Pathogenic role of mast cells in experimental eosinophilic esophagitis

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Niranjan R, Mavi P, Rayapudi M, Dynda S, Mishra A. Pathogenic role of mast cells in experimental eosinophilic esophagitis. Am J Physiol Gastrointest Liver Physiol 304: G1087–G1094, 2013. First published April 18, 2013; doi:10.1152/ajpgi.00070.2013.—Eosinophilic esophagitis (EoE) is a chronic allergic disease characterized by esophageal intraepithelial eosinophils, extracellular eosinophil granule deposition, induced mast cell accumulation, and epithelial cell hyperplasia. However, the processes involved in the development of a number of these characteristics are largely unknown. Herein, we tested the hypothesis whether induced mast cell accumulation in the esophagus has a role in promoting EoE pathogenesis. Accordingly, we induced experimental EoE in wild-type mice, mast cell-deficient WWv mice, and mast cell-reconstituted WWv mice. We report that esophageal mast cell numbers increase in parallel with eosinophils in a dose- and time-dependent manner following the induction of allergen-induced EoE. The induced mast cells are localized in the esophageal lamina propria and muscular mucosa but have no influence on promoting esophageal eosinophilia. The 5’-bromodeoxyuridine (BrdU) incorporation analysis indicated that mast cells have a significant role in muscle cell hyperplasia and hypertrophy. In addition, the wild-type and mast cell-reconstituted WWv mice showed a comparable number of BrdU+ cells in the esophageal muscular mucosa following allergen-induced EoE. In conclusion, we provide for the first time direct evidence that mast cells promote muscle cell hyperplasia and hypertrophy and may have a significant role in promoting esophageal functional abnormalities in EoE.

Eosinophilic esophagitis (EoE) is an increasingly recognized chronic inflammatory disease of the esophagus that clinically mimics gastroesophageal reflux disease. EoE is induced by food or aeroallergen exposure, characterized by intraepithelial eosinophil accumulation, eosinophil granule deposition in the extracellular tissue, and epithelial cell hyperplasia and is resistant to acid suppression therapy (10, 11, 14, 20, 27, 31). Eosinophils and mast cells are increased in the esophageal mucosa of experimental and human EoE (2, 9, 17, 19, 25, 30), but their role in disease pathogenesis is unclear. Mast cells express the high-affinity receptor for IgE on their surface (3, 4, 15, 16) that initiates a complex process of signal transduction that releases proinflammatory mediators, cytokines, and chemokines (3, 4, 12, 16). Mast cells produce the eosinophil chemoattractant eotaxin-1 that influences allergen-induced eosinophil accumulation (29) in select tissues, raising the possibility that they may mediate eosinophil accumulation in the esophagus.

Additionally, it has been shown that mast cells have a clear-cut pathological role in a number of allergic diseases (6, 7, 13, 18), and increased esophageal mast cells may have the potential to influence multiple aspects of the EoE pathogenesis. We recently reported that esophageal peristaltic dysfunction in experimental EoE is independent of eosinophils in experimental EoE (19). Therefore, we hypothesized that allergen-induced mast cell accumulation in the esophagus may have an important role in some of the esophageal abnormal function in EoE. Accordingly, we monitored the influence of mast cells on allergen-induced inflammation in the esophagus. We demonstrate that the esophageal mast cells increase in parallel with eosinophils; however, the esophageal eosinophilia is independent of the esophageal mast cells in EoE. Importantly, mast cell-deficient WWv mice are protected from the induction of muscle cell hyperplasia in allergen-induced EoE. Although using WWv mice has some limitations, as they have some reduced neutrophils and basophils as reported earlier (28), our experimentation, however, with reconstituted mast cells ruled out any effect on our study conclusions. Taken together, we provide direct evidence that mast cells may have a significant role in esophageal functional abnormalities, particularly the development of esophageal peristaltic dysfunction.

MATERIALS AND METHODS

Mice. Specific pathogen-free wild-type, mast cell-deficient WWv mice with their respective controls were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a barrier facility, and animals were handled according to institutional guidelines; all animal protocols were approved by the Case Western Reserve University Animal Care and Use Committee. All of the experiments were performed on age- and sex-matched mice that were 6–8 wk old.

Induction of experimental EoE in mice. Experimental EoE was induced in mice following an established protocol described previously (20). In brief, mice were lightly anesthetized with isoflurane inhalation (methoxy-flurane; Pittman-Moore, Mundelein, IL), and 100 μg (50 μl) of Aspergillus fumigatus (Greer Laboratories, Lenoir, NC) or 50 μl of normal saline alone was applied to the nares using a micropipette with the mouse held in the supine position. After instillation, mice were held upright until alert. After three treatments per week for 3 wk, mice were killed between 18 and 20 h after the last intranasal challenge.

Mast cell analysis. The 5-μm esophageal paraffin tissue sections were deparaffinized and stained with hematoxylin with 4% sodium nitrate in naphthol-AS-D chloroacetate (Sigma-Aldrich, St. Louis, MO) and phosphate-buffered saline solution for 30 min and counterstained with hematoxylin. In addition, Giemsa and Toluidine blue staining was also performed to identify degranulated and activated mast cells in the tissue sections. The tissue sections were stained for 15 min with either Giemsa or Toluidine reagent (Fisher Scientific, Pittsburgh, PA) followed by rinsing with running water of the tissue slides for 7–10 min. The histological analysis was performed using light microscopy.

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Eosinophil analysis in the esophagus. The 5-μm esophageal paraffin tissue sections were immunostained with antisera against mouse eosinophil major basic protein (anti-MBP) as described earlier (20–22). In brief, endogenous peroxidase in the tissues was quenched with 0.3% hydrogen peroxide in methanol followed by nonspecific protein blocking with normal goat serum. Tissue sections were then incubated with rat anti-MBP (1:2,000) overnight at 4°C (kindly provided by Drs. James and Nancy Lee), followed by a 1:200 dilution of biotinylated goat anti-rat IgG secondary antibody and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min each. These slides were further developed with nickel diaminobenzidine-cobalt chloride solution to form a black precipitate and counterstained with nuclear fast red. Negative controls included replacing the primary antibody with normal rabbit serum to check endogenous biotin and peroxidase activity.

Quantification of eosinophils and mast cells. Eosinophils and mast cells were quantified by counting the positively stained cells on each tissue section with the assistance of digital morphometry and expressed as cells/mm² tissue area as described earlier (21, 23).

Mast cell protease-1 analysis. Mast cell protease (MMCp)-1 plasma and tissue levels were measured by using commercially available ELISA (Moredun Scientific, Midlothian, UK) following manufacturer’s instructions and protocol as reported previously (6). Esophageal 10% homogenates were prepared by homogenizing each esophagus of saline and Aspergillus-challenged mice in phosphate buffer, pH 7.2; the tissue homogenates were centrifuged at 100,000 g for 2 min, and supernatants were collected for ELISA assay. The ELISA plate (DYNEX Technologies, Chantilly, VA) was coated with capture antibody diluted to 2 μg/ml in 0.1 M carbonate buffer pH 9.6 overnight at 4°C. Mouse serum, tissue samples, and purified mouse standards were applied to the plate. The plate was incubated 2 h at room temperature, washed, and incubated with optimally diluted conjugate. Finally, tetramethylbenzidine substrate solution (BD Biosciences, San Diego, CA) was added to each well, and the color was developed in the dark at room temperature. The reaction was stopped using 0.25 M H₂SO₄, and optical density was determined at 450 nM. The MMCp-1 concentration of each sample was calculated based on a standard curve.

Mast cell culture and reconstitution in WW⁺ mice. Mouse bone marrow cells were harvested in RPMI media from both the femur and tibia. Cells were centrifuged at 250 g for 5 min; cell pellets were resuspended and counted. The total cells were cultured with 30 ng/ml of IL-3 (eBioscience, San Diego, CA) for 4 wk. The cultured mast cells (96% pure at 10⁷ cells/mouse) were reconstituted into the mast cell-deficient mice by tail vain injection. Three months after mast cell injection in mast cell-deficient mice along with sex- and age-matched wild-type mice, the mice were challenged with saline or Aspergillus following the experimental EoE protocol described above. The mast cell reconstitution in each mouse was confirmed by performing chloroacetate staining of the paraffin-embedded ear sections. The numbers of mast cells in the ear sections were detected in mast cell-reconstituted WW⁺ mice.

Analysis of muscle cell hyperplasia in the esophagus. To determine the degree of cellular hyperplasia, 5'-bromodeoxyuridine (BrdU) (Zymed Laboratories, San Francisco, CA) incorporation analysis was performed as previously reported (20, 23). In brief, saline- or Aspergillus-challenged mice were injected intraperitoneally with 0.25 ml of 5'-BrdU (0.75 μg), 24 h before death. The esophagus was fixed with 10% neutral buffered formalin (Sigma-Aldrich) for 24 h. After fixation, the tissue was embedded in paraffin, and a 5-μm section was processed using standard histological approaches. Tissues were digested with trypsin (0.125%) for 3 min at 37°C followed by incubation for 45 min at room temperature. Sections were washed with PBS three times for 2 min and further incubated with monoclonal biotinylated anti-BrdU antibody for 60 min at room temperature. Negative controls included replacing the primary antibody with PBS and manufacturer-provided positive control. BrdU nuclear incorporated-positive cells were detected with streptavidin-peroxidase and DAB substrate (Zymed Laboratories) followed by counterstaining with hematoxylin. The muscle cell hyperplasia was analyzed by counting the percentage of BrdU⁺-positive cells in the muscle cell mucosa of the esophagus.

Statistical analysis. Data are expressed as means ± SD. Statistical significance comparing different sets of mice was determined by unpaired InStat GraphPad t-test software (InStat Software, San Diego, CA). A P value <0.05 was considered statistically significant.

RESULTS

Mast cell numbers are increased in experimental EoE. It is well reported that mast cells and eosinophils are induced in human and experimental EoE; however, it is not yet clear whether mast cell recruitment in EoE is an eosinophil response or an allergen-induced response. Therefore, we were interested in determining whether mast cell levels increase in parallel with eosinophilia following aeroallergen exposure. Accordingly, a kinetic analysis of eosinophils and mast cells in the esophagus was performed following intranasal allergen challenge to the mice. Mice received intranasal saline or Aspergillus fumigatus extract separated by 24 h and were killed 18–20 h after zero, two, three, four, five, six, and nine doses. Eosinophil and mast cell numbers in the esophagial tissue sections were determined following anti-MBP and chloroacetate staining, respectively. Both eosinophil and mast cell levels in the esophageal tissue sections were increased following four doses of allergen and continued to rise following subsequent allergen exposure (Fig. 1). Mast cell numbers were increased from 12.5 ± 4/mm² in saline to 28.8 ± 5/mm² (means ± SD, n = 10 mouse/data point, P < 0.001) following nine allergen challenges. Mice given intranasal saline did not show any increase in the levels of mast cells at any time point. The histopathological analysis of esophageal tissue sec-
tions identified mast cells in the lamina propria of allergen-challenged mice along with the eosinophils. Both eosinophils and mast cells were detected gathering in the same region of esophageal lamina propria of allergen-challenged mice (Fig. 2, A and B). Interestingly, a number of mast cells was detected in the muscle cell mucosa of allergen-challenged mice compared with the saline-challenged mice (Fig. 2C). Additionally, intraepithelial mast cells and eosinophils including extracellular granules of eosinophils and mast cells were noted (Fig. 2, D–F). Activated degranulating mast cells were detected in the muscular mucosa of allergen-challenged wild-type mice (Fig. 2G). Interestingly, no significant change in the number of mast cells was observed in the other segment of the gastrointestinal tract (i.e., stomach or jejunum) in the mouse model of allergen-induced EoE (data not shown).

Mast cell recruitment in the esophagus is independent of IL-5. Allergen-challenge induces IL-5 in the esophagus (24), and eosinophil accumulation in the esophagus is dependent on IL-5 (20); therefore, we were interested in testing the hypothesis whether IL-5 also influences mast cell accumulation in the esophagus following allergen-induced experimental EoE. We examined mast cell numbers in wild-type and IL-5 gene-deficient mice following allergen challenge. A comparable level of mast cells in allergen-challenged IL-5 gene-deficient and wild-type mice were observed (Fig. 3). The number of mast cells in the esophagus of allergen-challenged wild-type and IL-5 gene-deficient mice was 25.6 ± 8 and 21.2 ± 6/mm² (mean ± SD, n = 10) compared with their saline-challenged controls, 12.6 ± 7 and 11 ± 5/mm², respectively (mean ± SD, n = 10). In contrast, the number of eosinophils in the esophagus of allergen-challenged wild-type and IL-5 gene-deficient mice was 30.5 ± 10.8 and 0.2 ± 0.4/mm² (mean ± SD, n = 8) compared with their saline-challenged controls, 0.3 ± 0.3 and 0.2 ± 0.2/mm², respectively (mean ± SD, n = 8). These data indicate that IL-5 and eosinophils are not required for allergen-induced esophageal mastocytosis.

Fig. 2. Esophageal eosinophil and mast cell localization in experimental EoE. A: histopathological representative photomicrograph of the localization of eosinophils and mast cells in the lamina propria of allergen-challenged wild-type mice (original magnification is ×100, eosinophils are represented by the arrow and mast cells by the arrow head). B: mast cell and eosinophil tissue colocalization in a high magnification (×400) of inserted area. C: allergen-induced chloracacetate-stained mast cell accumulation in the muscular mucosa. The intraepithelial mast cells (D), eosinophils (E), eosinophil degranulation (granules pointed by arrow) (F), and degranulating muscular mucosal mast cell are shown following Toluidine blue staining, ×400 (G). LU, lumen; EP, epithelium, LP, lamina propria; MM, muscular mucosa.
Esophageal eosinophil recruitment is independent of mast cells. Earlier, it has been shown that mast cells influence eosinophil recruitment (29); therefore, we next tested the role of mast cells in the recruitment of eosinophils in allergen-induced experimental EoE. We induced experimental EoE in wild-type and mast cell-deficient WWv mice, and esophageal eosinophilia was examined. Eosinophil localization was observed in each segment of the esophagus, including the muscular mucosa of allergen-challenged wild-type and mast cell-deficient mice (data not shown). The quantification of eosinophils in wild-type and WWv mice showed similar levels of esophageal eosinophilic inflammation (Fig. 4). The number of eosinophils in the esophagus was 44 ± 11/millimeter squared and 1.3 ± 0.2/millimeter squared (mean ± SD, n = 10 –12, P < 0.001) in allergen- and saline-challenged wild-type mice, respectively. Similarly, the eosinophil numbers in WWv allergen- or saline-challenged mice were 40.6 ± 13.7/millimeter squared and 1.3 ± 2.1/millimeter squared (mean ± SD, n = 12/group, P < 0.001), respectively. No mast cells were detected by performing chloroacetal staining in saline- or allergen-challenged WWv mice.

MMCP-1 level is increased in the blood and esophagus following allergen-induced experimental EoE. Because degranulated mast cells were detected in the esophagus in experimental EoE, we next examined the mast cell mediators in the mice following the induction of EoE. The MMCP-1 levels in the blood and esophageal homogenate of saline- and allergen-challenged mice were measured. We detected ~12-fold increase of MMCP-1 levels in the blood (Fig. 5A) and only ~2-fold increase in the esophageal homogenates (Fig. 5B) of allergen-challenged mice compared with saline-challenged mice. The levels of MMCP-1 in the blood were 11.9 ± 1.6 and 138.1 ± 59.3 µg/ml (mean ± SD, n = 10, P < 0.001), whereas MMCP-1 in the esophagus was 1.4 ± 0.3 and 2.4 ± 0.6 µg/ml (mean ± SD, n = 10/group, P < 0.05) following saline and allergen challenge, respectively. The approximately twofold increase in MMCP-1 in the esophagus is consistent with the greater than twofold increase of mast cells in the esophagus.

Esophageal muscular cell hyperplasia is mediated by mast cells in experimental EoE. We observed an increased number of mast cells in the esophageal lamina propria and mast cell infiltration into the muscular mucosa. Therefore, we next tested the hypothesis whether mast cells have a role in the induction of muscular cell hyperplasia in experimental EoE. We observed an increased number of mast cells in the esophageal lamina propria and mast cell infiltration into the muscular mucosa. Therefore, we next tested the hypothesis whether mast cells have a role in the induction of muscular cell hyperplasia in experimental EoE.
of muscle cell hyperplasia, resulting in hypertrophy in EoE. Accordingly, we induced experimental EoE in wild-type and WW\(^v\) mice. A thickened muscular mucosa and a number of BrdU\(^+\) cells were detected in muscular mucosa of allergen-challenged wild-type mice. The BrdU\(^+\) cells were commonly detected in the epithelial mucosa and rarely found in muscular mucosa of saline-challenged mice (Fig. 6, A and B). The allergen-challenged wild-type mice showed a significant increase in BrdU\(^+\) muscle cells and the muscular mucosa thickness compared with the saline-challenged wild-type and WW\(^v\) mice (Fig. 6, C and D). The histological photomicrograph clearly demonstrates muscular hypertrophy following allergen challenge as a bundle of smooth muscles separated by fibrous septa and smooth muscle fibers with enlarged and irregular nuclei in the mucosa (Fig. 6B). The morphometric analysis revealed that BrdU\(^+\) cells in the muscular mucosa of allergen-challenged wild-type and WW\(^v\) mice were 17.5 ± 12.0/mm\(^2\) and 2.1 ± 4.4/mm\(^2\) compared with a nondetectable value in saline-challenged wild-type and WW\(^v\) mice (mean ± SD, \(n = 12\)).

Reconstitution of mast cells in the WW\(^v\) shows comparable muscle cell hyperplasia and hypertrophy. Furthermore, to establish whether mast cells have a critical role in esophageal muscle cell hyperplasia and hypertrophy in EoE, we generated bone marrow-derived mast cells and adoptively transferred in mast cell-deficient WW\(^v\) mice. Both wild-type mice and mast cell-reconstituted WW\(^v\) mice were challenged with Aspergillus extract to induce experimental EoE. Each mast cell-reconstituted WW\(^v\) mouse was examined for mast cells before being included in the experiment as stated in MATERIALS AND METHODS, and, following the allergen challenge, both wild-type and mast cell-reconstituted WW\(^v\) mice indicated mast cells in the esophagus (Fig. 7, A and B). Our analysis showed comparable BrdU\(^+\) esophageal muscle cells and muscular mucosa thickness in allergen-challenged wild-type and mast cell-reconstituted WW\(^v\) mice (Fig. 7, C and D). The morphometric analysis revealed that BrdU\(^+\) cells in the muscular mucosa of wild-type and mast cell-reconstituted WW\(^v\) mice were 20.8 ± 7.4/mm\(^2\) and 17.6 ± 8.3/mm\(^2\) compared with 1.1 ± 2.3/mm\(^2\)
and 3.3 ± 4.7/mm² in saline-challenged wild-type and mast cell-reconstituted WWv mice (mean ± SD, n = 6–8). Furthermore, the muscular mucosa thickness in wild-type and mast cell-reconstituted WWv mice was 119.3 ± 22.8/µm and 98.6 ± 20.7/µm compared with 78.4 ± 18.2/µm and 61.7 ± 5.9/µm in saline-challenged wild-type and mast cell-reconstituted WWv mice (mean ± SD, n = 6–8).

**DISCUSSION**

The pathogenesis of EoE is largely focused on the role of allergens, allergen-induced Th2 cytokines, chemokines including esophageal eosinophils, and antigen-induced antibodies (20, 21, 23). Herein, we report that esophageal mast cells increase in parallel with eosinophils in a dose- and time-dependent manner in experimental EoE, which is in accordance with the a number of earlier reported studies of human EoE (2, 9, 17, 19, 25, 30). We earlier showed that the esophagus is devoid of eosinophils (22), but mast cells are resident cells in the esophagus. Our present study demonstrated that two different pathways are operational in mast cells and eosinophil recruitment in EoE. Earlier, few reports indicated that mast cells produce eosinophil chemotactic factor, eotaxin-1 (8, 32); therefore, our study tested whether esophageal mast cells are responsible for the esophageal eosinophilia in allergen-induced EoE. We demonstrated that the recruitment of allergen-induced esophageal eosinophilia is independent to the mast cell function in EoE. Additionally, we also demonstrated that mast cell induction in allergen-induced EoE is independent to IL-5, in contrast to esophageal eosinophilia that is largely dependent on IL-5 (20). However, the tissue distribution of eosinophils and mast cells in the EoE indicated a possible crosstalk between these two types of inflammatory cells. Our data provide the evidence and demonstrate that mast cells localize near the eosinophils in the lamina propria, and we show extracellular mast cell granules and MBP immunoreactivity along with the increase of mast cell product MMCP-1 in
In conclusion, this study suggests an important role for mast cells in EoE pathogenesis in a number of ways. First, in experimental EoE, esophageal mast cell numbers increase and their accumulation is independent of IL-5. Second, an increase of mast cell mediators in the blood and esophagus can be seen, at least in the setting of allergen-induced experimental EoE. Third, mast cells infiltrate and degranulate into the esophageal muscular mucosa. Lastly, mast cells have a critical role in muscular cell hyperplasia and possibly esophageal functional impairment in EoE. Taken together, the present study directly implicates mast cells in the pathogenesis of EoE.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: R.N., P.M., and M.R. performed experiments; R.N. and S.D. prepared figures; S.D. analyzed data; A.M. conception and design of research; A.M. drafted manuscript.

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