

Characterization of mast cell subtypes, distribution, and antigen-induced activation in the guinea pig esophagus

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SUMMARY. The number of esophageal mucosa mast cells (MCs) increases in allergic and inflammation conditions in the esophagus, but their role in these conditions is less clear. MCs are derived from bone marrow, migrate and mature in the peripheral tissues. Two subsets of MCs have been characterized as mucosal MC (MMC) and connective tissue MC (CTMC) defined by anatomic location, granule contents, and functions. Whether esophageal MCs share typical features with either MMC or CTMC has yet to be determined. This study characterized esophageal MCs subtypes, distribution, antigen-induced sensitization, and degranulation as measured by MC staining and histamine release assay. Immunofluorescent double staining of MC tryptase and chymase were performed in the esophagus, intestine, and skin from normal and ovalbumin (OVA) actively sensitized guinea pigs. Histamine release was measured in the esophagus from OVA-sensitized guinea pigs following *in vitro* antigen challenge. Similar to the MCs in the intestine and skin, esophageal MCs contained three subtypes, which included 62% MCTC (tryptase+/chymase+), 17% MCC (chymase+/tryptase–), and 21% MCT (tryptase+/chymase–). In contrast to the ileal MCs, which were distributed all over the mucosa, submucosa, and serosa, MCs in the esophagus almost all (more than 98%) lined along the lamina propria. OVA active sensitization significantly increased the esophageal MC subtype MCTC. OVA *in vitro* challenge of the esophagus from sensitized guinea pig significantly decreased tryptase-positive MC subtypes MCTC and MCT, and released a significant amount of tissue histamine content. In conclusion, MCs in the guinea pig esophagus have unique features in immunophenotypes, distribution, and degranulation response to OVA challenge with the release of significant amounts of proteases and histamine into the tissue. These characteristics may indicate that OVA *in vitro* challenge in OVA-sensitized guinea pig esophagus could be a good model to study the role of esophageal MCs in allergic and inflammation conditions.

KEY WORDS: chymase, esophagus, histamine, mast cell, ovalbumin, tryptase.

INTRODUCTION

Mast cells (MCs) are heterogeneous tissue cells found in the host defense system and participate mainly in the immediate-type hypersensitivity reaction, as well as in the normal physiological function.^{1,2} Derived from bone marrow, MC progenitors migrate to the peripheral tissue from the circulation, differentiate *in situ*, and mature in the settled microenvironments in the presence of stem cell factor. The different microenvironment determines MC heterogeneity.

There are two classical subsets of MCs in humans and rodents defined by anatomic location, granule contents, and functions: mucosal MC (MMC) and connective tissue MC (CTMC). MMC are mainly found in intestinal mucosa with low histamine content and high T cell dependence. In contrast, CTMC are predominately seen in the skin and have high histamine content and little T cell dependence.³ Immunostaining of MCs granule contents of tryptase and chymase in rodents⁴ and humans⁵ revealed that three immunophenotypes of MCs are found: tryptase positive only (MCT), chymase positive only (MCC), and positive for both (MCTC). Tryptase is present in all human and most rodent MCs, but not in any other cell type. When activated, MCs release tryptase into the tissue. At this moment, the MCs themselves do

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not disappear; the ability to stain the MC granules disappears. Therefore, the changes in the number of tryptase-positive MCs are well accepted as a biomarker for MC activation.

MCs are present throughout the gastrointestinal (GI) tract. They are mainly located in the lamina propria of the mucosal layer, but have also been identified in the epithelium of mucosal layer and in the submucosal layer.⁶ In general, MCs located in the mucosal layer are MMC, whereas in the submucosal layer are CTMC. All three immunophenotypes of MCs have been identified in humans,⁷ rats,⁸ and cats⁹ in the stomach, intestine, and colon. There are numerous investigations of the roles of MCs in the stomach, intestine, and colon in physiological and pathological conditions. Little is known about esophageal MCs with respect to their phenotypes, distribution, and functions.

The number of esophageal mucosa MCs increase in allergic and inflammation conditions.^{10,11} Our previous studies^{12,13} showed that activation of esophageal MCs in the guinea pig by OVA challenge increased esophageal vagal nociceptive C fibers' mechano-excitability, and OVA inhalation induced MC and eosinophils infiltration in the esophageal epithelium. These results indicate that MCs play an important role in esophageal inflammatory reaction and nociception. The esophagus is a unique part of the GI tract with respect to embryological origin, anatomic location, and epithelial histology. This unique microenvironment suggests that esophageal MCs may have features that resemble either MMC or CTMC. MC granule contents in the guinea pig are mainly histamine and tryptase, which more closely resemble those in the human than the rat and mouse.¹ Thus, OVA challenge to induce MC activation in OVA-sensitized guinea pig has been applied as a reliable and clinical relevant asthma model to study airway sensory nerve and smooth muscle functions. Whether MCs in the guinea pig esophagus have distinct morphological (immunophenotypes) and functional (release granule contents) features has yet to be determined. The present study aimed to characterize esophageal MCs with respect to subtype, distribution, and response to antigen-induced degranulation and histamine release in the guinea pig.

MATERIALS AND METHODS

Active immune sensitization and allergen challenge

The study protocol was approved by the Animal Care and Use Committee (ACUC) at the Penn State University College of Medicine. Adult male guinea pigs (200–400 g, Hilltop, Scottsdale, PA, USA) were actively sensitized and challenged by allergen as previously described.^{12,13} Briefly, guinea pigs were given three intraperitoneal injections with ovalbumin

(OVA, 10 mg/kg) every 48 h. Three weeks after the last injection, guinea pigs were euthanized by intraperitoneal administration of excess sodium pentobarbital and exsanguination. The esophagus (4 cm from esophageal–gastric junction), stomach, intestine (10 cm from pylorus), and skin were then removed and challenged with OVA (10 µg/mL, 20 min). Control tissues were treated with phosphate-buffered saline (PBS). Histamine release assay was conducted in the esophageal tissue according to the reported method.¹² Immunofluorescent double-staining of MC tryptase and chymase were performed on selections from the esophagus, intestine, and skin. All procedures were performed according to the methods reported previously^{12,13} and briefly described below.

Immunofluorescent double staining of MC tryptase and chymase

The dissected tissues were immersed in the Krebs bicarbonate solution (KBS) containing antigen (OVA, 10 µg/mL) for 20 min to induce MC degranulation by antigen–antibody response and compared with control tissues that were immersed in KBS without antigen. After challenge, the tissues were fixed in 4% formaldehyde in PBS overnight at 4°C. The tissues were rinsed in PBS and cryoprotected with 18% sucrose in PBS for 18–24 h. The tissues were then covered with optimum cutting temperature mounting medium and frozen on dry ice. Tissues were cut in serial sections of 12-µm thickness on a cryostat, mounted on silane-coated slides, and air dried for 30 min.

Sections were incubated with blocking solution containing 1% bovine serum albumin (BSA), 10% normal goat serum, and 0.1% Tween 20 in PBS for 60 min. Immunofluorescent double labeling of MC chymase and tryptase was performed by first labeling chymase, and then tryptase. Sections were incubated overnight at 4°C with mouse antihuman MC chymase monoclonal antibody (diluted at 1 : 100, Chemicon International, Temecula, CA, USA) in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (PBS–TX–BSA). Slides were washed in PBS–TX–BSA and incubated with goat antimouse labeled with Alexa 594 (diluted 1 : 100, Molecular Probe, Eugene, OR, USA) in PBS–TX–BSA for 2 h in room temperature. After washing with PBS and TBS, sections were then incubated overnight with biotinylated mouse antihuman tryptase monoclonal antibody (diluted 1 : 300, Promega, Madison, WI, USA). Slides were then washed in PBS–TX–BSA and incubated with Alexa 488-conjugated antibiotin mouse monoclonal antibody (diluted 1 : 300, Molecular Probe) in PBS–TX–BSA for 2 h in the room temperature. The sections were rinsed with PBS and with saline, buffered with phosphate to pH 8.6, coverslipped. The slides were analyzed and counted by a

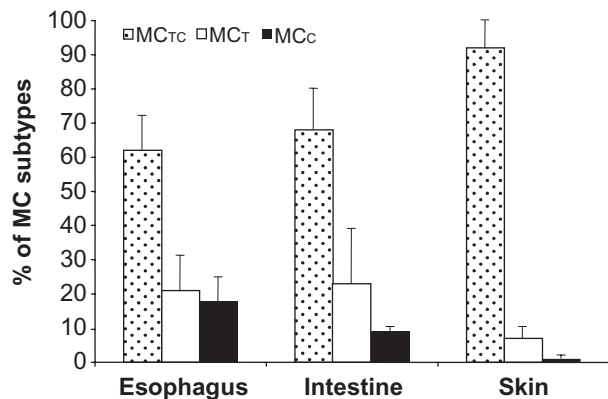


Fig. 1 Mast cells (MCs) in the esophagus included three subtypes as classified by immunostaining of MC tryptase and chymase as: $62 \pm 10\%$ MCTC (tryptase+/chymase+), $21 \pm 10\%$ MCT (tryptase+/chymase-), and $17 \pm 7\%$ MCC (tryptase-/chymase+) ($n = 7$). Similar subtypes were also observed in the intestine ($68 \pm 12\%$ MCTC, $23 \pm 16\%$ MCT, and $9 \pm 1\%$ MCC, $n = 7$) and the skin ($92 \pm 8\%$ MCTC, $7 \pm 3\%$ MCT, and $1 \pm 1\%$ MCC, $n = 7$).

conventional epifluorescence microscopy (Olympus DX60, Olympus Imaging America, Inc., Center Valley, PA, USA), with the filters set to allow separate visualization of Alexa 488 (fluorescein) or Alexa 594 (rhodamine) in a computer-aided digital image system.

Histamine release assay

Six OVA-sensitized animals were used for histamine release assay. The whole esophagus was removed and dissected. Specimens were first immersed in 2 mL of oxygenated KBS solution (37°C) for 20 min to measure the spontaneous release of histamine. The specimens were then transferred into the KBS containing antigen (OVA, 10 µg/mL) for another 20 min to measure histamine release following OVA challenge. One hundred fifty microliter of supernatant from all samples were aliquoted and stored at -70°C. Supernatant was incubated with Tris HCl buffer containing 0.5 mM/L substrate to measure histamine release by a microtiter plate reader (absorbance 405 nm) and normalized to the wet weight of the specimens by the end of the experiment.

Data analysis

Seven nonsensitized animals were used to study MCs subtypes and distribution in the esophagus, intestine, and skin. Seven OVA-sensitized animals were selected to compare esophageal MCs subtypes before and after OVA challenge. The average numbers of MCs from each animal were counted and calculated from three sections (upper, middle, and lower parts) of the esophagus. The numbers of MCT, MCC, and MCTC were counted in the cross-section of the

tissues. The densities of MCs were presented as total numbers per cross-sections of the esophagus. The percentage of each subtype of MCs was also calculated as the number of each cell subtype divided by the combined numbers of the three subtypes. Six OVA-sensitized animals were used for histamine release assay. Histamine release was expressed as the percentage of the total histamine content of the tissue. Values were expressed as mean $\pm$ standard error of the mean. Differences between the values were determined by Student's *t*-test, and $P < 0.05$ was considered statistically significant.

RESULTS

MCs subtypes

The total numbers of MCs that stained positively for tryptase, chymase, and both in cross-sections of the sampled tissues from unsensitized guinea pigs were counted. Three immunophenotypes of MCs were thus found in the guinea pig esophagus, intestine, and skin as: tryptase positive/chymase negative (MCT), chymase positive/tryptase negative (MCC), and tryptase positive/chymase positive (MCTC). By subtracting total tryptase-positive MCs with MCTC, we had the number for MCT – so did for the MCC. In the esophagus, 62% MCs were MCTC with 17% MCC and 21% MCT. This was similar to the subtype percentages found in the intestine, where 68% were MCTC with 9% MCC and 23% MCT, but different from the skin, where 92% were MCTC with 1% MCC and 7% MCT (Figs. 1 and 2). This indicates that esophageal MCs shared more features with respect to immunophenotype with intestinal MCs rather than skin MCs.

MCs distribution

MCs distribution was studied by examining all three immunosubtypes of MCs in the cross-sections of the esophagus from unsensitized guinea pigs. These three subtypes of MCs in the esophagus were almost all (>98%) distributed in the lamina propria of the

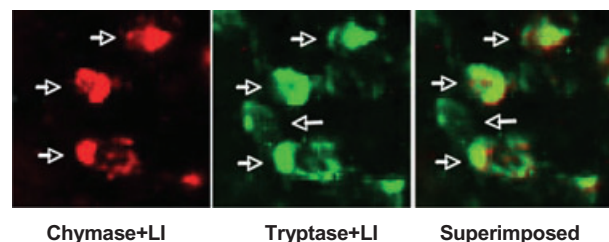


Fig. 2 Typical immunostaining of mast cell (MC) subtypes as shown here: MCTC (superimposed three tryptase+/chymase+ labeled cells as indicated by '→') and MCT (one tryptase+/chymase- labeled cell as indicated by '←').

mucosal layer, with a few labeled cells in the serosa. There were no immunofluorescent-labeled cells in the squamous epithelium and the submucosa. This was similar to the MCs in the skin, where more than 98% were located in the dermis, but differed from the intestine, where MCs were found in all layers with 53% in the mucosa (epithelium and lamina propria) and 47% in the submucosa/serosa. This data demonstrated that MCs in the esophagus, unlike classical 'mucosal' MCs in the intestine, were almost all located in the lamina propria of the mucosal layer, but not present in the mucosal epithelial and submucosal layers.

MCs degranulation

MC degranulation was studied by comparing the changes of MC subtypes in the esophagus before and after *in vitro* OVA challenge in OVA-sensitized animals. Repeated injection with OVA induced active sensitization, resulting in significant increases in MC subtype MCTc in the esophagus (10 ± 3 vs. 34 ± 10 /cross-section, $P < 0.05$, $n = 7$). The tissues from sensitized animals were then challenged *in vitro* with OVA to induce MC degranulation. We observed that OVA challenge (10 $\mu\text{g/mL}$, 20 min) caused significant decreases in MCTs in the esophagus (34 ± 10 vs. 5 ± 1 /cross-section in MCTc; 4 ± 1 vs. 0.7 ± 0.3 /cross-section in MCT, $P < 0.05$, $n = 7$) (Fig. 3).

Histamine release

MC activation was studied by measuring percent releases of the total histamine contents from OVA-sensitized guinea pig esophagus after *in vitro* challenge with OVA. OVA *in vitro* challenge released a

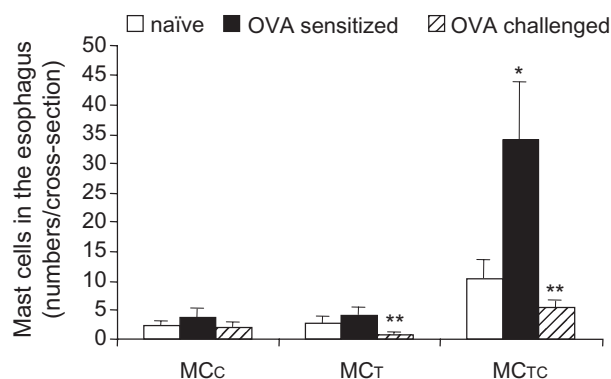


Fig. 3 Ovalbumin (OVA) active sensitization (10 mg/kg i.p. three times) increased mast cells (MCs) in the esophagus, but only significant in the subtype of MCTc (naïve vs. OVA sensitized: 10 ± 3 vs. 34 ± 10 , $*P < 0.05$, $n = 7$). OVA challenge (10 $\mu\text{g/mL}$) *in vitro* significantly induced MC degranulation as reflected by the decreases in immunostaining of MCTc (OVA sensitized vs. OVA challenged: 34 ± 10 vs. 5 ± 1 , $**P < 0.05$, $n = 7$), and MCT (OVA sensitized vs. OVA challenged: 4 ± 1 vs. 1 ± 0.3 , $**P < 0.05$, $n = 7$).

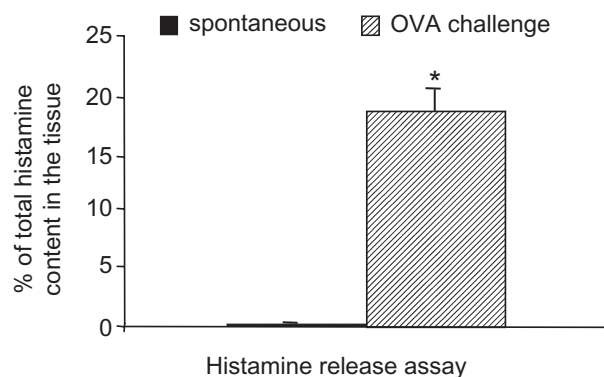


Fig. 4 Ovalbumin (OVA) challenge (10 $\mu\text{g/mL}$ for 30 min) in sensitized guinea pig esophagus significantly induced mast cell degranulation to release histamine in the esophagus ($17.4 \pm 1.3\%$ vs. spontaneous $0.15 \pm 0.03\%$ of total content of histamine in the esophagus, which is $4.9 \pm 0.5 \mu\text{g/g WW}$, $P < 0.001$, $n = 6$).

significant amount of the histamine content from the esophagus compared with spontaneous release ($17.4 \pm 1.3\%$ vs. $0.15 \pm 0.03\%$, $P < 0.001$, $n = 6$) (Fig. 4).

DISCUSSION

This study demonstrated that MCs in unsensitized guinea pig esophagus are almost all distributed in the lamina propria of the mucosal layer. They express three immunophenotypes: MCTc, MCT, and MCC. Active sensitization with OVA increased the number of MCs, especially MCTc subtypes, in the esophagus. OVA challenge *in vitro* induced MCs degranulation as measured by the decreased number of MCs containing tryptase (MCTc and MCT) and released significant amount of histamine from the OVA-sensitized guinea pig esophagus. These indicate that esophageal MCs could be actively sensitized by repeat antigen exposures *in vivo* and activated by antigen challenge *in vitro*. MCs activation releases a significant amount of granule content tryptase and histamine into the adjunctive tissue, which may play important roles in inflammation and inflammatory nociception.

MCs are heterogeneous tissue cells found at the body sites exposed to the external environment, such as skin, airway, and GI tract. The presence of MC in the esophagus had been reported in humans,⁷ rats,^{14,15} mice,¹⁶ and opossums.¹⁷ In the human esophagus, MCs were not usually present in normal human esophageal epithelium, but increased in idiopathic eosinophilic esophagitis,¹⁸ and MCs occurred in esophageal epithelium and lamina propria in reflux esophagitis and in intestinal metaplasia of Barrett's esophagus.¹¹ Consistent with our previous studies,^{12,13} the present study has shown that in the normal guinea pig esophagus, almost all three MC subtypes are located in the lamina propria of the mucosal layer,

with a few scattered in the serosa. There are no MCs in the esophageal epithelium. This distribution pattern is in agreement with previous studies that revealed none or only scattered MCs occurred in the esophageal epithelium in humans,⁷ rats,¹⁵ and mice.¹⁶ In comparison with the MCs' distribution in the intestine and skin, we also demonstrate that esophageal MCs' distribution pattern is unlike mucosal (intestine) MCs, where MCs are identified throughout the mucosal (epithelium and lamina propria), submucosa, and serosa. The embryological origin of the esophagus is different than the intestine. This may determine that the esophagus has its unique anatomic and histological features. When MC progenitors migrate into this distinct microenvironment, they may therefore mature and distribute differently from MCs in the intestine.

The major granule contents of MCs are histamine and neutral proteases. The neutral proteases include trypsin, chymase, carboxypeptidase, and cathepsin G. The contents of proteases in MCs are varied in different body sites in human and rodents because the expression of these granule proteases is regulated by local microenvironment. Three subtypes of MCs have been identified in humans and rodents by immunostaining of proteases in the tissues named as trypsin and chymase in human, and MC proteinase in rodents.^{19,20} Thus, MCs can be classified into three immunophenotypes as MCTC, MCT, and MCC. MCs from human intestine, skin, and lung encompassed all three immunophenotypes, whereas MCTC and MCT had distinct ultrastructural features.²¹ Another study showed that almost all MCs in human gut contained trypsin, and only a limited number of MCs, mainly in the submucosa, were positive to chymase.²² Human MCs phenotypes from different body sites were varied: in skin, more than 95% MCs were MCTC with rare MCT and MCC; in bowel mucosa, 58% MCs were MCT with 35% MCTC and 7% MCC; in bowel submucosa, 83% MCs were MCTC with 17% MCC; in esophageal epithelium, 38% MCs were MCT with 62% MCTC; and all MCs in esophageal subepithelium were MCTC.⁷ In rats and mice, MCTs were not detected in the bowel mucosa but in the typical CTMCs, such as skin, lung, and peritoneal cells, whereas there were also chymase positive staining appearing in parts of these MCTs.⁸ In the present study, we demonstrated that all three immunophenotypes of MCs were detected by immunostaining method in guinea pig esophagus, intestine, and skin.

MC activation may release mediators and cause subsequent immediate hypersensitivity. The released major granule contents, histamine and trypsin, are usually applied as the markers of MC activation, and histamine content is correlated well with MC number in the tissue. A previous study on MCs in rat GI tract¹⁴ showed that MCs in the esophagus were CTMCs with respect to their histamine content and

response to compound 48/80. This type of MCs contained higher histamine levels (1.29 pg/cell) than mucosal MCs (0.15 pg/cell) from glandular stomach and small and large intestine. Compound 48/80 caused decreases in both the number of MCs and histamine in the tissue from connective MCs, but not from mucosal MCs. The results from the present study are consistent with such findings in rats. *In vitro* antigen challenge in actively sensitized guinea pig induced MC activation as demonstrated by MC degranulation and histamine release in the tissues. The numbers of MCs subtypes (MCTC and MCT) from the esophagus significantly decreased after OVA challenge. When challenged with antigen *in vitro*, the esophagus released a significant amount of tissue histamine content. These two features in MC activation indicated that MCs in the guinea pig esophagus functionally act like typical CTMCs.

In summary, MCs in normal guinea pig esophagus are mainly distributed in the lamina propria of the mucosal layer with three immunophenotypes: MCTC, MCT, and MCC. OVA-active sensitization increased MC subtype MCTC in the esophagus. OVA challenge induced MC degranulation as indicated by a significant decrease in MCTC and MCT, and a significant increase in histamine release from the esophagus. These characteristics may help to study the role of esophageal MCs in allergic and inflammation conditions.

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