted in eosinophil infiltration of the esophagus accompanied by increased epithelial cell proliferation and accumulation of mast cells (Mishra et al. 2001; Rajavelu et al. 2012; Rubinstein et al. 2011). The eosinophils were predominantly localized in the submucosa and lamina propria and occasionally in the basal layer of the esophageal epitheliumin contrast to the more superficial localization of eosinophils in human patients with EoE. Using these models, it was shown that eosinophil accumulation was dependent on T cells, whereas B cells were dispensable (Mishra et al. 2007). Mice deficient in either $CD8^+$ T cells or $CD4^+$ T cells still developed esophageal eosinophilia and recent studies suggest a role for NKT cells (Rajavelu et al. 2012; Rayapudi et al. 2014).In another mouse model, transgenic mice with overexpression of IL5 in the esophageal epithelium were sensitized cutaneously and challenged via gavage with a hapten (Masterson et al. 2014). Eosinophils accumulated in the esophageal connective tissue and the epithelium and formed superficial microabscesses similar to human EoE (Masterson et al. 2014).

SHANK-associated RH domain-interacting protein (SHARPIN) is a widely expressed protein and a component of the linear ubiquitination assembly complex that plays a critical role in the NFKB signaling pathway (Walczak et al. 2012; Wang et al. 2012). SHARPIN is also a negative regulator of the beta1 integrin and decreases the activity of the tumor suppressor protein PTEN (He et al. 2010; Jung et al. 2010; Rantala et al. 2011). SHARPIN-deficient mice carry a spontaneous mutation resulting in a premature stop codon in exon 1 of the *Sharpin* gene (Seymour et al. 2007). These mice develop a chronic proliferative dermatitis that becomes clinically manifest at about four weeks of age (HogenEsch et al. 1993). The dermatitis is characterized by epidermal hyperplasia, hyperkeratosis, scattered keratinocyte apoptosis, and accumulation of eosinophils and fewer macrophages, mast cells, and neutrophils in the dermis and epidermis (HogenEsch et al. 1993). The esophagus of mice is lined by stratified squamous cell epithelium similar to the skin. Here, we report on the pathogenesis of the esophagitis in SHARPIN-deficient mice. We investigated whether the morphologic changes and gene expression were similar to those in the skin and we determined the role of B and T lymphocytes in the development of the inflammation.

2. Materials and methods

2.1. Mice

In this study, C57BL/KaLawRij-*Sharpin*^{cpdm}/*Sharpin*^{cpdm}RijSunJ (JR# 7599; referred to hereafter as *cpdm*) and CByJ.Cg-*Sharpin*$^{cpdm\text{-}Dem}$/*Sharpin*$^{cpdm\text{-}Dem}$ (hereafter *cpdm-Dem*) mutant and wild type (WT, +/+) mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in specific pathogen-free conditions (http://jaxmice.jax.org/genetichealth/health_program.html) on a 12:12 h light:dark cycle with constant temperature and humidity. Each double-pen polycarbonate cages (330 cm^2 floor area) housed a maximum of four per pen. Commercial autoclaved food (NIH 31, 6% fat; LabDiet 5 K52, Purina Mills, St. Louis, MO) and acidified water (pH 2.8–3.2) were fed ad libitum. Mice were genotyped by PCR as previously described to distinguish −/− or +/+ mice at day 10 after birth (Potter et al. 2014). *Sharpin*$^{cpdm\text{-}Dem}$, *Rag1*$^{-/-}$ double mutant mice were generated by intercrossing homozygous male BALB/c-*Rag1*^{tm1Mom}/J mice with heterozygous C.OcB3-*Sharpin*$^{cpdm\text{-}Dem}$ females. Progeny that genotyped as heterozygous for both alleles were then intercrossed until the *Rag1*^{tm1Mom} allele was fixed to homozygosity. The colony was maintained by mating mice homozygous for the *Rag1*^{tm1Mom} allele and heterozygous for the *Sharpin*$^{cpdm\text{-}Dem}$ allele. All work was approved by The Jackson Laboratory and Purdue University Animal Care and Use Committees.

2.2. Esophagus collection

Age and gender matched mice were euthanized by CO_2 asphyxiation at 4, 6, 8, and 10 weeks of age. Euthanized mice were placed ventral side up. A small incision was made along the ventral midline. The skin and peritoneal wall were reflected back to expose the internal organs. Lifting up the sternum with forceps, the diaphragm cut followed by the ribs at the costo-chondral junction. The liver was removed to expose the esophagus and the stomach. The esophagus was then gently dissected free of the trachea to the hyoid apparatus, removed from the mouse, placed on a piece of aluminum foil, and coiled to form a “Swiss roll” with the distal segment, near the stomach, being in the center of the coil. The esophagus was fixed by immersion in Fekete's acid-alcohol-formalin (for routine histology and immunohistochemistry). Tissues were processed routinely, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E), toluidine blue, Masson's trichrome, or processed for immunohistochemistry. Alternatively, esophagus was embedded in Optimal Cutting Temperature (OCT) medium, snap frozen in liquid nitrogen, and stored at −80 °C, or collected in cold HBSS for culture.

2.3. Immunohistochemistry

Serial paraffin sections from tissues fixed in Fekete's acid alcohol formalin were processed using a Ventana autostainer (Tucson, AZ). Cleaved caspase 3 (Cell Signalling Technologies; Danvers, MA) and cleaved caspase 9 (Novus Biologicals; Littleton, CO) were used to evaluate apoptosis in the esophagus, and anti-major basic protein (MBP) antibody (obtained from J. Lee, Mayo Clinic, Scottsdale, AZ) to identify eosinophils. Diaminobenzidine (Sigma, St. Louis, MO) was used as chromogen.

For immunofluorescent labeling, frozen sections of esophagus were fixed in 100% acetone and incubated with fluorochrome-labeled antibodies against CD11B (Biolegend) or rabbit antibodies against CHIL3/4 proteins (Stemcell Technologies, Vancouver, CA) followed by Alexafluor 488-labeled donkey anti-rabbit IgG (Jackson Immunoresearch, Westgrove, PA). The sections were coverslipped with ProLong® Gold Antifade solution (Thermo Fisher Scientific, Grand Island, NY) with 4′,6-diamidino-2-phenylindole (DAPI).

2.4. Histopathological scoring

To quantify the severity of esophageal lesions, three criteria were used to measure the cellular changes: (1) mucosal thickness, (2) number of apoptotic keratinocytes, and (3) number of eosinophils. Each criterion was measured on ten randomly selected areas of the esophagus as described below.

2.4.1. Epithelial thickness

The esophageal epithelium was measured from the basement membrane to the top of the stratum granulosum (malphigian layer) and to the top of the stratum corneum (full thickness) using the 40× objective on an Olympus BH-2 microscope with a DP70 digital camera and DP Controller (3.2.1.276 version software, Olympus Corp., Tokyo, Japan).

2.4.2. Apoptotic keratinocytes

The number of apoptotic keratinocytes was counted within a field using a 63× high dry objective on an Olympus BH2 microscope. The field had a total area of 139 μm^2.

2.4.3. Eosinophil scoring

All lesions were subjectively graded using a 63× high dry objective on an Olympus BH-2 microscope (0 = normal, 1 = few widely scattered eosinophils, 2 = small clusters of eosinophils below the basement membrane, 3 = a linear arrangement of eosinophils under basement membrane, and 4 = rows of eosinophils below the basement