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Cathepsin *G* in the Immune Defense of the Human Duodenum: New Sources for Biosynthesis

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Abstract—Proteases play a key role in the physiological processes of the small intestine, supporting its normal physiological functions as a part of the digestive system, in which hydrolysis and assimilation of nutrients are implemented. A high concentration of antigens in the intestinal lumen activates immunity and stimulates a chronic weakly expressed inflammatory response in a normal gastrointestinal tract (GIT). Cathepsin *G*, a serine protease controlling the functional state of immune cells, directly participates in the complicated system for the regulation of balance between physiological and pathological inflammations. To determine the role of cathepsin *G* in the small intestine, an immunofluorescent investigation of biopsies from the human duodenal mucosa were investigated using the confocal immunofluorescence microscopy method and human antibodies to cathepsin *G*. It has been shown for the first time that cathepsin *G*, which was regarded conventionally as one of the effectors of the inflammatory process, is a constitutive enzyme of the human duodenum and is constantly present in its normal mucosa. The new cell sources for the cathepsin *G* biosynthesis identified: intraepithelial lymphocytes (IELs), lamina propria lymphocytes, *CD*14-positive intestinal macrophages, and Paneth cells, which are specialized epitheliocytes of intestinal glands. Our data on the cathepsin *G* expression by immunocytes and Paneth cells in the duodenum allow us to attribute cathepsin *G* to the main proteases of intestinal immunity, which indicates the important role of this enzyme in the regulation of human GIT functions.

Keywords: cathepsin *G*, duodenum, lymphocytes, Paneth cells, intestinal glands

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In the physiological processes occurring in the small intestine, a key role belongs to proteases that participate in all mechanisms of monitoring the functional and vital activities of this central part of the digestive system. Proteases participate not only in the function of the digestive conveyer implementing the hydrolysis of nutrients, but also control a diversity of cellular processes, providing the renovation and remodeling of tissues, the contractility of smooth musculature, hormonal regulation, and protection for the gastrointestinal tract (GIT). The close contact of the intestinal mucosa with antigens (viruses, bacteria, parasites, toxins, etc.) stimulates therein the development of a strong gut associated lymphoid tissue (GALT) forming the intestinal immunocompetent system composed of lymphocytes, plasmocytes, granulocytes, macrophages, and mast cells. The proteases synthesized in immune cells promote detoxication, removal of allergens and other xenobiotics, initiate apoptosis of mutant and virally infected cells, and participate in inflammatory reactions, which is necessary for the initial protection of the intestinal mucosa from pathogens [1]. The multifunctional serine protease of immunocytes, cathepsin *G*, possessing the unusual dual trypsin-like and chymotrypsin-like specificity, is synthesized in neutrophils, monocytes, and mast cells. Cathepsin *G* participates in the regulation of immune response and is supposed to be the factor to maintain a delicate equilibrium between the defense of a tissue and its lesion in inflammatory conditions. Cathepsin *G* influences the functional state of immune cells and promotes neutrophil migration and neutralization of antigens [2].

According to the previous study [3], cathepsin *G* was firstlocalized in nonmyeloid type cells, that is, in the specialized secretory epitheliocytes of intestinal glands, Paneth cells. Considering the functional importance of cathepsin *G* for the defensive reactions of the body and, at the same time, the insufficiency of data about the role of this protease in the intestinal

immunity, we carried out an immunohistochemical investigation on the localization of cathepsin *G* in the human duodenum without clinically manifested inflammation and in the inflamed mucosa (duodenitis of II–III degree). New data have been obtained about the synthesis and secretion of cathepsin *G* by lymphoid and epithelial cells of the duodenal mucosa.

METHODS

We used the fluorescein isothiocyanate (FITC) antibodies to cathepsin *G* (Novus Biologicals, United States) and anti-*CD*14 antibodies, labeled with R-Phycoerythrin (PE) (Sorbent, Moscow, Russia). Biopsies from the duodenal mucosa were obtained during endoscopic investigations of patients diagnosed with superficial proximal gastritis and duodenogastral reflux upon their informed consent.

The biopsies were fixed with 4% paraformaldehyde in a phosphate buffer (PB) during 30 min at 37°C. After the fixation, the tissue was washed in PB, immersed overnight in a 30% sucrose solution, and embedded in a Killik medium for cryostat sectioning (Advanced Research Systems, USA). Cryosections 7 μm in thickness were produced with a cryotome (Termo Fisher Scientific, United States) and placed on slides with poly-*L*-lysin (Termo Fisher Scientific, United States). The bioptic specimens were incubated with normal human serum to block a nonspecific binding. The antibodies were added at a concentration of 1 μg/sample for 2 h at room temperature. Counterstaining was performed with the Mito-Tracker-Red dye for mitochondria (Invitrogen, United States). The nuclear *H*33342 dye (Sigma) was introduced 30 min prior to the end of incubation. Then, the specimens were washed and embedded in a polymerized Mowiol 4.88 medium (Calbiochem, Germany). The specimens were analyzed using a confocal scanning Nikon Eclipse TE 2000 microscope (Japan).

RESULTS

The immunohistochemical study of the cathepsin *G* localization in the human duodenal mucosa was based on biopsies isolated from different segments of the duodenum, that is, pars superior, pars descendens, and pars horizontalis. The cathepsin *G* expression was studied in the normal (without clinical signs of inflammation) and in the inflamed mucosa (the diagnosed duodenitis of II–III degree). Cathepsin *G* was detected throughout the entire duodenum in norm in free cells of the lamina propria in the villi stroma and near intestinal glands (Lieberkühn's crypts) (Figs. 1a–1c). The cathepsin *G*-specific immunofluorescence was observed in the secretory granules of mast cells, macrophages, and intraepithelial lymphocytes (Figs. 1d, 1e). The amount of cathepsin *G*-containing cells in villi and intercryptic stroma increases three-fourfold in inflammatory processes because of the infiltrate (Figs. 1g–1l). Cathepsin *G*-positive cells are mainly concentrated in lamina propria and almost absent in the submucosa and the submucosal (Brunner's) glands (Fig. 1l).

To identify neutrophils and macrophages in the duodenal mucosa, the biopsies were stained with antibodies to the *CD*14 protein of a receptor complex of monocytes, macrophages, and neutrophils [4]. The dual staining of biopsies from inflamed mucosa (the diagnosed duodenitis of II–III degree) with the antibodies specific for cathepsin *G* and to *CD*14, helped to determine the co-localization of cathepsin *G* and *CD*14 in a significant part of mucosal immunocytes identified as neutrophils and macrophages (Fig. 2). No product of the immunochemical reaction with *CD*14-specific antibodies has been found in the epitheliocytes of intestinal glands (Lieberkühn's crypts), including Paneth cells.

Cathepsin *G*-specific fluorescence has been detected near the apical part of the villi in free cells having morphological signs of lymphocytes in the duodenal mucosa without clinical manifestations of inflammation (Figs. 3a, 3b). Cathepsin *G*-specific antibodies binding also observed with the lymphoid cells near the basement membrane of epitheliocytes in the apical part of the villi and the epithelial layer covering the intestinal glands (Figs. 3b, 3c). The localization and appearance of these cells allowed them to be referred to intraepithelial lymphocytes. It should be noted that the localization of cathepsin *G* in intraepithelial lymphocytes was unknown until this study.

An intensive cathepsin *G*-specific immunofluorescence was observed in Paneth cells located on the bottom of intestinal glands, predominantly in the subnuclear region (the rough endoplasmic reticulum zone), between the secretory granules and in the secretory duct (Figs. 3d–3f). The secretory granules located closer to the basal part of the cell appeared stained in some cases (Fig. 3d). The release of secretory granules from Paneth cells into the intestinal glandular lumen is well distinguishable in Figs. 3g–3i. On this cryosection (Fig. 3g), we can see that only a single prosecretory granule is stained and its contours are hardly distinguishable on the visualized specimen within the red spectral region (Fig. 3h). Immature prosecretory granules with the protein packed less densely can, probably, interact with labelled antibodies, which can explain the fact of fluorescence in some of these granules. No binding of cathepsin *G*-specific antibodies has been recorded in enterocytes, goblet cells, and secretory epitheliocytes of the submucosal glands.

Figure 4 gives data about the localization of cathepsin *G* in the duodenal mucosa with clinical and histological signs of inflammation (duodenitis of II–III degree). The cathepsin *G*-positive granulocytes are present in the edematous stroma of villi in the composition of the infiltrate (Figs. 4a, 4b). The free cathepsin *G*-containing granules that obviously emerged due to the degranulation of neutrophils are well distinguishable below

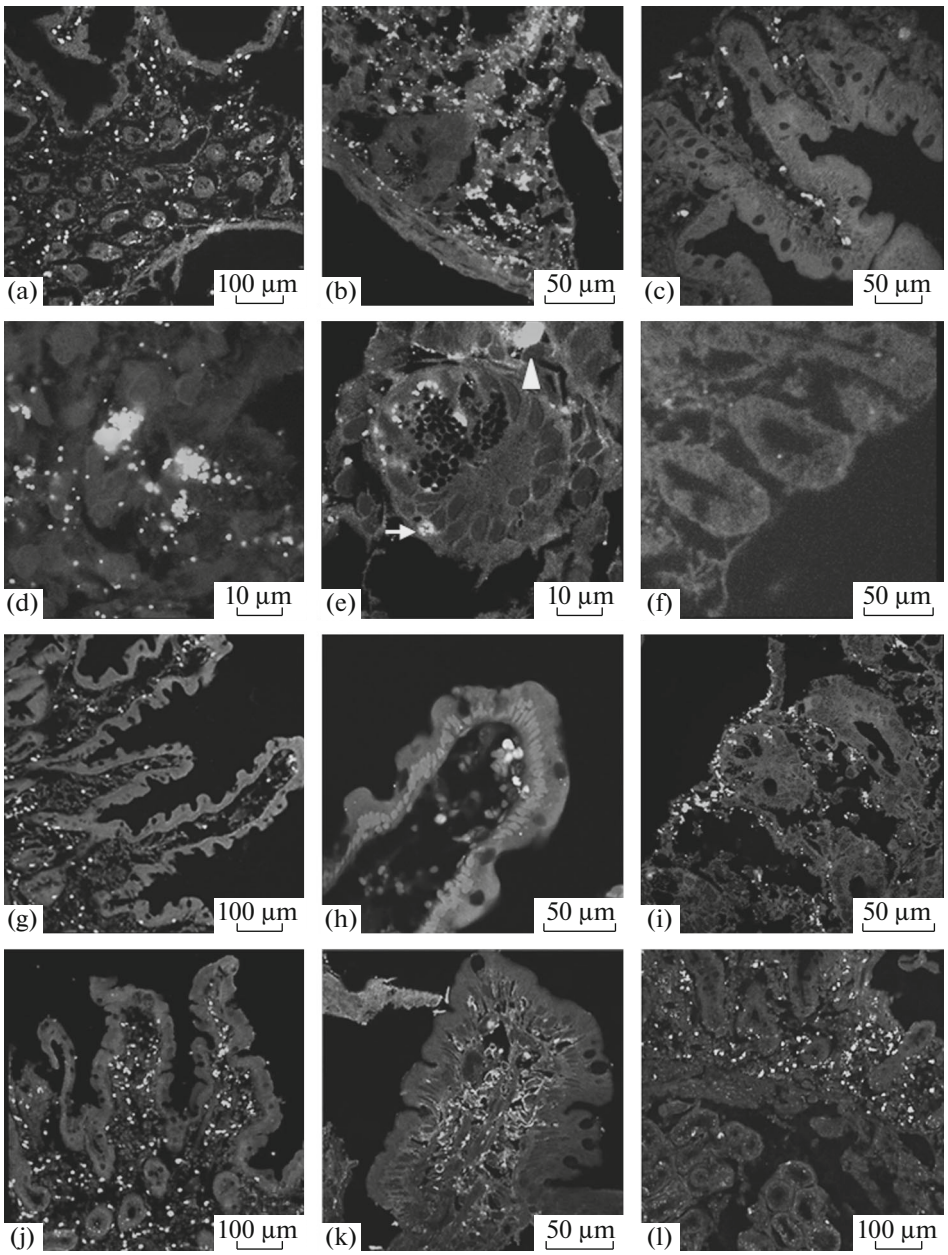

Fig. 1. Cathepsin *G* expression in the normal and the inflamed human duodenal mucosa. (a–e) Cathepsin *G* immunolocalization (FITC-antibodies, the lightest hue) in different regions of the duodenum without clinical manifestations of inflammation, such as (a) pars superior, (b) pars descendens, and (c) pars horizontalis; (d) cathepsin *G*-specific fluorescence in the secretory granules of mast cells; (e) the macrophage (the short arrow), and the intraepithelial lymphocyte (arrow) on the basal membrane of the intestinal gland; (f) the control, the nonspecific FITC antibodies. (g–l) the cathepsin *G* expression increases in inflammation: the (g–i) normal and (j–l) inflamed mucosa. Cell nuclei and cytoplasm are stained with specialized dyes, as recommended ("Methods").

the basement membrane in the apical part of the villus (Fig. 4a). An intensive cathepsin *G*-specific immunofluorescence was recorded in the secretory granules of Paneth cells, which points to the active synthesis and secretion of this enzyme by intestinal glands in inflammation (Fig. 4c). The dual staining with the cathepsin *G*- and *CD*14-specific antibodies helped to identify, in the infiltrate, the lamina propria lymphocytes binding only the cathepsin *G*-specific antibodies and the cathepsin *G*/*CD*14-positive cells identified as neutrophils (Fig. 3d). The types of lymphoid cells in which cathepsin *G* was localized in the duodenal mucosa are given in Figs. 4e–4i. Cathepsin *G* is contained in the granules of not only mature (segmented), but also of young (band) neutrophils (Figs. 4e, 4f). Large (up to 1.1 μm) granules binding cathepsin *G*-specific antibodies were detected in mononuclear cells with the beanlike or round nucleus and were identified

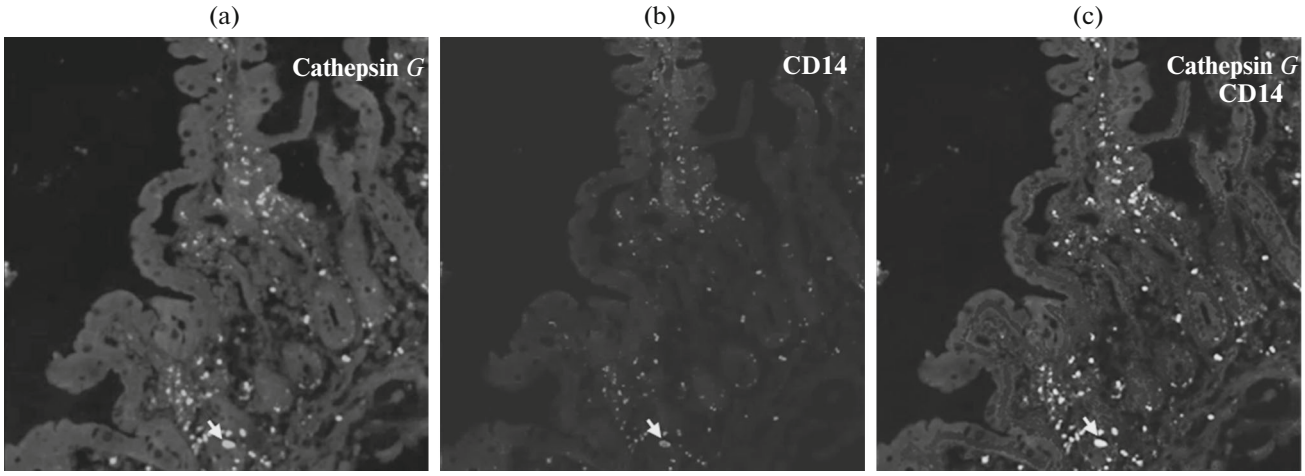

Fig. 2. Dual staining of the inflamed mucosa biopsy (the diagnosed duodenitis of II–III degree) with cathepsin *G*- and *CD*14-specific antibodies. Binding of the (a) cathepsin *G*-specific and (b) *CD*14-specific antibodies and (c) the co-localization of cathepsin *G* and *CD*14 in free cells of the villi stroma; the arrow indicates the macrophage. Magnification: 200×.

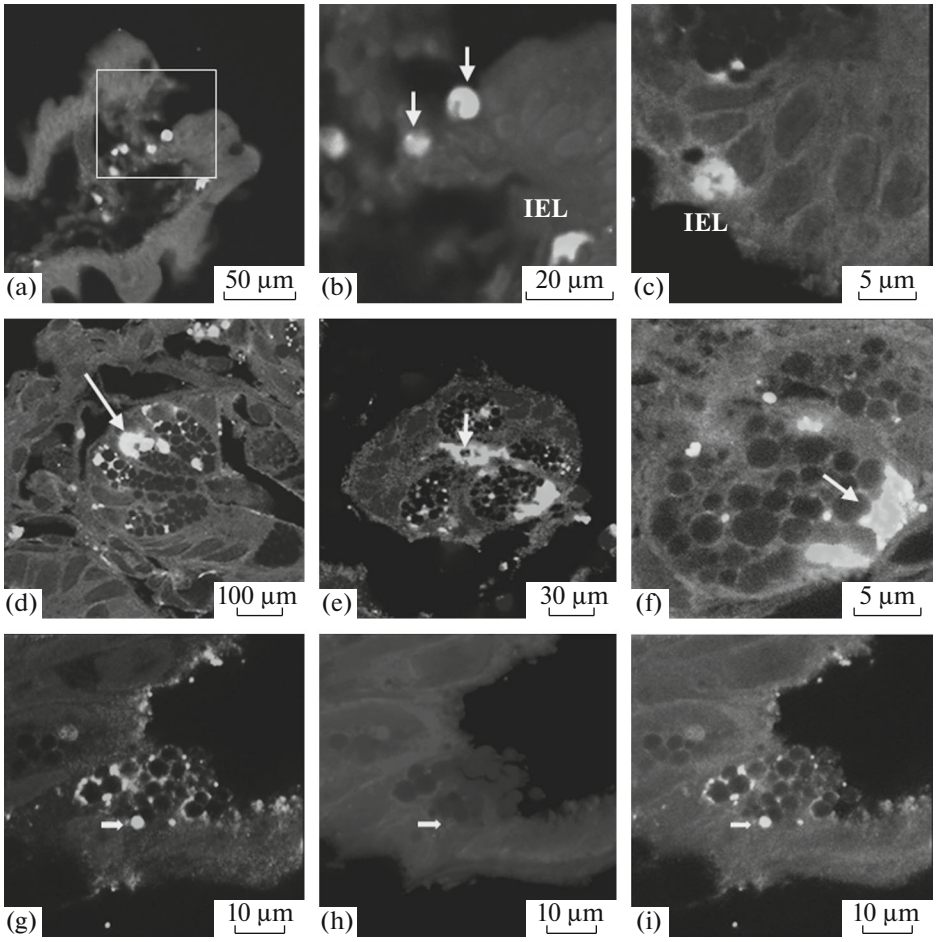

Fig. 3. Types of cells synthesizing cathepsin *G* (the lightest hue) in the normal duodenum. (a) Cathepsin *G* in lymphocytes near the apical part of the villi; (b) the enlarged box on the image 3a; lymphocytes are indicated by the arrows, IEL is the intraepithelial lymphocyte; (c) the intraepithelial lymphocyte (IEL) near the bottom of the intestinal gland; (d) the cathepsin *G*-specific fluorescence (arrow) in the secretory granules of Paneth cells; the secretory duct (e) of the intestinal gland and (f) in the area of the rough endoplasmic reticulum; (g–i) release of secretory granules from the Paneth cell into the lumen of the intestinal gland, the arrow indicates the prosecretory granule.

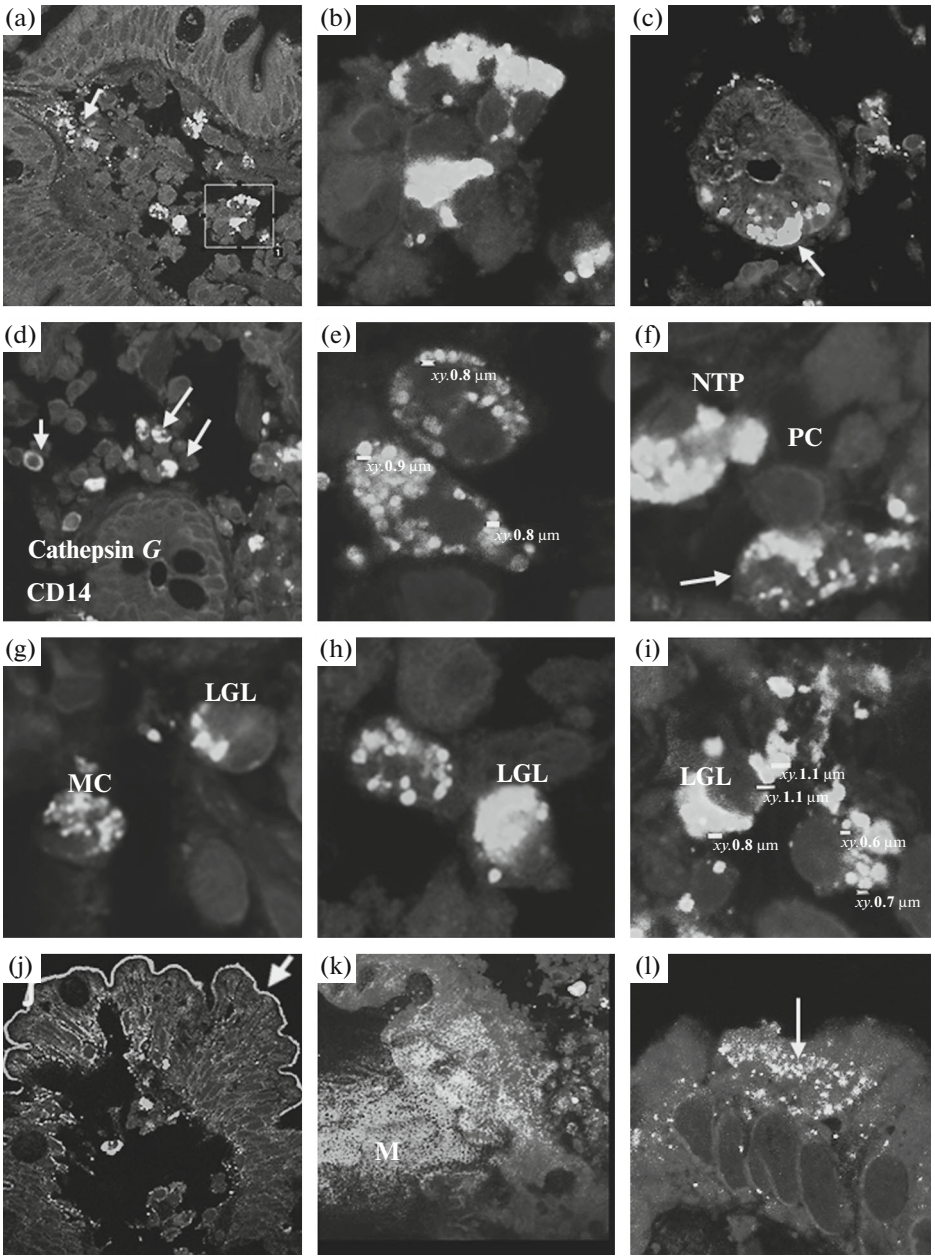

Fig. 4. Cathepsin *G*-specific immunofluorescence on the cryosection of the inflamed mucosa (duodenitis of II–III degree). (a) Immuno-positive to cathepsin *G* of the cell in the villi stroma; degranulation below the basal membrane of cathepsin *G*-containing cells (the arrow); (b) cathepsin *G* in granulocytes (the box) is shown with a large magnification; (c) cathepsin *G* in the secretory granules of Paneth cells (the arrow). (d) Binding the cathepsin *G*- and *CD*14-specific antibodies in the interglandular space of lamina propria; the co-localization of cathepsin *G* and *CD*14 in neutrophils (the elongated arrows in the middle of the image); the cathepsin *G*-specific immunofluorescence in lymphocyte (the short arrow). Cathepsin *G* in (e) mature (segmented) and (f) yong (arrow) neutrophils (neutrophil, NTP; plasmocyte, PC; scale bars, 0.8 μm); (g–i) cathepsin G in mast cells (MC) and large granular lymphocytes (LGL), scale bars, 0.6–1.1 μm (i). Binding of cathepsin *G*-specific antibodies (j) on the brush border (arrow), (k) on the mucous secretion of goblet cells (designated by M), and (l) in the cytoplasm of enterocytes (arrow). Magnification: (a, k, j) 1000×; (c, d) 600×; (b, e, g, h) 5000×; (f) 6000×; (i) 7000×; (l) 4000×.

by us as large granular lymphocytes due to their morphological signs (cell sizes, nucleus diameter, and the presence of granules). Cathepsin *G*-positive granules are also present in the cytoplasm of mast cells (Figs. 4g–4i). Plasmocytes do not contain cathepsin *G* (Fig. 4f).

In some cases, a cathepsin *G*-specific fluorescence was found on the brush border of enterocytes and in

the secretion of goblet cells (Figs. 4j, 4k). Small granules stained with cathepsin *G*-specific antibodies were episodically met in the cytoplasm of enterocytes, which possibly was a result of the absorption of cathepsin *G* adsorbed on the brush border of enterocytes through endocytosis (Fig. 4l).

DISCUSSION

We have shown for the first time that cathepsin *G* conventionally regarded as one of the inflammatory process effectors is constitutively synthesized in the normal duodenal mucosa having no clinical manifestations of inflammation (Fig. 1). Cathepsin *G* is identified throughout the duodenum in the gut-associated lymphoid tissue (GALT) cells, such as intraepithelial lymphocytes (IELs), lamina propria lymphocytes, macrophages, and mast cells. The alternative source of cathepsin *G* in the normal duodenal mucosa is the specialized epitheliocytes of intestinal glands,—Paneth cells (Fig. 3).

IELs are the first line of defense of the intestinal epithelium from the penetration and a further spread of pathogens. Apart from the local immune control, IELs help to preserve the integrity of the epithelial layer, preventing the uncontrolled infiltration of immune cells and the excessive immune response mediated by the systemic *T* cells [5]. The localization of cathepsin *G* in IELs, which was established for the first time by this study, can elucidate the insufficiently known mechanisms underlying the functioning of these cells in maintaining the necessary balance between the hazard of destruction to the epithelial barrier, due to the excessive immune response, and its preservation from damage.

The lamina propria lymphocytes are the most important element of GALT and normally constitute 35–40% of the total immunocyte count in the duodenal stroma [6]. We registered cathepsin G-specific fluorescence in lamina propria small lymphocytes, whichsuggests their belonging to the *B* or *T* type. The lymphocytes have been unconsidered until recently as a source of the cathepsin *G* biosynthesis. Cathepsin *G* has only recently been localized in *T* lymphocytes of atherosclerotic plaques [7] and the antigen-presenting cells, including *B* lymphocytes, and based on this fact, researchers supposed its role in both the breakdown of foreign proteins and the presentation of antigens [8].

We observed the cathepsin *G* expression in macrophages in the normal duodenal mucosa. The intestinal macrophages manifest minimal anti-inflammatory activity despite their strong phagocytic and bacteriocytic properties and do not belong to a type of antigen-presenting cells, unlike the macrophages of other tissues [9]. Cathepsin *G* of macrophages in the duodenal mucosa can participate in regulating the functional activity of these cells.

As has been shown, the cathepsin *G*-positive mast cells are normally present in the duodenal mucosa. The proteases of mast cells participate in the intestinal mechanisms of protection from pathogens, as well as in the regulation of epithelial renewal, permeability of the epithelial barrier, and induction of inflammation [10]. The role of cathepsin *G* in the immune activity of mast cells remains so far unclear.

The cathepsin *G* synthesis and secretion are implemented in the normal human duodenal mucosa not only by innate immune cells, but also by Paneth cells which are specialized secretory epitheliocytes located at the base of intestinal glands (Fig. 3). Paneth cells secrete bactericidal substances, such as defensins, lysozyme, phospholipase 2, immunoglobulin A (IgA), etc., into the crypt lumen, which provides sterile conditions in the growth area of the epithelium and protects stem cells from a bacterial invasion [11, 12]. We have established that Paneth cells are the constant sources of cathepsin *G* in the duodenal mucosa both in norm and inflammation. The release of cathepsin *G*-containing granules by Paneth cells into the secretory duct of intestinal glands maintains the constant presence of the protease in the area of epithelial stem cells. Cathepsin *G* in intestinal glands may play a significant role in the antibacterial protection of epithelial cells. We can note a certain functional similarity between Paneth cells and neutrophils, which are the classical source of cathepsin *G*, since both cell types participate in nonspecific immunity and synthesize similar antimicrobial factors, such as defensins, lysozyme, and cathepsin *G*. At the same time, cathepsin *G* of neutrophils is released into the intercellular space predominantly in inflammation during degranulation in response to a receptor-mediated stimulation, whereas Paneth cells continuously secrete antimicrobial factors, including cathepsin *G*, into the crypt lumen. The obtained results indicate the presence of cathepsin *G* in the intestinal glands as one of the factors of antibacterial protection for the intestinal epithelium.

An enormous antigen load in the intestinal lumen stimulates the immune activation and constitutes the cause for a chronic weakly expressed inflammatory response in the normal GIT. The stimulation of the mucosal lamina propria immune cells by the antigens of the transitory microflora microorganisms present in the intestinal lumen are detected in the small intestine, including the duodenum in norm. The innate immune cells participate in the complex regulatory system responsible for the balance between physiological and pathological inflammations. The localization of cathepsin G in the intestinal immune cells constantly present in the normal intestinal mucosa, such as lymphocytes, macrophages, and mast cells, allows us to characterize this enzyme as one ofthe main proteases of innate immunity.

The level of cathepsin *G* expression significantly increases in inflammation (stage II–III duodenitis).

Localization of cathepsin *G* in lymphoid and epithelial cells of the human duodenal mucosa

Cell types	Cathepsin *G* localization
Duodenal immune system cells	
Intraepithelial lymphocytes	Cytoplasm
Lamina propria lymphocytes (small, medium)	Cytoplasm
Large granular lymphocytes (duodenitis)	Cytoplasmic granules (0.6–1.1 μm)
Neutrophils (duodenitis)	Cytoplasmic granules (0.4–0.8 μm)
Mast cells	Secretory granules
Macrophages	Cytoplasm
Plasmocytes	–
Intestinal epithelium	
Enterocytes (apex of a villus)	Brush border, cytoplasm (endocytosis)
Paneth cells (intestinal glands)	Rough endoplasmic reticulum, secretory granules
Goblet cells	–
Epitheliocytes of submucosal (Brunner's) glands	–

The count of lymphoid cells in lamina propria is known to increase significantly in duodenitis, due to the infiltration of lymphocytes, plasmocytes, and eosinophils. In addition, a large amount of segmented neutrophils, almost completely absent in norm, is characteristic of the inflamed mucosa [13]. The binding of cathepsin *G*-specific antibodies was observed in the granules of young and mature neutrophils (Fig. 4). The macrophage-like cathepsin *G*/*CD*14-positive cells identified by dual staining of cryosections with the anti-cathepsin *G* and anti-*CD*14 receptor antibodies may belong to the recently-identified *CD*14-positive macrophage population of the intestinal mucosa—the producers of the proinflammatory cytokines, the count of which may significantly increase in inflammation [14].

This study has also obtained the first data about the localization of cathepsin *G* in large granular lymphocytes (LGLs) (Figs. 4g–4i). Duodenal LGLs are a special variety of natural killer (*NK*) cells, which phenotypically differ from the circulating *NK* cells, expressing serine proteases such as granzymes *A* and *M* [15]. The duodenal LGLs produce different cytokines (*IL*-8, *GM-CSF*, *TNF*-α), exhibit cytotoxic properties in relation to tumor cells, which underlines a special role of these cells in the intestinal defensive reactions [16]. Cathepsin *G* localized in cytoplasmic granules of duodenal LGLs may, like granzymes, participate in the cytotoxic activity of these immunocytes.

Our results indicate the constant presence of cathepsin *G* in the area of intestinal epithelium, which may introduce significant corrections into the understanding of this enzyme's role in the physiology of intestinal mucosa. It has been established that the protease-activated receptors (*PAR*4 type), possibly activated by cathepsin *G*, emerge in the epithelium of colon intestinal glands in inflammation (ulcerative colitis) [17]. The authors associated the destroyed integrity of the epithelial barrier in ulcerative colitis with the activation of the *PAR*4 type receptors by cathepsin *G* of the neutrophil origin in the epithelium of intestinal glands, but did not explain the pathway through which the enzyme could enter the lumen of intestinal glands. The detected cathepsin *G* secretion by Paneth cells provides the emergence of this protease in the epithelial area of intestinal glands, where the enzyme can interact with the respective protein substrates, including *PAR*4.

Cathepsin *G* is known to have angiotensin-converting properties and, furthermore, to be capable to activate renin, the key enzyme of the renin-angiotensin system (RAS), which is synthesized as an inactive precursor, prorenin [18]. Renin is synthesized by goblet cells in the intestinal epithelium of mice and enters, in the content of the secretion, the area of microvilli on the apical enterocyte surface [19], where angiotensinogen and other factors of the intestinal RAS are localized [20]. The observed sorption of cathepsin *G* on the brush border and the mucous secretion of goblet cells (Fig. 4) indicate the possibility of a direct contact between this protease and the potential substrates in this area, including prorenin and angiotensinogen, which presupposes the existence of cathepsin *G*-dependent pathway for the activation of the local RAS.

CONCLUSIONS

Thus, we have shown in this study that cathepsin *G* is a constitutive enzyme of human duodenum and typically present in the duodenal mucosa. The cathepsin *G* biosynthesis has been identified in a variety of lymphoid cells, such as lymphocytes (IELs, and lamina propria lymphocytes), macrophages, neutrophils, mast cells, and the specialized secretory epitheliocytes, the Paneth cells of intestinal glands (table). Cathepsin *G* was localized for the first time in intraep-

ithelial lymphocytes and large globular lymphocytes. The data obtained by us in this study on the expression of cathepsin *G* by various types of immunocytes and Paneth cells in the normal and inflamed duodenal mucosa allow us to regard cathepsin *G* as the main protease of intestinal immunity, which indicates the role of this enzyme as an important bioregulator of physiological functions in the human gastrointestinal tract.

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