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ORIGINAL ARTICLE

Submucosal connective tissue-type mast cells contribute to the production of lysophosphatidic acid (LPA) in the gastrointestinal tract through the secretion of autotaxin (ATX)/lysophospholipase D (lysoPLD)

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Abstract Lysophosphatidic acid (LPA) is involved in a broad spectrum of biological activities, including wound healing and cancer metastasis. Autotaxin (ATX), originally isolated from a melanoma supernatant as a tumor cell motility-stimulating factor, has been shown to be molecularly identical to lysophospholipase D (lysoPLD), which is the main enzyme in the production of LPA. Although ATX/lysoPLD is known to be widely expressed in normal human tissues, the exact distribution of ATX-producing cells has not been fully investigated. In this study, we evaluated ATX/lysoPLD expression by immunohistochemical staining using a rat anti-ATX mAb in the human gastrointestinal tract and found that submucosal mast cells (MC) highly expressed this enzyme. This was confirmed by immunofluo rescent double staining using mAbs to tryptase and chymase. Then, we isolated MC from human gastric tissue by an immunomagnetic method using CD117-microbeads and showed that a subpopulation of CD203c-positive MC showed positive staining for intracellular ATX/lysoPLD on flowcytometry. This was confirmed by Western blotting of the isolated cells. Moreover, a significant level of ATX/lysoPLD release could be detected in the culture supernatants of human MC by Western blot analysis. Our data suggest that submucosal MC play significant roles in various aspects of pathophysiology in the gastrointestinal tract by locally providing bioactive LPA through the production of ATX/lysoPLD.

Keywords Autotaxin · Lysophpsphatidic acid · Mast cell · Gastrointestinal tract

Abbreviations

ATX	autotaxin
lysoPLD	lysophospholipase D
LPA	lysophosphatidic acid
MC	mast cell
NPP	ecto-nucleotide pyrophosphatase / phosphodiesterase

Introduction

Autotaxin (ATX) was originally isolated from a melanoma cell supernatant as a 125-kD glycoprotein that stimulates tumor cell motility in a pertussis toxin-sensitive manner [55, 57]. Thereafter, more evidence for a positive role of ATX in tumor metastasis has been demonstrated by both in vivo and in vitro studies [36–38, 56, 70]. ATX is strongly detected in the culture media of various cancer cells, and its mRNA is overexpressed in various human malignancies, such as lung, breast, and renal cell carcinoma [53, 70, 71]. These findings support that ATX functions as an autocrine motility factor in the step of tumor progression. ATX was initially identified as a member of the ecto-nucleotide

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pyrophosphatase/phosphodiesterase (NPP) family and was termed NPP2 [56]. The NPP family is composed of three ecto-enzymes that hydrolyze phosphodiester and pyrophosphate bonds in nucleotides [9, 18]; however, its molecular structure relating to stimulation of cell motility is an enigma. On the other hand, lysophospholipase D (lysoPLD), which converts lysophosphatidylcoline (LPC) to lysophosphatidic acid (LPA, 1- or 2-acyl-*sn*-glycero-3-phosphate), was found to be abundant in plasma and serum [3, 62]. LPA is a lipid mediator of a broad range of cellular responses, including smooth muscle contraction, platelet aggregation, neurite retraction/cell rounding, regulation of proliferation, protection from apoptosis, and modulation of chemotaxis and transcellular migration [19, 35]. LPA elicits most of its cellular responses via transduction cascades downstream of its specific G protein-coupled receptors, LPA1/Edg-2, LPA2/Edg-4, and LPA3/Ede-7, which belong to the endothelial cell differentiation gene (Edg) family [2, 4, 11, 24]. Some of these cellular responses implicate LPA as a mediator of tumor progression [31, 34, 50–52]. Recently, molecular cloning of lysoPLD revealed that this enzyme was identical to ATX [63, 66]. As LPC is abundantly present in plasma and tissues (exceeding 100 μM), ATX/LysoPLD is considered to be a key enzyme in the production of LPA in vivo [3, 12, 61]. Therefore, it is generally accepted that the effects of ATX/LysoPLD in cancer progression (including proliferation, migration, and angiogenesis) are mostly attributable to the production of LPA from LPC or more complex lysophospholipids [66, 67]. Although the mechanism of catalysis of ATX/lysoPLD is well characterized, its post-translational processing, regulation of expression, and mechanism of release from cells are not well understood. ATX is widely expressed, with highest mRNA levels detected in brain, placenta, intestine, and ovary [36]. However, the cellular expression of ATX/lysoPLD in each tissue remains unclear. Herein, by immunohistochemical study using a specific monoclonal antibody, we assessed the cellular distribution of ATX/LysoPLD in the human gastrointestinal tract, and found that submucosal mast cells (MC) constitutively express ATX/lysoPLD.

Materials and methods

Reagents and human samples

Rat anti-ATX/lysoPLD monoclonal antibodies (2A12, 4F1) were generated by the immunization of rat with a polypeptide (amino acids 58–182 of human autotaxin) at the Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan. The specificity [60] and immunoreactivity for tissue section of these mAbs were evaluated previously [5, 46]. Antihuman tryptase mAb (AA1) and antihuman chymase mAb (CC1) were purchased from Dako (Carpinteria, CA) and Serotic (Oxford, UK), respectively, and both of their subclasses were IgG1.

Surgical specimens were obtained from seven patients with primary gastric carcinoma treated by total gastrectomy and seven patients with primary colon cancer treated by right hemicolectomy in the University of Tokyo, with written informed consent. Normal parts of the stomach or colon in those specimens, more than 5 cm from the tumor edge, were used for staining experiments. The ileum of the oral margin of right hemicolectomy specimens and the jejunal margin used for reconstruction after gastrectomy was used for evaluation of the small intestine.

Immunohistochemical study

Paraffin-embedded sections, 5 μm thick, were deparaffinized in xylene, hydrated through a graded series of ethanol, then immersed in 3% hydrogen peroxide in 100% methanol for 30 min to inhibit endogenous peroxidase activity. To activate the antigens, the sections were boiled in 10 mM citrate buffer pH 6.0 for 15 min. After being rinsed in phosphate-buffered saline (PBS), the sections were incubated with normal rabbit serum for 30 min and incubated overnight at 4°C in humid chambers with primary antibodies to tryptase (AA1, dilution 1:100), chymase (CC1, dilution 1:100), or ATX/lysoPLD (2A12, dilution 1:50). After washing with PBS, the sections were incubated with biotinylated rabbit anti-mouse immunoglobulin (Nichirei, Tokyo, Japan) for 20 min. After washing again with PBS, the slides were treated with peroxidase-conjugated streptavidin for 10 min and developed by immersion in 0.01% H_2O_2 and 0.05% diaminobenzidine tetrahydrochloride. Light counterstaining with Mayer's hematoxylin was performed. All cases had a negative control that was run simultaneously with the test slide, in which control rat IgG was used as the primary antibody. Tryptase-, chymase-, and ATX/LysoPLD-positive cells were counted in five different fields selected at random, using a microscope (and expressed as number per ×200 field).

Immunofluorescent double staining

To identify the two molecules in the same specimen, immunofluorescent double staining was performed on frozen sections. The surgically removed specimen was immediately fixed with acetone for 10 min and immersed in 3% hydrogen peroxide in 100% methanol for 30 min. Then, sections were incubated with normal rabbit serum for 30 min and incubated overnight at 4°C in shaded humid chambers with primary antibodies to tryptase or chymase. After washing with PBS, sections were incubated for 2 h with rhodamine-conjugated rabbit anti-mouse IgG (Chemicon International, Temecula, CA). Then, sections were incubated overnight with rat anti-

ATX antibody chemically conjugated with fluorescein isothiocyanate (FITC) (FITC-conjugated 4F1, dilution 1:10). The sections were observed with a confocal laser microscope (Fluoview, Olympus, Tokyo, Japan).

Buffer for cell preparation

Tris–ethylenediaminetetraacetic acid (EDTA) (TE) buffer is Eagle's minimum essential medium (MEM, Gibco, Berlin, Germany) containing 2 mM EDTA. TGMD buffer is TE buffer supplemented with 1 mg/ml gelatin, 1.23 mM $MgCl_2$, and 15 μg/ml DNAse. Hepes buffer contains 20 mM Hepes, 125 mM NaCl, 5 mM KCl, and 0.5 mM glucose. HA buffer is Hepes buffer plus 0.25 mg/ml bovine serum albumin (BSA).

Cell preparation

The normal parts of the gastric wall were resected, and tissues containing the mucosal and submucosal layers were separated from the muscularis propria layer using scissors and used for cell separation. MC were isolated by a four-step enzymatic tissue dispersion method as described previously with some modifications [7, 8, 49]. The tissues were cut into fragments with scissors and incubated in TE buffer containing 1 mg/ml acetylcysteine for 10 min at room temperature to remove mucus and then in TE buffer containing 5 mM EDTA for 15 min at 37°C to detach epithelial cells. After washing in TE buffer, the tissue was incubated in TE buffer containing 3 mg/ml pronase and 0.75 mg/ml chymopapain at room temperature. Pronase and chymopapain were purchased from Roche (Mannheim, Germany). During this first digestion step, the tissue was cut into smaller pieces using scalpels. After 30 min, the free cells (fraction 1) were separated from tissue fragments by filtration through a polyamide Nybolt filter (pore size 300 μm). The remaining tissue fragments were washed in TE buffer, and the first digestion step was repeated at 37°C (fraction 2). The tissue fragments were then washed in TGMD buffer and incubated twice for 30 min at 37°C in TGMD buffer containing 1.5 mg/ml collagenase D (Roche, Mannheim, Germany) and 0.15 mg/ml elastase (Roche, Mannheim, Germany). The freed cells were separated from the digested tissue by filtration (fraction 3 and 4). Fraction 3 and 4 were filtered through a Nyblot filter (pore size 100 μm) again and resuspended in HA buffer. Cell viability was measured using trypan blue staining, and MC counts were performed using Alcian blue at each step.

Mast cell purification

Dispersed cells containing approximately 1% MC were purified as described previously with some modifications [7, 39, 49]. In brief, the dispersed cells were incubated with anti-CD117 mAb-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C. Then, CD117-positive cells were enriched by passing through a magnetic column twice at 1 ml/min using the "POSSEL DS" program of the AutoMACS system which enables the fast and efficient positive selection of rare population with low immunogenic epitope (Miltenyi Biotec, Bergisch Gladbach, Germany). "POSSEL DS" means "double sensitive positive selection".

The purified MC were dispersed on slides by centrifugation for 10 min (×500 rpm) and fixed with acetone. Then, the slide samples were incubated with biotinylated anti-tryptase (AA1) or anti-chymase (CC1) mAbs followed by incubation with peroxidase-conjugated streptavidin and development by diaminobenzidine tetrahydrochloride as described above. Under the microscope, 100 cells were randomly selected in five to seven different fields, and cells positive for tryptase or chymase in those cell populations were calculated (per ×200 field).

Detection of ATX/lysoPLD by flow cytometry

Dispersed cells obtained after the four enzymatic steps were washed twice with PBS and fixed with acetone for 10 min at 4°C. Then they were permeabilized with 4% paraformaldehyde (PFA) for 10 min at 4°C. For two-color staining, cells were incubated with FITC-conjugated anti-ATX mAb and PE-conjugated anti-CD203c antibody (Immunotech, Marseille, France). Data were collected in a fluorescent activated cell sorting (FACS) Calibur (Becton Dickinson, Mountain View, CA) and analyzed using CellQuest software (Becton Dickinson).

Detection of ATX/lysoPLD by Western blot analysis

Immunoselected MC (2×10^6), which contained more than 70% Alcian blue-stained cells, were cultured in 8 ml Roswell Park Memorial Institute (RPMI) supplemented with 0.1% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY). As a control, the same numbers of negatively selected cells were also cultured in the same medium. After culture for 48 h, the proteins of the culture supernatants were extracted as described previously [66]. Then, 8 ml culture supernatant was concentrated to 100 μl by a Centricon Plus-20 (Millipore, Bedford, MA), and electrophoresed in sodium dodecyl sulfate (sodium dodecyl sulfate (SDS)) 7.5% polyacrylamide gel for 45 min at 200 V. Then, the protein was transferred onto an Immobilon transfer membrane (Millipore, Bedford, MA) for sequential incubation with 5% reconstituted nonfat milk powder to block nonspecific sites, dilutions of anti-ATX antibody, and horseradish peroxidase-labeled rabbit polyclonal anti-rat IgG, before development with a standard enhanced chemiluminenscence kit (Amersham, Inc., Buckinghamshire, United Kingdom).

Results

Immunostaining of MC in the gastrointestinal tract

MC were clearly identified in the stomach, small intestine, and colon by immunostaining with anti-tryptase antibody (AA1), as reported previously [27, 69]. Granules in the MC cytoplasm were stained brown. In the stomach, tryptase-positive MC were diffusely present from the mucosal to the muscular layer, but the number of MC in the submucosal layer was significantly greater than that in the mucosal or muscular layer (Fig. 1b). MC were also diffusely present in the small intestinal and colonic tissues (Fig. 1d).

Immunodetection of ATX/lysoPLD in the gastrointestinal tract

As shown in Fig. 1, there were many large oval cells whose cytoplasm was clearly stained by anti-ATX/lysoPLD antibody (2A12). The positive cells were detected in the mesenchymal area in the submucosal layer of the gastrointestinal tract, while they were rarely observed in the mesenchymal area of the mucosal or muscular layers (Table 1). Interestingly, in serial sections, most of the ATX-positive cells were morphologically similar to MC detected by anti-tryptase antibody (Fig. 1e,f).

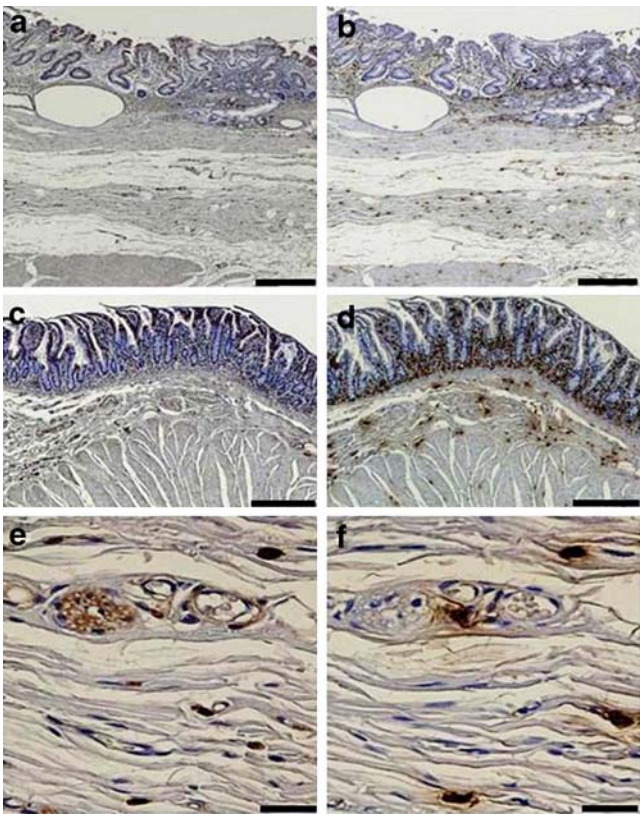

Fig. 1 Immunohistochemical staining of the stomach (**a**, **b**, **e**, **f**) or terminal ileum (**c**, **d**) with anti-ATX/lysoPLD mAb (2A12) (**a**, **c**, **e**) or anti-tryptase mAb (AA1) (**b**, **d**, **f**). Serial sections of submucosal area of the stomach with high magnification (**e**, **f**). *Bar* indicates 1,000 μm (**a**, **b**), 500 μm (**c**, **d**), and 50 μm (**e**, **f**). Immunoreactivity for ATX/lysoPLD was clearly detected in a subpopulation of tryptase positive cells

ATX/lysoPLD-positive cells were identified as a subtype of MC

To confirm that ATX/lysoPLD-positive cells are identical to MC, we performed immunofluorescent double staining in surgically removed gastric tissue. As shown in Fig. 2, when tryptase-positive MC were stained red and ATX/LysoPLD-positive MC green, ATX/LysoPLD-positive cells were detected with yellow fluorescence in merged views (Fig. 2c). On the other hand, some MC were detected as red in merged views, suggesting that some MC did not express ATX/LysoPLD (Fig. 2f). No cells were stained green in these frozen sections. This clearly indicates that the cells expressing ATX/LysoPLD in merged views are confined to a subpopulation of MC in the gastrointestinal tract.

Then, we performed double immunofluorescent staining with anti-chymase antibody (CC1). Most of the ATX/lysoPLD-positive MC were stained yellow in the merged view (Fig. 2i). When the number of ATX/lysoPLD-positive MC in tryptase- or chymase-positive MC was counted in 20 fields selected at random, 92% (82–96%) of chymase-positive cells expressed ATX/lysoPLD, whereas 68% (61–86%) of tryptase-positive cells expressed ATX/LysoPLD (Table 2). A previous study on human intestine showed that tryptase-positive MC exist in both the mucosal and submucosal layers, while most chymase-positive MC exist in the submucosal layer [27]. This appears to be consistent with our result that most of the MC in submucosal, but not mucosal layers, highly expressed ATX/lysoPLD.

Proportion of ATX/lysoPLD-positive MC in the gastrointestinal tracts

The proportion of ATX/lysoPLD-positive cells in tryptase-positive MC was comparatively evaluated in the stomach, intestine, and colon. As shown in Table 1, more than 50% of MC in the submucosal layer expressed ATX/lysoPLD in all organs examined (stomach: 52%, small intestine: 63%, colon: 56%). In contrast, only a small proportion of MC in the mucosal or muscular layer expressed ATX/lysoPLD (stomach: 7.1%, 8%, small intestine: 6.7%, 8%, colon: 14%, 4.1%).

Detection of intracellular ATX/lysoPLD in MC isolated from gut tissue

Enzymatic isolation provided at least 2×10^8 single cells containing about 1% MC, and their viability was at least 90%. After positive selection using magnetic affinity purification with anti-CD117, the majority of the cells were

Table 1 Distribution of tryptase and autotaxin (ATX/lysoPLD)-positive cells in each layer of gastrointestinal tract

Tryptase- and ATX/LysoPLD-positive cells were counted in five different fields using a microscope (×200 field), and the mean (min–max) of seven different samples were expressed.

Parts of the gastrointestinal tract	Layer	Tryptase(+)	ATX(+)	ATX(+)/Tryptase(+)
Stomach	m	16 (13–27)	1 (0–5)	7.1% (0–27%)
	sm	40 (17–77)	20 (3–35)	52% (18–69%)
	mp	56 (6–94)	4 (0–6)	8% (0–17%)
Small intestine	m	33 (10–54)	2 (1–4)	6.7% (2.7–10%)
	sm	41 (16–48)	26 (10–30)	63% (32–95%)
	mp	18 (3–35)	2 (0–6)	8% (0–50%)
Colon	m	20 (18–45)	3 (1–5)	14% (4–18%)
	sm	27 (18–29)	16 (14–36)	56% (47–90%)
	mp	15 (2–28)	1 (0–4)	4.1% (0–27%)

stained blue by Alcian blue (Fig. 3a) and anti-tryptase mAb (Fig. 3b). In contrast, cells stained with anti-chymase mAb were fewer than tryptase-positive cells (Fig. 3b). Then, to detect intracellular expression of ATX/lysoPLD, we performed double staining of the dispersed cells with anti-ATX/lysoPLD and anti-CD203c, which was determined as a specific cell surface antigen of human MC and basophils [17]. FACS analysis showed that more than 70% of the cell population positively expressed CD203c (Fig. 3c). When the CD203c-positive population was gated, 22% (13–27%) of CD-203c positive cells were clearly positive for intracellular ATX/lysoPLD, whereas the CD203c-negative population lacked expression of ATX/lysoPLD (Fig. 3d).

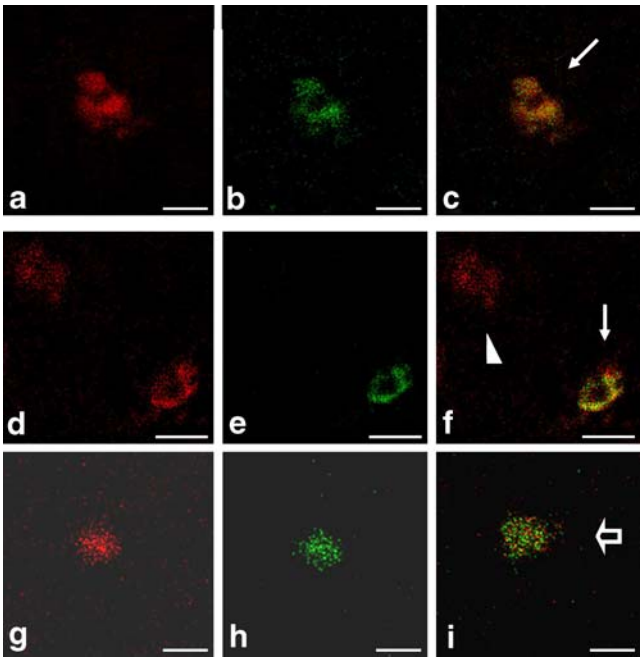

Fig. 2 Immunofluorescent double staining with anti-tryptase (AA1), anti-chymase (CC1), and anti-ATX/lysoPLD (4F1) mAbs. A frozen section of normal gastric tissue was stained as described in the Materials and Methods section. The section was observed with a confocal laser microscope (Fluoview, Olympus, Tokyo, Japan). The *bar* indicates 10 μm **(a–f)**. Double staining for tryptase in *red* (**a**, **d**) and ATX/lysoPLD (**b**, **e**) in *green*. In the merged view, a double-positive cell is stained *yellow*, indicating that the cell expresses ATX/lysoPLD and tryptase (*white arrows* in **c**, **f**). In contrast, a cell stained *red* in the merged view expresses tryptase but not ATX/lysoPLD (*white arrow head* in **f**). No cells are stained green in the merged view, indicating that ATX/lysoPLD-producing cells are restricted to a subtype of MC. **(g–i)** Double staining for chymase in *red* (**g**) and ATX/lysoPLD (**h**) in *green*. In the merged view, most of the cells are stained *yellow* [*open arrow* in (**i**)], indicating that most chymase-positive MC coexpress ATX/lysoPLD

Expression of tryptase or chymase in cell population isolated from gut tissue

As shown in Fig. 3b, 82% of the cells were positive for tryptase, while only 17% MC were positive for chymase (Table 3). This result is consistent with Gebhardt's report, showing that about 20% of purified MC expressed both tryptase and chymase [16]. From these results, it is supposed that the cell isolates from gut tissue by this method contain relatively more mucosal MC than submucosal MC.

Detection of the release of ATX/lysoPLD from MC by Western blot analysis

Finally, we examined the protein expression of ATX/lysoPLD and tryptase and chymase in MC-rich population.

Table 2 Proportion of ATX/lysoPLD-positive cells in tryptase- or chymase-positive cells

Cells	ATX/lysoPLD-positive cells	ATX/lysoPLD-negative cell	Percentage of ATX/lysoPLD-positive cells
Tryptase-positive cells	30 (27–44)	16 (5–19)	68% (61–86%)
Chymase-positive cells	26 (23–38)	2 (1–5)	92% (82–96%)

Frozen section of normal gastric or intestinal tissues were immunostained red by anti-tryptase or anti-chymase mAbs and green, by anti-ATX/lysoPLD mAbs. Positive cells were counted in 20 different fields, and the mean (min–max) in five different samples were expressed.

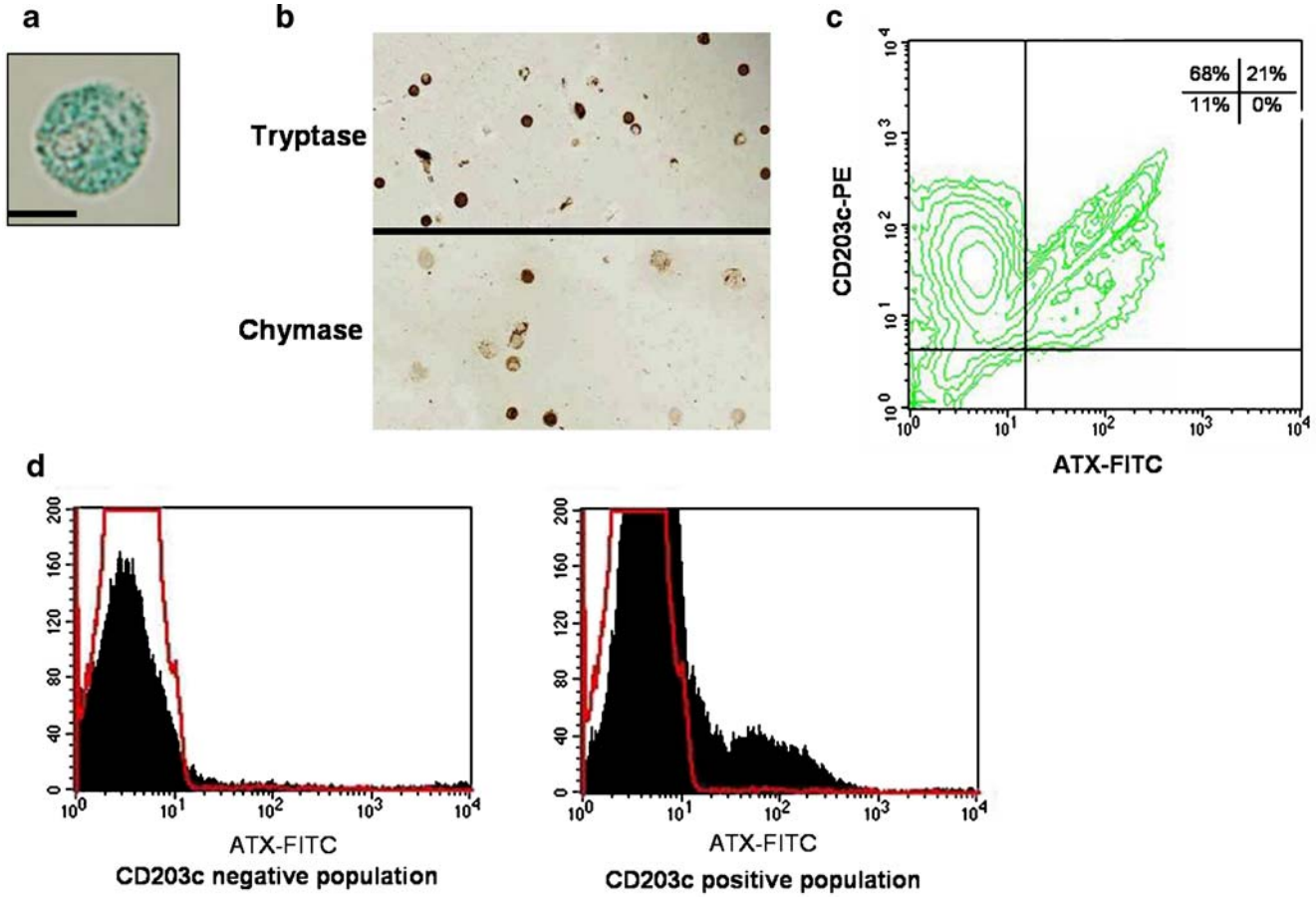

Fig. 3 Characterization of MC isolated from gastrointestinal tissue. The cells recovered after enzymatic digestion of gastrointestinal mucosal tissue were positively selected using anti-CD117 mAb. **a** A representative MC stained by Alcian blue (**b**); Immunochemical staining with anti-tryptase (*upper*) and anti-chymase (*lower*). The majority of cells are positive for tryptase, but not for chymase. **b** Flowcytometric profile of double staining by CD203c and ATX/lysoPLD. The MC-enriched fraction was fixed, permeabilized, and double stained with PE-conjugated anti-CD203c mAb and FITC-conjugated anti-ATX/lysoPLD mAb (**c**). The CD203c-negative (*left*) and -positive (*right*) areas were gated, and the FITC level was examined in each gated fraction. The *red line* represents negative control. The figure shows the representative data from four different experiments (Table 4)

Positively selected cells expressed ATX/lysoPLD, whereas negatively selected cells did not (Fig. 4a). As shown in Fig. 4a, tryptase and chymase were also detectable in cell lysates of MC-rich population.

Then, we evaluated whether MC extracellularly secrete ATX/lysoPLD. As shown in Fig. 4b, ATX/lysoPLD protein was detected in the concentrated culture supernatant of MC-enriched population cells isolated from gastric tissue, but not in the supernatant of negatively selected cell populations. This finding indicates that a subpopulation of the human MC in gastric tissue release soluble ATX/lysoPLD into the culture medium.

Discussion

LPA is involved in the etiology of a variety of diseases such as atherosclerosis, obesity, and cancer and in physiological responses such as wound healing, vascular tone, vascular integrity, and reproduction [34, 48]. However, the molecular mechanism of the production of this bioactive phospholipid is poorly understood, although a large amount of LPA is known to be produced by activated platelets or stimulated fibroblasts in pathological conditions. Recently, it has been postulated that at least two pathways exist for LPA production, intracellular synthesis from phosphatidic acid and

Table 3 Distribution of tryptase- and chymase-positive MC in the immunomagnetically purified population

Tryptase (+)	Chymase (+)	Chymase (+)/Tryptase(+)
82 (79–90)	17 (12–25)	21% (13–29%)

The purified MC were stained with avidin–biotin peroxidase method using biotinylated anti-tryptase or anti-chymase mAbs. Under the microscope, a hundred cells were randomly selected in five to seven different fields and positive cells for tryptase or chymase in those cell populations were calculated. Mean (min–max) in five different samples were expressed (Fig. 3b).

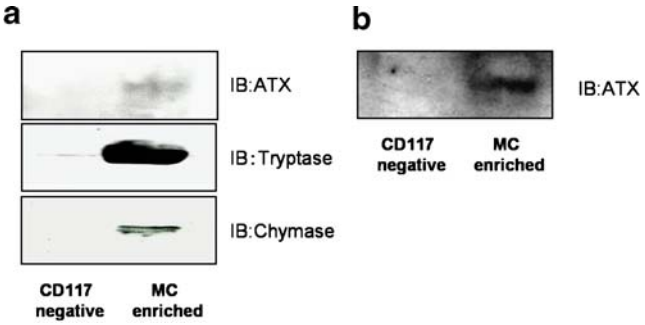

Fig. 4 **a** Western blotting of lysates of MC-enriched cell population from gastrointestinal tract for ATX/lysoPLD, tryptase, and chymase. **b** Detection of release of ATX/lysoPLD from MC-enriched population. MC-enriched cells (2×10^6) were cultured. Then, the supernatant was concentrated and the protein expression of ATX/lysoPLD was evaluated by Western blotting. For negative control, negatively selected cells with anti-CD117 mAb were cultured in the same conditions, and the culture supernatant was assessed for the presence of ATX/lysoPLD. The figure shows representative data from three different experiments

extracellular conversion from other lysophospholipids. In the latter pathway, ATX/lysoPLD is shown to be a key enzyme in the production of LPA by the catalysis of LPC, which is known to be abundantly present in plasma and tissue fluids [3, 64, 66]. In this study, we found a subtype of MC in the human gastrointestinal tract that showed strong staining for ATX/lysoPLD. This reports characterized the cellular producer of ATX/lysoPLD in normal tissue, while a recent study has demonstrated that ATX/LysoPLD is overexpressed in Epstein–Barr virus-infected Hodgkin's lymphoma cells [5]. That study used the same antibody as us and showed similar cytosolic staining of ATX/LysoPLD in lymphoma cells, suggesting that ATX/lysoPLD is stored in the cytosol in contrast to the other NPP family proteins, which are predominantly distributed in the plasma membrane [54]. By Western blotting of culture supernatants, we confirmed the secretion of ATX/lysoPLD from the MC-enriched fraction. These data clearly indicate that those MC undergo membrane-proximal cleavage to yield soluble ATX/lysoPLD, and thus, have important physiological roles through the local supply of a high concentration of LPA in gastrointestinal mucosal tissue.

Tissue MC are known to derive from specific bone marrow progenitor cells and migrate into tissues, where they mature depending on the microenvironment and acquire the capability to produce a wide range of mediators including histamine, heparin, prostaglandins, leukotrienes, and many inflammatory cytokines such as interleukin-8, vascular endothelial cell growth factor (VEGF), and platelet-derived growth factor (PDGF), and exert their biological effects by releasing such mediators [6, 15, 21, 26, 30]. In rodents, MC are phenotypically divided into two subtypes, mucosal-type MC (MMC) and connective tissue-type MC (CTMC), that produce distinct types of mediators. MC in the human gastrointestinal tract are also subdivided by immunohistochemical detection of two proteases; CTMC that contain tryptase and chymase, and MMC containing tryptase but not chymase [1, 27–29]. Those studies have shown that MMC are preferentially located in the mucosal surface, while CTMC are detected mainly in submucosal tissue in the gut. Our immunostaining results revealed that MC in the submucosal layer highly expressed ATX/lysoPLD, but MC located in the mucosal and muscular layers did not. Moreover, double staining experiments showed that most of the chymase-positive MC also expressed ATX/lysoPLD, while about half of the tryptase-positive cells lacked the expression of ATX/lysoPLD. This is consistent with previous findings and indicates that ATX/lysoPLD is constitutively produced mainly by submucosal CTMC in the gastrointestinal tract.

Using flowcytometry, we confirmed that intracellular staining of ATX/lysoPLD was detected by CD203c-positive MC isolated from gut tissue. In FACS analysis, however, only 10–20% CD203c-positive MC are positive for ATX / lysoPLD, which does not agree with the immunostaining data indicating that most of the submucosal MC are positive for ATX /lysoPLD (Table 4). In fact, cells separated by this immunomagnetic method contained many tryptase-positive MC but fewer chymase-positive MC. This suggests that submucosal MC are collected less efficiently than mucosal MC by this isolation process, which may contribute to the discrepancy between FACS analysis and immunohistochemistry.

Table 4 Percentages of each quadrant for different four FACS plots

Quadrant	Ex.1 (%)	Ex.2 (%)	Ex.3 (%)	Ex.4 (%)	Mean (%)	(Max–min) (%)
Left upper	68	66	60	55	62	(55–68)
Right upper	21	10	22	17	18	(10–22)
Left lower	11	24	18	28	20	(11–28)
Right lower	0	0	0	0	0	0

The MC-enriched fraction was fixed, permeabilized and double stained with PE-conjugated anti-CD203c mAb and FITC-conjugated anti-ATX/lysoPLD mAb. Percentages of each quadrant for different four FACS plots were shown.

The expression of ATX/lysoPLD in submucosal MC is considered to have physiological relevance. MC are essential for allergic and late phase reactions and T-cell-mediated immunity [14, 33, 42]. LPA has been reported to lead to overexpression of adhesion molecules and chemokines in endothelial cells, and thus, induces the recruitment of mononuclear phagocytic cells [40, 45]. For helper T cells, LPA has been shown to induce chemotactic migration of naïve CD4(+) T cells expressing the specific receptor Edg4 (LPA2), while enhancing proliferation and IL-2 production in activated CD4(+) T cells expressing Edg2 (LPA1) predominantly, but rather, inhibit the migration of these activated CD4 (+) T cells [20, 73]. This strongly suggests that the production of ATX/lysoPLD in submucosal MC has an important role in preparation of CD4(+) helper T cells' repertoire to respond to a variety of antigens in gastrointestinal mucosal tissue. Once T cells are stimulated with certain antigens, ATX/lysoPLD can augment T cell-mediated responses in mucosal tissue through the production of LPA.

On the other hand, LPA is considered to have suppressive effects on neutrophil-induced inflammation in vivo. LPA reduces IL-8-induced migration of human neutrophils in patients with pneumonia and inhibits the production of oxygen species in human neutrophils in response to stimulation with PMA [10, 43]. In the gastrointestinal tract, LPA reduces the degree of colonic inflammation induced by ethanol and trinitrobenzene sulfonic acid in a rat colitis model in vivo [59]. These findings suggest the possibility that ATX/lysoPLD released from submucosal MC plays a protective role against acute phase inflammatory bowel disease (IBD) [23]. Because the number of MC is markedly increased in IBD, it would be intriguing to examine the production of ATX/lysoPLD in MC in the intestinal mucosa in patients with IBD. In addition, recent studies have demonstrated that LPA also activates enteric glia cells, which are considered to play critical immunoregulatory roles in gut tissue [47, 68]. Taken together, these findings suggest that MC-derived ATX/lysoPLD has pivotal effects on mucosal immunity in the gastrointestinal tract.

LPA is also essential in wound healing. When superficial mucosal injury occurs, the mucosal defect is rapidly closed through a process termed epithelial restitution in physiological conditions [25, 58]. LPA is considered to play major roles in this step, as it strongly promotes epithelial cell migration and proliferation in vitro [41]. In fact, rectally applied LPA stimulates wound healing of the intestinal epithelium and reduces the size of ulcers in the rat [58]. Therefore, the production of ATX/lysoPLD in gastrointestinal CTMC is considered to be necessary for the maintenance of mucosal barrier function, as they can constitutively provide a considerable amount of LPA in the submucosal area, and thus, epithelial cells can rapidly respond to repair the mucosal injury.

Finally, ATX/lysoPLD has an essential role in the progression of malignant diseases in the gastrointestinal tract. Many studies have provided evidence for positive roles of LPA in the initiation or progression of malignancy, including melanoma, ovary, prostate, breast, head and neck, colon, and stomach cancer [13, 22, 72]. This is reasonable, because LPA can function as a growth factor for cancer cells and also stimulates angiogenesis [34]. More than 40 years ago, it has been reported that a large number of MC accumulate around solid tumors and a large number of tumor-associated MC were significantly correlated with high vascular density and poor outcome [32, 44, 65]. In those studies, MC-derived secretalogues, such as histamine, heparin, or VEGF are considered to be responsible for the new vessel formation. However, our results strongly support additional mechanisms of MC to promote the malignant potential of cancer through the production of ATX/lysoPLD.

In summary, we discovered that a subpopulation of submucosal CTMC constitutively secrete ATX/lysoPLD. MC are considered to play important roles in the pathophysiology of gastrointestinal diseases, such as allergic enteritis, inflammatory bowel disease and malignancy. The effects of MC may be partly attributable to ATX/lysoPLD by providing LPA in gastrointestinal mucosal tissue, although circulating ATX/lysoPLD synthesized in different locales may also modulate LPA levels. As ATX/lysoPLD is a relatively stable protein compared to the bioactive phospholipid product, further research on this enzyme may be useful for the evaluation of functional role.

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