Distribution of ghrelin-positive mast cells in rat stomach

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ABSTRACT
It is known that the gastrointestinal peptide hormone ghrelin is expressed in human and rodent B lymphocytes, T lymphocytes, monocytes and natural killer cells. However, there are no data about ghrelin expression by mast cells. These facts, as well as the common progenitor cells of mast cells and the above-mentioned immune cells, motivated us to undertake the current work in order to prove that like other granulocytes, rat gastric mast cells are capable of immunohistochemical expression of ghrelin. Gastric wall sections of Wistar rats were studied immunohistochemically for detection of ghrelin and tryptase and histochemically for toluidine blue in order to identify ghrelin-positive mast cells as well as to establish their localization and distribution. Results showed that mast cell granules expressed ghrelin. The ghrelin-positive mast cells were the least numerous as compared to tryptase-positive mast cells and toluidine blue-positive mast cells. Based on the observed expression of ghrelin in granules of mast cells localized in the rat gastric wall, we suggested that this type of cell can be regarded as an important source of ghrelin and suggested that ghrelin may exert different physiological functions, such as regulation of muscular, epithelial and glandular functions.

KEYWORDS
Ghrelin; mast cells; stomach; rat

Introduction
Mast cells (MCs) together with monocytes, basophils, eosinophils and neutrophils originate from the common pluripotent haematopoietic progenitor cells CD34+ [1]. However, the specific MC precursor CD34+/c-kit+/CD13 + helps to differentiate MCs from granulocytes [1]. MCs circulate in the blood in an immature form before migrating to vascularized tissues, where they undergo final differentiation and maturation influenced by stem-cell factor and other cytokines from endothelial cells and fibroblasts. MCs are found in most tissues of the body, particularly in locations that are in close contact with the external environment, such as skin, airways and intestines [2,3].

Many studies have characterized the morphology, function and localization of MCs in different organs of humans and animals [4–12,13]. In recent years, MCs have been described as highly specialized immune effector cells that take part in the maintenance of homeostasis as well as in modulating some pathological processes. It is important to note that in rodents and humans, MC heterogeneity depends on the different expression of two types of proteases: tryptase and chymase [4]. As a result, two different subpopulations of MCs are detected – those containing both tryptase and chymase (MCTryc+) and those containing only tryptase (MCTry+) [5]. In addition, two MC types depending on different localization sites are identified: connective tissue MCs (CTMCs) and mucosal MCs (MMCs) [6–12].

MCs can be activated in different ways. The most studied mechanism for the activation of these cells is via stimulation of the high-affinity immunoglobulin E (IgE) receptor FceRI. MCs can also be activated by directly interacting with pathogens through pattern recognition receptors, including the Toll-like receptors, NOD-like receptors, C-type lectins and the glycosylphosphatidylinositol-anchored protein CD48 [14]. MCs can participate in direct killing of organisms by phagocytosis and reactive oxygen species production [15], and are able to produce antimicrobial peptides, such as cathelicidins, both constitutively and in response to lipopolysaccharides [16]. MCs can modulate host innate immune responses through the release of granular and secreted mediators such as histamine and other vasoactive mediators.

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MC-produced chemotactic factors (IL-8, TNF-α, eotaxin) increase the recruitment of inflammatory cells including neutrophils, eosinophils and natural killer (NK) cells. MC products have also been implicated in the regulation of adaptive immune responses [3,4]. MC-derived cytokines and chemokines can enhance the migration of dendritic cells and effector T lymphocytes (T cells) to the site of infection and to the lymph nodes. Moreover, MCs were reported to act as antigen-presenting cells, particularly for CD8+T cells. This fact was also confirmed by Stefanov et al. [17] who established S-100 protein-immunoreactivity in canine MCs granules providing another evidence for the antigen-presenting function of MCs.

MCs are found primarily in the gastrointestinal tract, skin and nerve endings, where they exert their important role in host defence against particular parasites and bacteria. These cells are observed in all vascularized tissues except for the central nervous system and the retina [18]. In recent years, many authors have established the ability of MCs to synthesize and release different mediators and cytokines that control several pathological events. Some mediators are stored preformed, while others are produced after MC activation [19–22]. MC secreting histamine, substance P (SP), vasoactive intestinal polypeptide (VIP) and other factors are mobile that make contact with peptidergic nerve endings (secreting histamine, substance P (SP), vasoactive intestinal peptide hormone ghrelin have been reported in numerous studies. [27–40,41] Ghrelin is known to be an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) 1a, capable of stimulating growth hormone release from the pituitary gland. This peptide hormone has been obtained from rat and human stomachs [27]. It participates also in stimulation of appetite in mammals [28]. Additionally, ghrelin is involved in many physiological processes, including control of gastric motility and acid secretion in animals and humans [29,30]. Recent studies using immunohistochemistry, immunoelectron microscopy and in situ hybridisation analyses indicate that in the rat stomach, ghrelin is produced mainly by X cells [31–34]. Handjieva-Darlenksa et al. [35] investigated the effect of insulin and glucose administered in the third ventriculum on leptin and ghrelin expression in the hypothalamus, and elucidated the mechanisms of leptin resistance in obese rats. Over the past 14 years, ghrelin has also been shown to mediate other biological activities including promoting central memory, improving neurogenesis, the survival of cardiomyocytes and regulating ovarian and testicular functions [36–40].

Earlier studies on ghrelin (Ghr) expression by different immune cells have mainly focused on human, rodent and goldfish B lymphocytes (B cells), T cells, monocytes and NK cells [42–44].

Factors influencing the smooth muscle contraction in different organs were established by several authors [45–47]. Some of these factors are MC mediators such as histamine and eicosanoids which role in modulating smooth muscle contraction is well known [45,46]. Other studies reported the involvement of ghrelin, produced by gastric endocrine cells as a motilin-related peptide in gastrointestinal contraction [47]. Because of the lack of data about the MC-produced ghrelin it is important to know if the ghrelin is also expressed by MC granules in order to define one more type of cells which may be a source of ghrelin.

Bearing in mind that the common haematopoietic progenitor cells CD34+ of MCs and granulocytes as well as the ghrelin (Ghr) expression by B cells, T cells, monocytes and NK cells, we supposed that an additional feature of MCs was the ghrelin expression. That fact allowed us to presume the ability of MCs to produce, store and release ghrelin similarly to other immune cells. As a result, MC-produced ghrelin is supposed to be involved in the maintenance of the local immune cell homeostasis, regulation of gastric motility and epithelial function as well as modulation of inflammatory processes in the stomach.

Based on the significant role of MCs and ghrelin in physiological and pathological processes, we undertook the present study to determine the ghrelin-immunoreactivity of MCs.

Materials and methods

Animals

Five seven-month-old male Wistar rats were used in our study. They were housed under a 12:12 h light dark cycle and given a standard diet (TopMix, KF 70-1, HL-TopMix Ltd, Bulgaria, with ingredients: crude protein – 20.0%; crude fat – 2.46%; crude fibre – 5.50%; calcium; sodium; phosphorus; lysine; methionine; Vit. A – 12,000 UI/kg; Vit. D3 – 3000 UI/kg; Vit. E – 30 UI/kg) and tap water ad libitum. All procedures were performed in accordance with the Bulgarian laws regarding animal care (Ordinance 20 of 01.11.2012 on the minimum requirements for the protection and welfare of experimental animals and requirements to objects for use, cultivation and/or delivery) and with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes [48]. The permit (№ 110) from the Bulgarian Food Safety
Agency and from the University Commission for animal care was obtained to use the rats in the current study. For light microscopic examination, the animals were euthanized by a combination of ketamine and xylazine (overdose, IP). Then, the stomach was removed immediately and a segment (0.5 cm²) from the gastric body was sampled.

**Material**

Tissue samples collected from the body of the stomach were fixed in 10% neutral buffered formalin solution for 48 h, then washed in running tap water, dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin.

**Peroxidase-immunohistochemistry for detection of ghrelin and mast cell tryptase**

Serial sections of 4 μm thickness were cut and mounted on glass slides, deparaffinized in xylene and placed in a series of descending ethanol concentrations. The next step was incubation of sections with Peroxidase Blocking Reagent (S2001, DAKO) for 10 min to block unspecific binding of the antibodies. After that, the slides were incubated with primary antibody for 1 h and then incubated with labelled polymer for 30 min and washed again. Then, tissue samples were incubated with DAB substrate-chromogen, washed and counterstained with Mayer’s haematoxylin. The following antibodies were used for immunohistochemistry: ghrelin (H-40) rabbit polyclonal antibody at a dilution of 1:100 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and monoclonal mouse antihuman MC tryptase (Clone AA1, M7052, DAKO Denmark) at a dilution of 1:100, as well as detection system EnVision™ FLEX, High pH (Link) (K8000, DAKO Denmark). Ghrelin-positive endocrine cells in the rat stomach glands were used as positive controls. Sections incubated with non-immune sera instead of the primary antibodies were used as negative controls.

**Light microscopic histochemistry for detection of metachromasia in mast cells**

Some of the serial sections were used for histochemical staining using 0.1% solution of toluidine blue in McIlvane’s buffer, pH 3 to detect metachromasia in the MCs.

**Statistical analysis**

The number of MCs was determined on five microscopic fields (X200) from five serial sections of the stomach from each animal using a light microscope (Leica DM2500, Germany), camera (Leica DFC 290) and software analysis programme (LAS Leica Microsystems CMS GmbH). The data for MCs density (number/field) are presented as mean ± SD. Statistical data processing was done by using Student’s t-test. The difference was considered as significant when *P* < 0.05.

**Results and discussion**

Several studies have established the expression of ghrelin and its receptor GHS-R in different tissues and cells [42–44,50,51]. So far, we have not found any data about ghrelin expression in MCs. Therefore, in our study, the light microscopic histochemical detection of metachromasia, immunohistochemical and double immunofluorescent staining for ghrelin with tryptase provided the first evidence that MCs expressed ghrelin in the *tunica mucosa*, *tela submucosa*, *tunica muscularis*, *tunica serosa* of rat gastric wall (Figures 1–3). Since tryptase is well-known marker for MCs [52], we performed double immunofluorescent staining for ghrelin with tryptase to confirm that both CTMCs and MMCs express ghrelin (Figure 1(a,b)). It was clearly visible that both ghrelin and tryptase were expressed by the same MCs. Comparing
Ghr+ cells with TB+ and Try+MC MCs, we also found out that Ghr+ cells had similar morphology (granular cells of similar size and shape) and localization as TB+ and Try+MC (Figure 2(a–c)). This finding allowed us to suggest that MCs are another cell type within the rat gastric wall capable to produce ghrelin similarly to A/X cells [31,32,34].

The number of Ghr+MC, TB+MC and Try+MC was compared, as well. The statistical analysis showed that the number of Ghr+MC (12.20 ± 5.20) was lower ($P < 0.001$) than those of TB+MC (24.13 ± 6.22) and Try+MC (19.13 ± 7.85). The number of TB+MC was the highest. It was found that Ghr+MC were 61.7% of the Try+MC and 47.15% of the TB+MC. Try+MC were 76.41% of the TB+MC. The greatest number of TB+MC established in our study correlates with the results of Pearce [49] who reported that toluidine blue is a universal marker for all MCs [49]. As mentioned above, tryptase is also used as a marker for MCs to differentiate them from leukocytes [52]. The smaller number of Try+MC versus that of TB+MC established in our study correlates with the results of Pearce [49] who reported that toluidine blue is a universal marker for all MCs [49]. As mentioned above, tryptase is also used as a marker for MCs to differentiate them from leukocytes [52]. The smaller number of Try+MC versus that of TB+MC in the rat stomach can be explained by the probable presence of chymase-positive MCs, which were not studied in this work. The ghrelin positivity in MCs was possibly due to their ability to act as immunomodulators, i.e. in contrast to leptin, ghrelin-inhibited proinflammatory cytokines similarly to anti-inflammatory cytokines secreted by T-helper cells and monocytes/macrophages [44]. Several studies have described ghrelin as an anti-inflammatory mediator both in vitro and in vivo in different types of immune cells, but not in MCs [42–44,53,54]. Ghrelin is involved in the inhibition of TNF-α-induced IL-8 production and inhibition of NF-kB signalling in human endothelial cells [53]. Having these properties, ghrelin could be used successfully in modulating of some inflammatory conditions [42,43,52]. Dixit et al. [44] suggested that ghrelin and leptin, similarly to their antagonistic effects on food intake in the hypothalamus, exerted also reciprocal regulatory effects on inflammatory cytokine expression in the immune system. Thus, the variations in circulating levels of leptin and ghrelin may significantly influence the production of some cytokines by immune cell populations. Given these findings, we suggested that such reciprocal immunoregulatory effects could exist in the rat stomach that play a role in maintaining MC homeostasis and modulating some pathological processes. Apart from its well-studied metabolic effects, ghrelin also exerts many regulatory effects on the cardiovascular, central nervous and immune systems [55]. So far, among the immune cells, ghrelin mRNA has been found in human, rodent and goldfish lymphoid organs, B cells, T cells, monocytes and NK cells [42,44]. Dixit et al. [44] established that growth hormone secretagouge receptor (GHS-R) and ghrelin were expressed in human T lymphocytes and monocytes, where ghrelin acts via GHS-R to inhibit the expression of proinflammatory cytokines such as IL-1, IL-6 and TNF-α. It is well known that MCs produce a broad range of cytokines. Proinflammatory cytokines include cytokines associated with type 2 T-helper cell (Th2) responses such as IL-4, IL-5, IL-6 and IL-1 and cytokines associated with T-helper 1 responses including interferon-gamma (IFN-γ), IL-2, IL-3, IL-12, IL-18 and TNF-α [19–22]. Taildeman et al. [56] established the expression of leptin and leptin receptors in MCs in different human organs but did not provide any information on the presence of ghrelin-immunoreactivity in these cells. Based on their results, the authors suggested that leptin could influence MC activities using both paracrine and autocrine signalling.

Figure 1. Double fluorescent microscopy for tryptase (a) with ghrelin (b) in rat stomach. The immunofluorescence staining shows the colocalization of tryptase (a) with ghrelin (b) expression in mast cells (arrows) located in the serosal and muscle layers of the organ. Bar = 15 µm.
The paucity of data about ghrelin expression by MCs makes our findings relevant. The role of ghrelin in the control of immune responses and inflammatory responses has been explained by few authors [44]. The current work allowed us to hypothesize that other immune cells such as MCs could be able to produce and store ghrelin in their granules. This finding allowed us to assume the presence of similar interactions between ghrelin and leptin synthesized in MCs. However, it is necessary to clarify the roles of ghrelin and leptin, produced by MCs, in regulation of immune cell activation and inflammation. According to Hirayama et al. [57], ghrelin may be involved in MC activation during inflammatory responses.

In the current study, the presence of MCs with ghrelin- and tryptase-immunoreactivity in the serosal, muscular, submucosal and mucosal layers of the rat stomach was established. In the lamina propria mucosae MCs were located near the base of the proper gastric glands,
in the interglandular space and around the capillaries (Figure 3). In the submucosa, MCs were found predominantly near the microcirculatory blood vessels (arterioles, capillaries and venules). In the tunica muscularis, the MCs were also located mainly near the blood vessels and between smooth muscle layers. Some of the MCs were observed in the muscle bundles close to smooth muscle cells. In the serosa, ghrelin-positive MCs were found near the autonomic nerves as well as in the adventitia of the small arteries, veins, arterioles and venules. In this regard, new information about the immunohistochemical characteristics of gastric MCs in rats was added to that described by other authors who reported mainly alcian blue, toluidine blue and histamine-positive MCs [58,59]. Role of ghrelin in the stomach is controversial. Several researchers reported that ghrelin can influence gastric activity, especially its motility and acid secretion [60–63]. According to Masuda et al. [58], ghrelin increases stomach motility and acid secretion while Sibilia et al. [61] reported that ghrelin inhibits acid secretion. This role of ghrelin is important because during fasting, increased ghrelin serum level and decreased acid secretion protect the gastric mucosa. Based on assumptions of Sibilia et al. [62], we suggested that ghrelin-positive MCs in the gastric smooth muscle and near the proper gastric glands of the rat stomach could assist in regulation of stomach motility and acid secretion. Thus, we suggested that similarly to histamine and eicosanoids, ghrelin is another MC mediator participating in the regulation of smooth muscle contraction [45,46]. In addition, ghrelin was also described as a motilin-related peptide [47]. It is well known that motilin is an intestine-derived factor modulating gastrointestinal motility in humans. It also regulates gallbladder emptying by stimulating smooth muscle contraction [64].

Dixit et al. [44] reported a wide tissue distribution of GHS-R in the lymphoid system and suggested that ghrelin and GHS-R ligands may function as signal modulators among not only the immune and endocrine systems but also the nervous system. Inflammatory cytokines released by immune cells have been shown to act on the central nervous system to control food intake and energy homeostasis [65]. The localization of ghrelin-positive MCs near the autonomic nerves of the rat stomach suggests that the possible existence of nerve-immune interaction is established in urogenital organs of humans [63]. It has also been reported that MCs are localized near the SP-positive neurons where SP promotes the release of histamine [64]. Ghrelin-positive MCs localization near the autonomic nerves in the rat stomach wall correlates with results detecting MCs near the peptidergic nerves in other organs and species [23–25]. According to earlier studies, the neuropeptides can influence the secretion of MC cytokines which exert their modulatory effect in neurogenic inflammation [66–68]. Moreover, in vivo studies have been performed to confirm that the catecholamines, norepinephrine and epinephrine act as direct ghrelin secretagogues. These results are supported by high levels of β1-adrenergic receptor expression in ghrelin cells of the stomach. Thus, sympathetic nerves stimulate directly ghrelin secretion [69–72]. According to Yang et al. [73], ghrelin may regulate gastric smooth muscle contraction induced by cholinergic neurotransmitters using GHS-R1s, which are expressed on myenteric nerve cells, interstitial cells of Cajal and smooth muscle cells. The mentioned features of ghrelin could be used for explanation of the observed Ghr⁺ MC localization in the muscular layers of rat stomach.

Taking into account our results and those cited above, we hypothesize that MCs are another source of ghrelin which perform the communication between nervous, endocrine and immune systems. Controlled interactions between these systems are believed to be critical for the maintenance of systemic homeostasis.

**Conclusions**

In conclusion, the presence of ghrelin-positive MCs in the wall of the rat stomach allowed us to presume the ability of these cells to synthesize and store ghrelin in their granules, which may be an important source of ghrelin. Future studies will be required to identify the molecular pathways responsible for the biosynthesis and release of MC ghrelin as well as to understand the effects of ghrelin on specific aspects of MC function, maintenance of immune cell homeostasis and susceptibility to specific infections. New strategies focused on increasing the beneficial roles of MCs may facilitate the early response to pathogens.

**Disclosure statement**

All authors have no financial or personal relationship with other people or organizations that could inappropriately influence this work.

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