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Short communication

METACHROMATIC, TRYPTASE- AND GHRELIN-POSITIVE MAST CELLS IN THE BLOOD VESSELS OF RAT'S LUNG

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Summary

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Mast cells and the mediators they release play a major role in the pathogenesis of lung diseases. They regulate the function of the smooth muscle layer of the pulmonary airways and the same layer of the blood vessels. There are no data about the distribution of metachromatic and tryptase-positive mast cells in the blood vessels' wall as well as on the content of ghrelin in mast cell granules in rat's lung. The aim of this research was to present an analysis of the number and localisation of tryptase-positive, ghrelin-positive and metachromatic mast cells in the blood vessels (arteries, capillaries, venules and veins) in rats of different age. Six male Wistar rats were used for each age group – 20 days, 3 months and 1 year. Tissue slices for histochemical examination were taken from the caudal lobe of the left lung and stained with toluidine blue. Immunohistochemical reactions were then performed to indicate the expression of tryptase and ghrelin, allowing comparing the presence of tryptase-, ghrelin-positive and metachromatic mast cells in the wall of the blood vessels of different diameters. The light microscopical study showed that in all types of blood vessels from the three age groups, the number of ghrelin positive cells was the largest, followed by tryptase-positive and metachromatic mast cells. The observed differences in the distribution of these cells are important for maintaining the lung homeostasis and can be used as reference values in experimental studies in order to obtain accurate results.

Key words: blood vessels, lung, mast cells, rat

Mast cells (MCs) release biogenic amines including serotonin, playing a key role in pulmonary arterial vasoconstriction and smooth muscle cell proliferation (Maclean & Dempsie, 2009) and histamine, which is a vasoconstrictor in pulmonary veins. They also activate the renin-angiotensin system which has been implicated in the pathogenesis of pulmonary hypertension (Ferreira *et al*., 2009) by releasing renin and chymase converting angiotensin I to angiotensin II (Miyazaki *et al*., 2006).

Presence of MCs in the vicinity of endothelial cells is one evidence for the MCs relationship with angiogenesis (De Souza Junior *et al*., 2017).

Regarding pulmonary vascular pathology, an increased number of lung MCs has been reported in plexogenic pulmonary arteriopathy (Heath & Yacoub, 1991), and congenital heart diseases associated with early pulmonary vascular diseases (Hamada *et al*., 1999).

Activated MCs produce several mediators including the biogenic amine serotonin, the cytokines interleukin (IL)-6 and IL-13, and serine proteases chymase and tryptase which are capable of activating matrix metalloproteases and are involved in the pathogenesis of pulmonary vascular remodelling (Gilfillan & Rivera, 2009).

The presence of ghrelin in mast cell granules in rat stomach was reported by Stefanov *et al*. (2017). The lack of data about the distribution of metachromatic (MCTBs), tryptase- (MCTr) and ghrelin- (MCGhr) positive mast cells in the wall of the blood vessels in rat's lung motivated us to undertake this study.

The aim of this research was to analyse the number and localisation of tryptase-, ghrelin- positive and metachromatic mast cells in the blood vessels of different calibers in rats of different age.

Experimental animals. In this study, 18 male Wistar rats at the age of 20 days, 3 months and 1 year (6 in each age group) were used. The procedures were performed according to the Scientific Project (№13/2017) of Medical Faculty of Trakia University, Stara Zagora, Bulgaria. Animals were anaesthetised with ketamine and xylazine (90 mg/kg + 10 mg/kg, IP), then transcardially perfused with 4% paraformaldehyde in phosphate buffer (PBS).

Material. The lungs of each animal were immediately removed, leaving the left lung in 4% paraformaldehyde for 24 hours, washed with 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 deparaffinised twice in xylene and rehydrated by a series of decreasing ethanol concentrations.

Histochemical method with toluidine blue for visualization of metachromatic mast cells. Tissue sections were mounted on gelatinised slides, twice placed in xylene and rehydrated by decreasing ethanol concentrations. The sections were stained in a 0.1% solution of toluidine blue in McLivane's buffer, pH 3 (Pearce,1960).

Immunohistochemical methods for visualisation 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 min. Antigen recovery in buffer (pH 9.0) was followed for 20 min. Between 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, monoclonal mouse antihuman mast cell tryptase – ready for use. After triple washing with PBS, the sections were incubated with EnVision detection system (DAKO) for 24 hours at 4 ° C. The immune reaction was visualised 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 all three age groups.

Statistical methods. The number of mast cells in the study was determined on

three microscopic fields (×200 with an area of 0.163 mm^2) of sections of the left lung of each animal using a light research microscope (LEICA DM1000) equipped with a digital camera (LEICA DFC 290). Numerical mast cell density data (number/field of view) were processed using one-way ANOVA, followed by Tukey-Kramer test (GraphPadPrism 6 for Windows; GraphPad Software, Inc., USA) for analysis of variations. Values of P less than 0.05 were considered statistically significant. The data are presented as mean ± standard deviation (SD).

In the wall of the pulmonary arteries accompanying the large bronchi, all types of MCs were localised mainly in the tunica adventitia, and in some cases in the tunica media (Fig. 1–3).

In capillaries, MCs were found near to the basal membrane of the endothelial cells. In the wall of the pulmonary veins accompanying the large bronchi, MCs were observed mainly in the tunica adventitia, adjacent to tunica media. In venules, MCs were located close to the basal membrane of the endothelial cells.

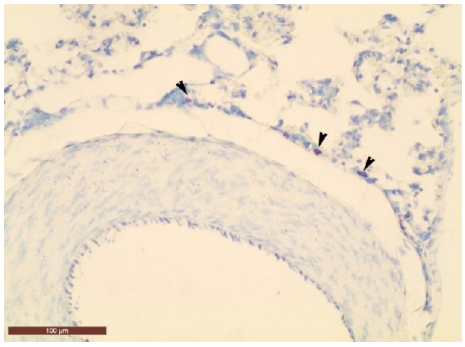

Fig. 1. Metachromatic mast cells (arrowheads) in arterial adventitia in 1-year-old rats. Bar=100 μm.

In all types of blood vessels from the three age groups, a significantly lower number of MCTBs was found compared to that of MCTr and ghrelin-positive cells (GhrC) ($P<0.0001$, Table 1). Only in the vein wall in all three age groups, GhrC prevailed, although with a small difference ($P<0.05$), compared to MCTr (Table 1).

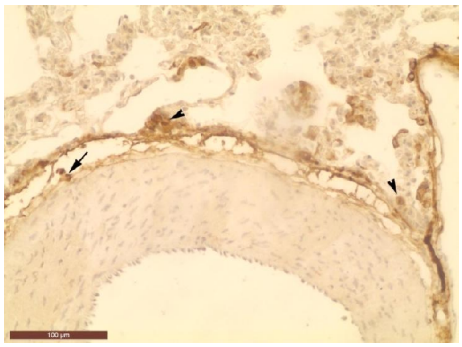

Fig. 2. MCTr (arrowheads) in the artery adventitia in 1-year-old rats. Arrow: MCTr adjacent to the muscular layer of the artery. Bar=100 μm.

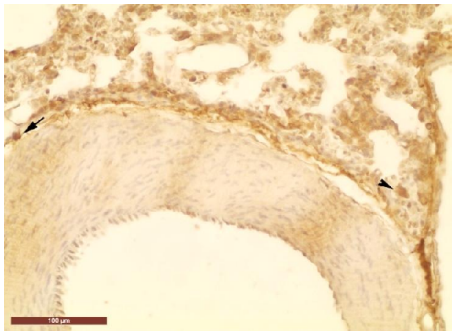

Fig. 3. GhrC (arrowhead) in the artery adventitia in 1-year-old rats. Arrow: GhrC adjacent to the muscular layer of the artery. Bar=100 μm.

In the venule wall, the number of MCTr and GhrC in animals at 3 months and 1 year of age, exceeded the number of both MCTr and GhrC at 20 days of age (Table 1) in contrast to that in the wall of veins, arteries and capillaries, where values at different ages were similar.

Table 1. Distribution (mean mast cells number/visual field ± standard deviation) of toluidine blue-positive mast cells (MCTB), tryptase-positive mast cells (MCTr) and ghrelin-positive mast cells (GhrC) in the wall of veins, venules, arteries and the capillaries in the caudal lobe of the left lung.

Parameter	Veins	Venules	Arteries	Capillaries
20 days of age				
MCTB	2.58±0.66	0.83 ±0.38	3.83±1.15	4.58±0.51
Min–max	2–4	0–1	2–5	4–5
MCTr	9.0± 0.95a	1.83 ±0.38a	9.08± 0.79a	7.50± 0.52a
Min–max/%	8–10 /29	1–2 /45	8–10 /42	7–8 /61
GhrC	9.91 ±0.66a,b	1.91 ±0.28a	9.25 ±0.75a	7.58± 0.51a
Min– max /%	9–11 /26	1–2 /43	8–10 /41	7–8 /61
3 months of age				
MCTB	3.17±0.72	1.00 ±0.42	5.33± 1.49	5.25± 0.45
Min–max	2–4	0–2	2–7	5–6
MCTr	8.42 ±0.51a	3.50 ±0.52a	8.66 ±0.65a	7.91± 0.79a
Min–max /%	8–10 /38	3–4 /29	8–10/62	7–9 /66
GhrC	9.58 ±0.51a,b	3.58 ±0.51a	8.68± 0.72a	7.83 ±0.71a
Min–max /%	9–10 /33	3–4 /28	8–10 /62	7–9 /67
1 year of age				
MCTB	3.50 ±0.52	1.16 ±0.38	5.42 ±1.56	5.58±0.51
Min–max	3–4	1–2	2–7	5–6
MCTr	9.50 ±0.67a	4.08 ±0.51a	8.67 ±0.77a	8.58 ±0.51a
Min–max /%	8–10 /37	3–5 /28	8–10 /63	8–9 /65
GhrC	10.67 ±0.78a,b	4.17 ±0.72a	8.75± 0.75a	8.58 ±0.51a
Min–max /%	10–12 /33	3–5 /28	8–10 /62	8–9 /65

a: $P<0.0001$ between the number of MCTBs and that of MCTr and GhrC; b: $P<0.05$ between the number of GhrC and that of MCTr; %: metachromatic MCTr and GhrC.

The highest percentage of MCTr and GhrC with metachromasia was found in the capillaries, followed by venules, arteries and veins (Table 1).

Most MCTBs were found in the artery wall and adjacent to the capillaries, followed by the veins, and the least number – in the venule wall in all three age groups. The comparative study of the number of MCTBs for each type of blood vessel showed a significant difference only in the artery wall. In 20-day-old animals the cell number was lower than that in 3-month-old ($P<0.01$) and 1-year-old ($P<0.001$) rats (Table 1).

The number of MCTr and GhrC in the venule wall was similar but significantly higher than that of MCTB (Table 1).

It is well-known that toluidine blue staining and tryptase immunohistochemis-

try were used for detection of mast cells in different mammalian species such as dogs (Stefanov, 2009; Stefanov *et al*., 2012; Stefanov & Vodenicharov, 2012), cats (Stefanov & Vodenicharov, 2008), swine (Stefanov & Vodenicharov, 2016; Stefanov *et al*., 2016) rats (Stefanov *et al*., 2017). The current study found out that in rat lung, the density of tryptase-positive mast cells was higher than that of the metachromatic ones, therefore tryptase can be consider as a better marker for lung mast cells than toluidine blue. Using these markers, ghrelin-positive mast cells were clearly identified in lung vessels. The presence of ghrelin-containing granules of rat mast cells corresponds to findings of Stefanov *et al.* (2017). The primary localisation of rat lung mast cells in the muscle layer and near the blood vessels of the adventitial layer can be explained by the ability of these cells to synthesise mediators such as serotonin (Keith *et al*., 1987) and nitric oxide (McCauley *et al*., 2005; Stefanov & Vodenicharov, 2012; Stefanov *et al*., 2012), responsible for the contraction and relaxation of smooth muscles. Serotonin has been reported to have a mitogenic effect on endothelial cells and pulmonary artery smooth muscle cells (Wang, 2004). Pulmonary vascular remodelling occurs when serotonin is bound by the serotonin transporter and thus, serotonin plays an important role in the development of pulmonary arterial hypertension (Mikulski *et al*., 2010). Penkova (2018) established that in the rat stomach and small intestine, ghrelin participated directly in the regulation of secretion and contractility by its receptor. This denotes that mast cells can regulate the function of the airways' and vascular smooth muscle layers.

The observed differences in the distribution of the mast cells in the blood vessels of different diameters in rat's lung are important for maintaining the lung homeostasis and can be used as reference values in experimental studies in order to obtain accurate results.

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