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Mast Cells in Rat Pulmonary Pleura

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The aim of the present immunohistochemical study is to determine the metachromatic, tryptase and ghrelin positive mast cells in pulmonary pleura of male rats at different ages. The detection on serial sections of tryptase and ghrelin immunoreactivity with metachromasy allowed us to estimate the number of these three types of cells in lung pleura of different aged male rats. We revealed that the amount of toluidine blue positive mast cells (MCTB) in the lung pleura was significantly less than that of tryptase (MCTr) and ghrelin positive mast cells (GhrMC). The number of tryptase and ghrelin positive mast cells showed similar values. In the comparative study on serial sections, we observed that some MCTr and GhrMC did not show metachromasia. In pleura's subserous layer the percentage of MCTr and GhrMC with manifested metachromasia varies in the different age groups: 58-59% in 1-year-old, 42% in 3-month-old and 20% in 20-day-old animals.

Key words: mast cells, ghrelin, tryptase, pleura, rat

Introduction

Mast cells are tissue-resident cells, which take part in the secretion of a variety of biologically active mediators, cytokines, and chemokines [9]. Their mediators can influence the biological activities of neighboring cells and tissues [10]. These cells are multifunctional effector cells involved in inflamation processes, hypersensitivity and allergic disease [1]. There are two main immunohistochemical types of mast cells (MCs) according to the protease content: tryptase positive mast cells (MCTr) and chymase positive mast cells [15]. Ertuğrul et al. [2] found that ghrelin and tryptase-positive cells are morphologically similar to each other. Matos et al. [8] in their study reported that the MC tryptase induces eosinophil recruitment into the pleural cavity and this effect dependens on MC tryptase proteolytic activity. Recently Giannou et al., [4] described the discovery of MCs in human and mouse malignant pleural effusion formations and the elucidation of their fate and role.

Mature and immature Wistar rats have been widely used to study the role of metachromatic and tryptase positive mast cells in pathological conditions of the lung, particularly the lung pleura. However, the knowledge of age-related features in the distribution of different phenotypes of mast cells in the lung pleura of healthy rats is scarce. The aim of our work was to perform immunohistochemical study to determine the metachromatic (MCTB), tryptase (MCTr) and ghrelin positive (GhrMC) mast cells in pulmonary pleura of healthy male rats at different ages.

Materials and Methods

Experimental animals

In our study 18 male Wistar rats at age of 20 days, 3 months and 1 year were used. Animals were anesthetized with ketamine and xylazine and were transcardially perfused with 4% paraformaldehyde in phosphate buffer. The animals were received from Project No. 13 / 2017, Medical faculty, Trakia University. All procedures were performed in accordance with the Bulgarian legislation regarding animal care (Ordinance 20 of 01.11.2012 on the minimum requirements for the protection and welfare of experimental animals and the requirements for the sites for use, breeding and/or delivery) and in in accordance with 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.

Material

The lungs of each animal were immediately removed, leaving the left and right lung lobes in 4% paraformaldehyde for 24 hours, washed with phosphate-buffered saline (PBS), dehydrated in an alcoholic beaker, clarified in xylene and included in paraffin. Serial tissue sections of 5 μm thickness from each animal were prepared from the included material, mounted on gelatin coated slides, then deparaffinized twice in xylene and rehydrated by series of decreasing ethanol concentrations.

Histochemical method with toluidine blue for visualization of metachromatic mast cells

Tissue sections were mounted on gelatinized slides, twice placed in xylene and rehydrated by decreasing ethanol concentrations. The sections were immersed in a 0.1% solution of toluidine blue in McLivane's buffer, pH 3.

Immunohistochemical methods for visualization of tryptase- and ghrelin-positive mast cells

The tissue sections were washed in 0.1 M PBS and placed in 1.2% hydrogen peroxide in methanol for 30 minutes, following by antigen recovery in buffer (pH 9.0) for 20 minutes. Between these steps, sections were washed with an EnVision Flex Wash Buffer, then incubated in a humidified chamber overnight at 4°C with primary antibodies: mouse antihuman ghrelin (2F4) at 1:50 dilution (sc-293422 Santa Cruz Biotechnology), monoclonal mouse antihuman mast cell tryptase – ready for use (MBS9510697, DAKO). After triple washing with PBS, the sections were incubated with EnVision detection system (DAKO) for 24 hours at 4°C. The immune reaction

was visualized with diaminobenzidine. PBS replacing the primary antibody was used as a negative control. The slices were dehydrated, washed, coated with glass slides and photographed with a research microscope (LEICA DM1000) equipped with a digital camera (LEICA DFC 290).

Of the three serial sections used, two were stained with tryptase and ghrelin antibodies, and the third was stained with toluidine blue for metachromasia in the three different age groups.

Statistical methods

The number of mast cells in the study was determined on three microscopic fields (X 200) of sections of the left lung of each animal using a light research microscope (LEICA DM1000) equipped with a digital camera (LEICA DFC 290). Raw mast cell density data (number / field of view) were processed using GraphPadPrism 6 for Windows (GraphPad Software, Inc., USA) for analysis of variations (one-way ANOVA), followed by Tukey-Kramer test. Values of $P < 0.05$ were considered statistically significant. The data are presented as mean ± standard deviation (SD).

Results

We revealed that the amount of toluidine blue positive mast cells in the lung pleura was significantly less than that of tryptase and ghrelin positive mast cells (**Table 1**).

Table 1. Distribution (mast cell count (MC) "mean"/field of view ± standard deviation "SD") of toluidine blue (MCTB)-, tryptase (MCTr)-positive mast cells and ghrelin-positive cells (GhrC) in the visceral pleura in the caudal lobe of the left lung. **a** ($P<0.0001$) – statistically significant difference between the number of MCTB and that of MCTr and GhrMC. **%** – MCTr and GhrMC, also showed metachromasia.

Age, Parameter	**Pleura**
20 days, **Number of MC**: MCTB Min-max MCTr Min-max /**%** GhrMC Min-max /**%**	 3.2 5± 0.87 2-4 16.08±0.90**a** 15-17/**20** 16.0±0.85**a** 15-17 /**20**
3 months, **Number of MC**: MCTB Min-max MCTr Min-max /**%** GhrMC Min-max /**%**	 7.0±0.89 6-8 16.45± 0.69**a** 15-17 /**42** 16.82± 0.87**a** 16-18 /**42**

Table 1.

1 year, **Number of MC**: MCTB Min-max	 9.75± 0.96 8-11
MCTr Min-max /**%**	16.67± 0.49**a** 16-17 /**58**
GhrMC Min-max /**%**	16.58± 0.51**a** 16-17 /**59**

The number of triptase and ghrelin positive mast cells showed similar values. In the comparative study on serial sections, we observed that some MCTr and GhrMC did not show metachromasia. In the subserous layer of the pleura, the percentage of MCTr and GhrMC with manifested metachromasia varies in different age groups: 58-59% in 1-year-old, 42% in 3-month-old and 20% in 20-day-old animals. Comparing the number of mast cells in each lobe in rats of different ages, we found an age-dependent difference in the number of mast cells in the subserosal layer of the three lobes. In the cranial right lobe, the greatest number of the searched cells was found in the 1-year-old rats, followed by the 3-month-old and the 20-day-old. The same distribution of mast cells was observed in the left lung. In the right caudal lobe, the fewest mast cells were found in the 20-day-old rats, and the number was approximately equal and greater in the 3-month-old and 1-year-old rats **(Fig. 1)**. Comparing the number of mast cells in the different lobes for each age, we found that in all three lung lobes examined in the 20-day-old rats, the number of cells was the same. In the 3-month-old and 1-year-old rats, the number of mast cells in the caudal right lobe was the greatest, followed by the cranial right lobe and the left lung **(Fig. 1)**. The present study showed a different distribution of MCTB compared to MCTr and GhrMC **(Table 1)**. The amount of MCTB in the lung pleura was significantly less than that of MCTr and GhrMC **(Fig. 2A, D)**. In the comparative study on serial sections, we found that part of MCTr

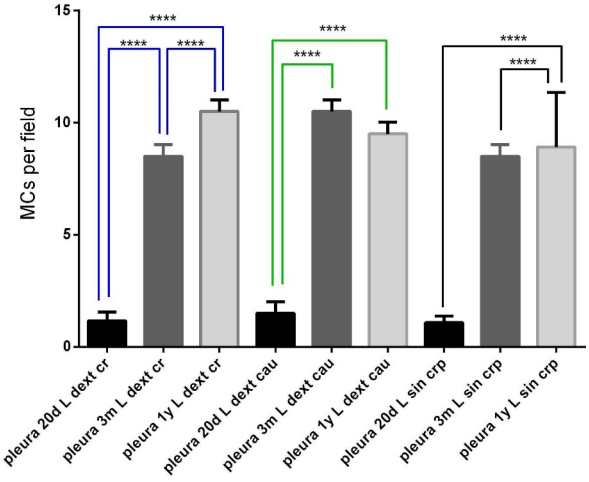

Fig. 1. Age-dependent difference in the number of mast cells in the subserosal layer of the three lobes of the lung- cranial lobe of the right lung, caudal lobe of the right lung and left lung.

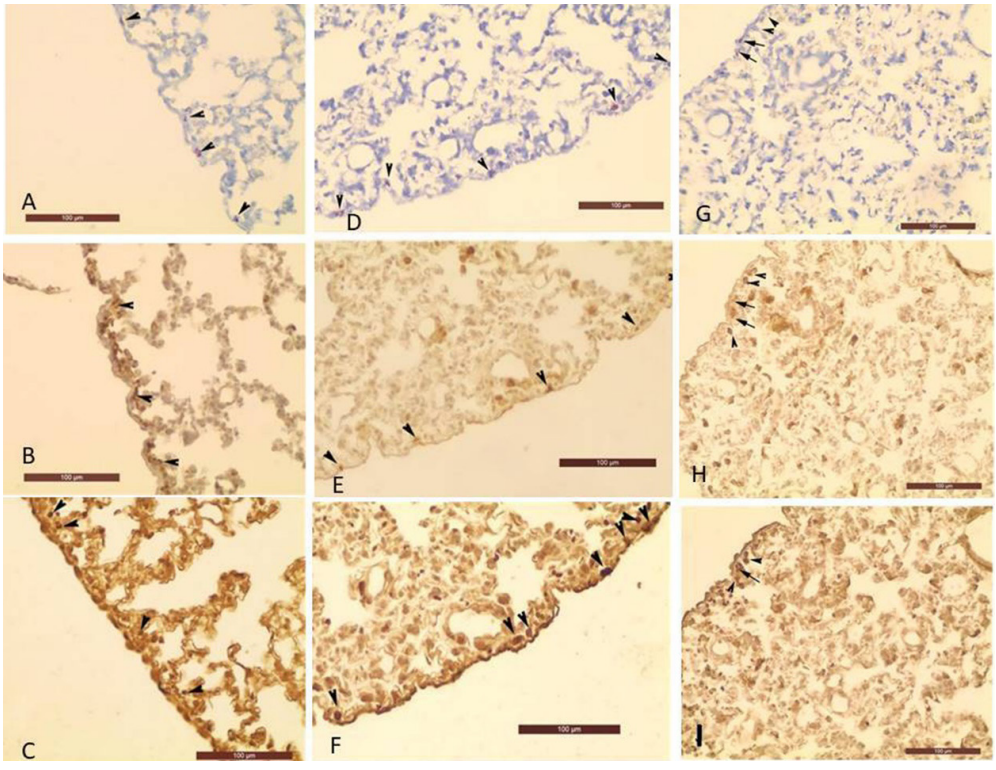

Fig. 2A. MCTB (arrowheads) in the subserosal layer of the visceral pleura in 20-day-old rats; **B.** MCTr (arrowheads) in the subserosal layer of the visceral pleura in 20-day-old rats; **C.** GhrMC (arrowheads) in the subserosal layer of the visceral pleura in 20-day-old rats; **D.** MCTB (arrowheads) in the subserosal layer of the pleura in 3-month-old rats; **E.** MCTr (arrowheads) in the subserosal layer of the pleura in 3-month-old rats; **F.** GhrMC (arrowheads) in the subserosal layer of the pleura in 3-month-old rats; **G.** MC (arrowheads) without metachromasia, but tryptase- and ghrelin-positive in the subserosal layer of the pleura. Arrows – MCTB. Age -1 year; **H.** MCTr (arrowheads) in the subserosal layer of the pleura without metachromasia but are ghrelin positive. Arrows – MCTr with metachromasia. Age -1 year; **I.** GhrMC (arrowheads) in the subserosal layer of the pleura without metachromasia, but are tryptase positive (arrow – GhrMC with metachromasia). Age -1 year. Bars = 100μm.

and GhrMC did not show metachromasia **(Figs. 2G, H, I)**. In the subserous layer of the pleura, the percentage of MCTr and GhrMC with manifested metachromasia varies in different age groups **(Table 1)**: it is the highest in 1-year-olds – 58-59%, followed by that in 3-month-olds – 42% and is the most -low in the 20-day-old animals – 20%. The number of GhrMC and MCTr in the pleura showed close values. At all three ages, no difference was found between the number of MCTr and GhrMC **(Figs. 2B, C, E, F).**

Discussion

This study presents original data regarding the normal density of the three types of mast cells: metachromatic, tryptase- and ghrelin positive mast cells in lung pleura of healthy

rats. The similar number of tryptase and ghrelin positive mast cells defines mast cells as a main source of ghrelin in pleura. The significance of our findings can be explained in several ways. Mast cell tryptase is a known smooth muscle mitogen participating in mast cell-mediated remodeling of the pleural vasculature but mast cell ghrelin may regulate contractility of vascular smooth muscle cells. In animal models was reported that ghrelin reduces the release of pro-inflammatory cytokines [7] and stimulates the release of the anti-inflammatory cytokine IL-10 by T lymphocytes and macrophages [16]. Gulubova et al., 2019, proved that ghrelin positive MCs number in the muscle layer of the stomach is significantly decreased in rats with metabolic disturbances which is related with lowered power of gastric muscle contraction [5]. It was found that that circulating ghrelin levels are deeply diminished in patients with gastric cancer related with the progression of gastric cancer [11]. On the other hand, it was shown that ghrelin is mitogenic having a promoting effect on neoplastic cell growth [14]. Mast cells as part of the innate immune system are involved in the pathogenesis of different tumors, exerting their pro- and antitumorigenic effects. Some studies have investigated the angiogenic role of MCs related to the increase of tumor growth and progression [6]. Mast cell mediators inducing angiogenesis are represented by basic fibroblast growth factor, tumor necrosis factor α and β, interleukin 4 (IL 4), interleukin 8 (IL 8) [13].

Other studies, however, reported the inhibitory role of MCs in tumor growth and progression, exerting a cytotoxic effect on tumor cells [3]. Both tryptase and chymase positive MCs have been detected in different solid tumors, including in lungs where tryptase positive MCs significantly predominated [6, 17]. In 2005, it has been reported that the frequency of the highly aggressive and relatively rare tumor malignant pleural mesothelioma (MPM) is increasing throughout the world [12]. Robinson et al., found that tryptase positive mast cells at tumor site were in higher number than chymase positive mast cells, even in some patients the chymase positive mast cells were absent [12]. Therefore, a high number of tryptase positive MCs can be assumed as independent favorable prognostic factor in pleural mesothelioma showing a correlation with a better prognosis. Robinson et al. revealed the importance of the immunologic analysis in the prognostic and therapeutic approach in patients with pleural mesothelioma [12].

Conclusions

It was found that immunohistochemical identification of mast cells by demonstration of tryptase in the rat lung is more reliable than toluidine blue staining. Mast cells are the main source of ghrelin in pleura. The knowledge of the distribution of different types of mast cells is of clinical importance for the development and prognosis of pathological processes of the pulmonary pleura.

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References

1. **Da Silva, E. Z. M., M..C. Jamur, C. Oliver.** Mast cell function: a new vision of an old cell. – *J. Histochem. Cytochem.*, **62**(10), 2014, 698-738.
2. **Ertuğrul, T., G. Sevilgen.** An immunohistochemical investigation of the effect of sambucus nigra on chymase-, tryptase- and ghrelin- positive cells in rat lung. **–** *Kocatepe Vet. J.,* **15**(2), 2022, 209-216.
3. **Ghiara, P., D. Boraschi, L. Villa, G. Scapigliati, C. Taddei, A. Tagliabue.** In vitro generated mast cells express natural cytotoxicity against tumour cells. **–** *Immunology,* **55**(2), 1985, 317–324.
4. **Giannou, A. D.,** A. **Marazioti, M. Spella, N. I. Kanellakis, H. Apostolopoulou, I. Psallidas, Z.M. Prijovich, M. Vreka, D.E. Zazara, I. Lilis, V. Papaleonidopoulos, C. A. Kairi, A. L. Patmanidi, I. Giopanou, N. Spiropoulou, V. Harokopos, V. Aidinis, D. Spyratos, S. Teliousi, H. Papadaki, S. Taraviras, L.A. Snyder, O. Eickelberg, D. Kardamakis, Y. Iwakura, T. B. Feyerabend, H. R. Rodewald, I. Kalomenidis, T. S. Blackwell, T. Agalioti, G. T. Stathopoulos.** Mast cells mediate malignant pleural effusion formation. – *J. Clin. Invest.,* **125**(6), 2015, 2317-2334.
5. **Gulubova, M. V., A. N. Tolekova, K. Ivanova, S. Hamza, M. Hadzhi, D. Chonov, J. Ananiev.** Fructose-induced metabolic disturbances in rats and its impact on stomach endocrine cell number and smooth muscle contractility. – *Archives of Physiology and Biochemistry*, **126**(3), 2019, 1-9.
6. **Ibaraki, T., M. Muramatsu, S. Takai**, **D. Jin, H. Maruyama, T. Orino, T. Katsumata, M. Miyazaki.** The relationship of tryptase- and chymase-positive mast cells to angiogenesis in stage I non-small cell lung cancer. – *Eur. J. Cardiothorac. Surg*., **28**(4), 2005, 617-621.
7. **Işeri, S. O., G. Sener, B. Saglam, F. Ercan, N. Gedik, B. C. Yeğen.** Ghrelin alleviates biliary obstruction-induced chronic hepatic injury in rats. **–** *Regul. Pept.,* **146**(1-3), 2008, 73-79.
8. **Matos, N. A., J. F. Silva, C. M Tamires, K. A. Damasceno, I. D. G. Duarte, V. S. Lemos, G. D. Cassali, A. Klein.** Mast cell tryptase induces eosinophil recruitment in the pleural cavity of mice via proteinase-activated receptor. – *Inflammation,* **36**(6), 2013, 1260-1267.
9. **Metcalfe, D., D. Baram, Y. A. Mekori.** Mast cells. **–** *Physiol. Rev*., **77**(4), 1997, 1033-1079.
10. **Mukai, K., M. Tsai, H. Saito, S. J. Galli.** Mast cells as sources of cytokines, chemokines, and growth factors. – *Immunol Rev.*, **282**(1), 2018, 121-150.
11. **Murphy, G., F. Kamangar, S.M. Dawsey, F. Z. Stanczyk, S. J. Weinstein, P. R. Taylor, J. Virtamo, C.C. Abnet, D. Albanes, N.D. Freedman.** The relationship between serum ghrelin and the risk of gastric and esophagogastric junctional adenocarcinomas. **–** *J. Natl. Cancer Inst.,***103**(14), 2011, 1123-1129.
12. **Robinson, B.W., A.W. Musk, R. A. Lake.** Malignant mesothelioma. – *Lancet*, **366**(9483), 2005, 397-408.
13. **Tharp, M. D.** The interaction between mast cells and endothelial cells. **–** *J. Invest. Dermatol.,* **93**(2), 1989, 107-112.
14. **Tian, P.Y., X. M. Fan.** The proliferative effects of ghrelin on human gastric cancer AGS cells. **–** *J. Dig. Dis.,* **13**(9), 2012, 453-458.
15. **Tütüncü, Ş., A.Ç. Torun, T. Ertuğrul**. The role of resveratrol on mast cell and chymase and tryptase expression in blunt – chesttrauma-induced acute lung injury in rats. – *Turk. J. Vet. Anim. Sci.*, **44**, 2020, 1260-1268.
16. **Warzecha, Z., P. Ceranowicz, A. Dembinski, J. Cieszkowski, B. Kusnierz-Cabala, R. Tomaszewska, A. Kuwahara, I. Kato.** Therapeutic effect of ghrelin in the course of cerulein induced acute pancreatitis in rats. **–** *J. Physiol. Pharmacol.,* **61**(4), 2010, 419-427.
17. **Welsh, T.J., R. H. Green, D. Richardson, D. A. Waller, J.K. O'Byrne, P. Bradding.** Macrophage and mast-cell invasion of tumor cell islets confers a marked survival advantage in non-small-cell lung cancer. **–** *J. Clin. Oncol*., **23**(35), 2005, 8959-8967.