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ANALYSIS OF LOCALISATION OF MEGAKARYOCYTES IN THE BONE MARROW OF THE RAT- POSSIBLE RELATIONSHIP WITH INNERVATIONS

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ABSTRACT

Within the bone marrow, megakaryocytes thrive in an environment rich in numerous growth factors that influences their development, maturation, and may play a role in signaling the start of platelet production. Up to now, experiments concerning localisation of various populations of cells in rat bone marrow showed their certain relationship to the blood vessels. There are strikingly few reliable sources of information available in the literature that confirm the relationship between the localisation of these cells in connection to nerve fibers. Therefore the purpose of the present study was to examine if megakaryocytes are located in a defined relation to sensory and sympathetic nerve fibers.

The study used immature 6 weeks rats of the Wistar breed of both sexes. The animals were kept under constant lighting conditions (12 hours light-dark cycle) and temperature, and had unrestricted access to water and food (standard laboratory rodent feed).Double immunostaining method was applied with usage of secondary antibodies conjugated with fluorochromes (Cy3 and DTAF) to identify cells. The antibody against CD42d was used to immunolocalise megakaryocytes, while in order to identify the distribution of nerves, antibodies anti-NPY (for detection sympathetic nerve fibers) and anti-PGP 9.5 (sensory nerve fibers) were applied.

These findings showed the presence of megakaryocyte and megakaryoblastic cells which were distributed with a close relationship to the blood vessels but some of them were located in parenchyma. It was shown that NPY-positive and PGP 9.5-positive cells were present in the vicinity of blood cells. Furthermore, some megakaryocytes were located near PGP 9.5-labelled cells. The relationship between sympathetic nerve fibers containing neuropeptide Y and megakaryocytes was also detected.

The presence of megakaryocytes with a close vicinity of nerve fibers suggests the influence of the released neurotransmitters on the location of the megakaryocytes in the bone marrow.

KEYWORDS: megakaryocytes, platelets, bone marrow, innervation, neuropeptide Y, PGP 9.5

INTRODUCTION

The bone marrow (BM) is the main haematopoietic organ in postnatal life of mammals. The red BM consists of two compartments - vascular and haematopoietic. The vascular compartment is made up of a few arteries, more numerous veins, and above all, BM sinusoids, the walls of which form tightly adjacent endothelial cells. The haematopoietic compartment forms a stroma composed of reticular connective tissue and free cells of the haematopoietic system (all lines in various stages of development) inherent in its meshes. Stromal cells form a specific microenvironment that determines the division, differentiation, and maturation of haematopoietic elements [1].

The haematopoietic environment consists of morphotic elements such as stromal cells and extracellular substance, i.a. in the form of fibers, as well as molecular factors: cytokines, chemokines, and growth factors secreted into the microenvironment by stromal cells. The bone marrow microenvironment is a well-organized structure that regulates haematopoiesis, amongst others through the occurrence of BM niches with different elements, depending on the stage of development and the type of haematopoietic cell line [1,2,3]. The concept of the BM niche was introduced by Schofield in 1978 [4], but the differences between niche structures, e.g. stem cells, and myeloid cell lines have been demonstrated in detail in the recent years. Niches of stem and progenitor (CD34 +) cells are located in the medullary canal, mainly in the area of inner periosteum (endosteum), while the maturing precursor cells of the granulocytic line are found close to the vessels [4]. Haematopoietic cell groups are not scattered randomly, but are arranged in the BM according to a certain pattern. Megakaryocytes are in direct contact with venous sinus walls, into which lumen they release platelets [2,5].

Erythropoiesis also occurs near the vessels, while cells of the granulopoetic line tend to occupy the space around the arterioles, as well as near the bone trabeculae [6].

Research on the innervation of the BM has a long history. Already back in the 30s of the last century De Castro showed that nerves penetrate into the medullary canal along with the nutrient artery, branch out, and run along the marrow arterioles, entwining them densely Nowadays it is known that some of the nerve fibers also reach the haematopoietic compartment, penetrating between haematopoietic cells. The bone marrow is innervated by myelin and non-myelin, autonomic and sensory nerve fibers, with only a few of them leaving the blood vessels and ending their course between the marrow cells [7,8]. All blood vessels receive a supply of sympathetic nerves, and the same blood vessels that supply a mineralized bone or periosteum continue their course in the BM. Similarly, putative sensory fibres releasing substance P (SP) and calcitonin gene-related peptide (CGRP) accompany noradrenergic sympathetic fibers along the same blood vessels that supply the surrounding bone and are further disseminated within the BM [9,10,11].

Due to the double immunohistochemical staining it was demonstrated that all SP-immunopositive cells contain CGRP, and the vast majority of CGRP-immunoreactive cells shows the presence of SP [11,12,13].

One of the conditions that proves the cooperation of the haematopoietic and nervous systems is the presence and synthesis of the neurotransmitter in the nerve endings, near haematopoietic cells. The bone marrow cells must thus have receptors for these relays. These are β2-adrenergic receptors on granulocytes, lymphocytes, and monocytes or CGRP receptors on mouse BM cells, respectively. Human megakaryocytes (Mks) account for less than 0.5% of cells that are present in the BM, although they have also been detected in the lungs and peripheral blood [14]. They are the largest (12-150 μm) BM cells appearing at the earliest in the yolk sac, then in the liver and spleen, and at around the third month of life in the BM. The megakaryocyte is easily identified within the BM due to the large, polyploid, multi-lobed cell nucleus. Unlike other cells, Mks are subject to a mitotic cell cycle during which they replicate DNA but do not undergo anaphase or cytokinesis. In the correct conditions, the most numerous are Mks with a nucleus of 8N or 16N, but the ploidy usually ranges from 2N to 64N [15,16]. Megakaryocytes develop in the cytokine-rich bone marrow environment, which affects their maturation, development, and signaling of the start of platelet production. When Mks mature in the BM space, they move close to the venous sinuses [17] Battinelli et al. (2007) suggested the role of PECAM-1 glycoprotein at the rate and direction of Mks migration in the myeloid environment. Megakaryocytes deprived of this glycoprotein show defective migration and altered BM localisation, which was confirmed in in vivo studies [18]. Patel et al. (2005) have shown that chemokines: stromal cell-derived factor (SDF-1) and fibroblast growth factor 4 (FGF-4) direct the Mks precursor cells to the appropriate medullary niches, allowing the localisation of Mks between BM endothelial cells. SDF-1 activity is important when transferring Mks precursor cells from "osteoblastic niches" to "vascular niches". FGF-4, however, affects the maturation of precursor cells and their adhesion to BMCS [16,18,19]. Platelets are created from Mks in the BM in a mechanism that is still poorly understood. Junt's et al. (2007) observations have shown that mature Mks in the BM routinely develop processes - blood proplatelets into blood vessels [20]. Stress resulting from blood flow divides the proplatelets, generating platelet formation [17,21].

There is not enough research on the distribution of nerve fibers in relation to the location of Mks, therefore it seemed advisable to carry out additional tests to check whether such dependence exists.

The aims of the present study were analysis of megakaryocytes distribution in rat bone marrow, detected with anti-CD42d antibody, investigate whether there is a relationship between the localization of Mks in the bone marrow rat, and the location of peripheral nerves detected by an antibody directed against PGP 9.5 and sympathetic nerves, detected with anti-NPY antibody, topographic characteristics of the Mks location in the marrow.

MATERIAL AND METHODS

Animals

The study used immature 6 weeks rats of the Wistar breed of both sexes. The animals were kept under constant lighting conditions (12 hours light-dark cycle) and temperature, and had unrestricted access to water and food (standard laboratory rodent feed).

IMMUNOHISTOCHEMISTRY

Reagents

Starting solutions:

- 0.05 M PBS (phosphate buffered saline) at pH = 7.4

1.53g of NaH2PO4 x2H2O, 14.4g of Na2HPO4 x12H2O, and 45g of NaCl were dissolved in 800 ml of redistilled water, and then filled up to 1 liter with redistilled water.

- 0.2 M phosphate buffer at pH = 7.4

8.06g of KH2PO4 and 37.75g of Na2HPO4 x7H2O were dissolved in 900 ml of redistilled water. To determine pH = 7.4, appropriate amounts of 2 N HCl and 2 N NaOH were added and then filled up to 1 liter with redistilled water.

Materials for perfusion and fixation

-Krebs-Singer fluid (physiological saline): NaCl - 133mM, KCl - 4.7 mM, MgCl2 - 1mM, CaCl2 - 2.5mM, NaH2PO4 - 1.4 mM, NaHCO3 - 16.3mM, glucose - 7.8mM.

Buffered paraformaldehyde: 4% paraformaldehyde solution in 0.1 M phosphate buffer at pH = 7.4

Materials for bone decalcifying and cryoprotection

-0.01 M PBS (prepared on the basis of an initial solution, i.e. 0.05 M PBS).

-0.3 M EDTA: 10% solution of disodium salt of ethylenediaminetetraacetic acid in 0.1 Tris/HCl buffer at pH = 6.9 (Sigma).

-25% sucrose solution in 0.01 M PBS with the addition of 0.01% of NaN3

Materials for immunohistochemistry:

-0.01 M PBS (prepared on the basis of an initial solution, i.e. 0.05 M PBS).

-PAD - primary antibody dilutor: 10% normal goat serum (Sigma), 0.1% bovine serum albumin (Sigma), 0.05% thimerosal (Sigma), 0.01% NaN3 (Sigma), 1% Triton X-100 (Sigma).

-primary antibodies:

- rabbit polyclonal immunoglobulins - antigen CD42d (Megakaryocytes) –Santa Cruz Biotechnology Sc-25807; working dilution 1:200,
- rabbit polyclonal immunoglobulins –antigen PGP 9.5 (Protein gene product)- Chemicon International AB1761; working dilution 1:2000,
- rabbit polyclonal immunoglobulins –antigen NPY (Neuropeptide Y) - Affiniti, Exeter NA1233; working dilution 1:1000

-secondary antibodies:

- goat anti-rabbit immunoglobulins conjugated with Cy3 fluorochrome (Jackson IR, West Grove, Pa, USA, 111-165-144; working dilution 1: 600),
- biotinylated goat anti-rabbit immunoglobulins (1: 500 dilution),
- streptavidin conjugated with DTAF (dilution 1: 500).

- glycerol solution in 0.01 M PBS (to close the preparations).

BONE PREPARATORY PROCEDURES

Perfusion and preservation

The animals were anesthetized with an excess of sodium pentobarbital (vetbutal) administered intraperitoneally and transcardially perfused with Krebs-Ringer fluid, and then, for good fixation, with 4% buffered paraformaldehyde. The perfusion pressure was regulated by gravity and maintained at a height of 60-80cm of water column. The corpses of perfused rats, from which femurs were dissected, were obtained from the Department of Neurophysiology after the brain removal. This establishment has the permission of the Local Ethical Committee for Animal Experiments to perform perfusion fixation.

After femurs bone removal, they were cleared from the skin and partly from the muscles, and additionally fixed by immersion in the same fixative for 17 hours (hrs), after which they were rinsed in 0.01 M PBS for a period of 2 hrs.

Decalcifying and cryoprotection

The bones were demineralized by decalcifying in 0.3 M EDTA solution for 10-14 days at 4°C. The decalcifying solution was changed every 2-3 days. The degree of demineralization was checked by puncturing the bone with a dissecting needle and controlling their elasticity. In order to protect the tissue from damage caused by freezing processes, the bones were transferred to a solution of 25% sucrose in 0.01 M PBS with 0.01% sodium azide and were saturated until they fell to the bottom of the vessel.

Section preparation

The bones were sunk in the Tissue Freezing Medium (Leica, Germany), frozen on a cryostat freeze plate (-40°C) (Leica, JUNG CM1800, Germany), and cut in the sagittal plane (longitudinal sections) into 14μm sections. The sections were collected on glass slides coated with a 0.1% poly-L-lysine solution (Sigma) and then dried at 37°C for 2 hrs.

Immunohistochemical staining

All immunohistochemical incubations were performed in moist chambers, at room temperature (RT). Primary and secondary antibodies were diluted in PAD solution.

Solutions of antibodies and other reagents were spotted on sections that were previously circled using a Novo Pen hydrophobic pen. It prevents mixing of antibodies administered to adjacent sections on the same slide, and also minimizes the amount of antibodies used.

The previously dried sections were hydrated in 0.01 M PBS and then pre-incubated in PAD solution for 30 minutes at RT. The preparations were then incubated in a solution containing primary antibodies which were dissolved in PAD for a period of 17 hrs. The preparations were rinsed sequentially (3 x 5 min), and then the secondary antibodies: goat anti-rabbit immunoglobulin (anti-PGP 9.5, anti-CD42d), coupled with Cy3 and biotinylated goat anti-rabbit immunoglobulins (anti-NPY) were applied for 1 h. After rinsing in PBS, a final hourly incubation with streptavidin conjugated with dichlorotriazinylamine fluorescein (DTAF) was conducted. The preparations were rinsed again in 0.01 M PBS (3 x 5 minutes) and closed in a glycerol solution in 0.01 M PBS.

Reaction control

Control reactions, in which the primary antibodies or secondary sera were replaced with non-immunogenic serum, did not show the presence of immunoreactive structures.

Microscopic observation and registering of preparations

The preparations were observed using an Olympus BX-50 microscope with the option of epifluorescence, and the images were recorded using the Olympus Camedia 5050 digital camera and saved on a PC computer in the form of JPG graphic files with a resolution of 2040 x 1536 pixels.

RESULTS

The obtained results are based on the interpretation of tissues photographs taken of the preparations observed under the fluorescence microscope that have been recorded in the form of JPG graphic files.

Immunohistochemical staining with the anti-CD42d antibody enabled to visualize the location of Mks /megakarioblasts in the rat bone marrow. The secondary antibody was conjugated with the Coch3 fluorochrome. In the pictures obtained (Fig. 1, 2), the cells of this line show red fluorescence. The images show single Mks with red fluorescence (Fig.1), as well as younger forms of megakaryocytic lineage - megakaryoblasts (Fig. 2), occurring mainly in the metaphyseal area of the bone and in larger clusters compared to labelled mature forms.

It was observed that the distribution of Mks was associated with the vascular compartment of the BM and some of the cells were present in the BM parenchyma. The percentage of stained cells of the megakaryocytic line was small due to the fact that in the normal rat myelogram this line constitutes about 2-3%.

Immunohistochemical staining with anti-PGP 9.5 antibody was performed to visualize the course of nerve fibers of all types. A secondary antibody against this nerve marker was conjugated with Cy3 fluorochrome, so PGP 9.5-positive nerves show red fluorescence (Fig. 3,4,5). It was observed that these nerves accompanied the BM blood vessels, coursing in their vicinity. We observed only a few Mks located at the blood vessels entwined with PGP-9.5-positive fibers (Fig. 6). The difficulty in observing this relationship was due to the fact that both the antibody detecting Mks and the anti-PGP 9.5 antibody we had at our disposal came from the same animal. Both of these antibodies were rabbit polyclonal and therefore the same secondary antibody was used, i.e. goat anti-rabbit immunoglobulins, conjugated with Cy3 fluorochrom, which exhibited red fluorescence. As a result, both antigens tested shone in the same color (in red) and it made the observation difficult.

Immunohistochemical staining with anti-NPY antibody was performed to visualize the course of

neuropeptide Y (sympathetic) nerves. The secondary antibody was conjugated with dichlorotriazinylamine fluorescein (DTAF), thus NPY-positive nerves show green fluorescence (Fig.7). Double immunohistochemical staining with the above mentioned antibodies was used to visualize the course of the nerves (anti-PGP 9.5, anti-NPY) in relation to the localisation of Mks /megakarioblasts (anti-CD42d). In addition, a correlation between the location of Mks and the course of NPY-positive fibers was recognized, as some of the labelled Mks were located in the vicinity of neuropeptide Y-containing fibers (Fig. 8, 9, 10). The mature Mks revealed in this double staining showed fluorescence between red and green. It is therefore believed that these Mks demonstrated both CD42d antigen (red fluorescence) and NPY (green fluorescence) expression. The green fluorescence could be due to the labeling of the neuropeptide Y, which occurs in Mks. In addition, it was observed that in mature Mks, green fluorescence prevailed over red, which in turn dominated in younger cells (Fig. 8).

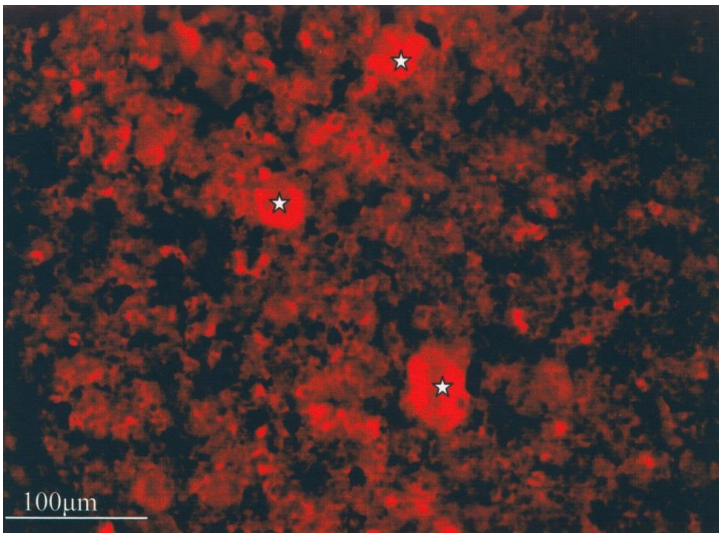

Fig. 1. Bone marrow of a rat. Immunohistochemical reaction using anti-CD42d antibody - detecting megakaryocytes. Visible single megakaryocytes (asterisks) are located in the marrow parenchyma.

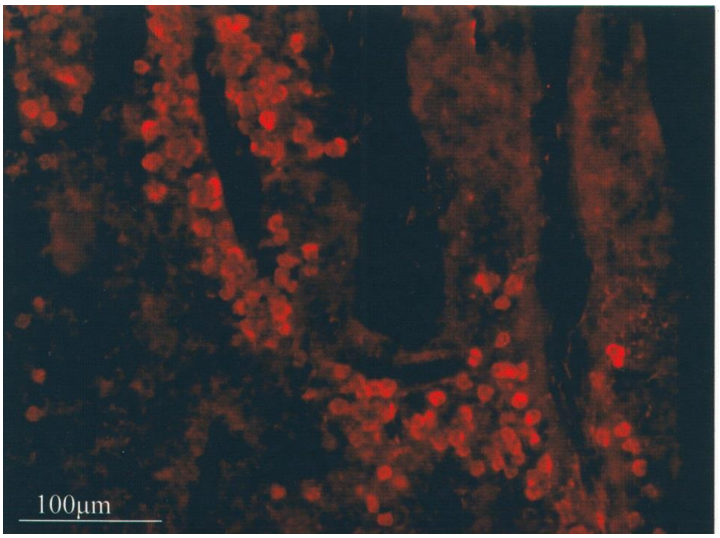

Fig. 2. Bone marrow of a rat. An immunohistochemical reaction using anti-CD42d antibody. Visible juvenile forms of megakaryocytes - megakaryoblasts occurring in clusters in the metaphysis of the bone.

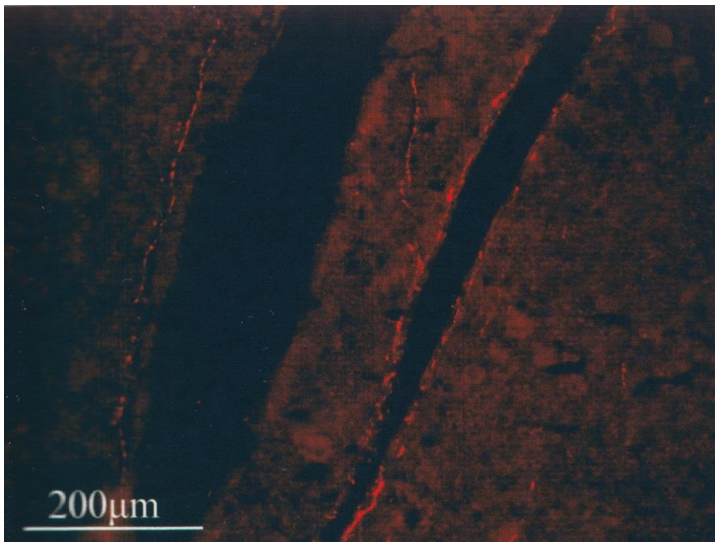

Fig. 3. Thin PGP 9.5-positive nerve fibers accompanying a large bone marrow nutrient vessel.

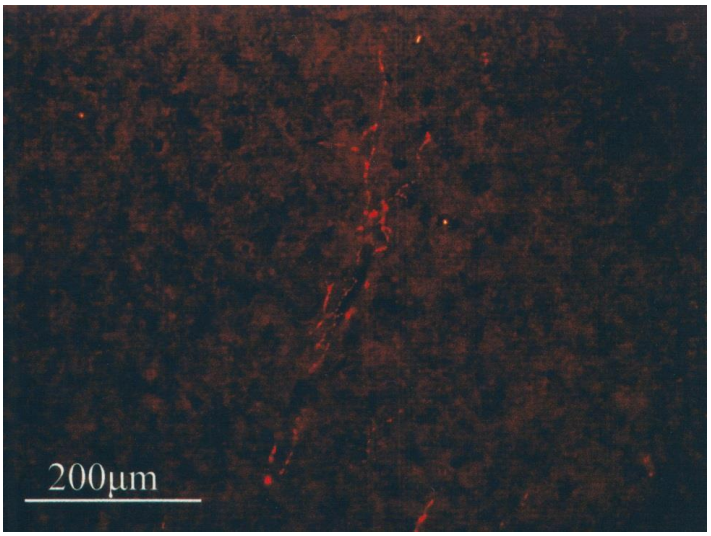

Fig. 4. Visible numerous thin 9.5-positive PGP fibers along the blood vessels.

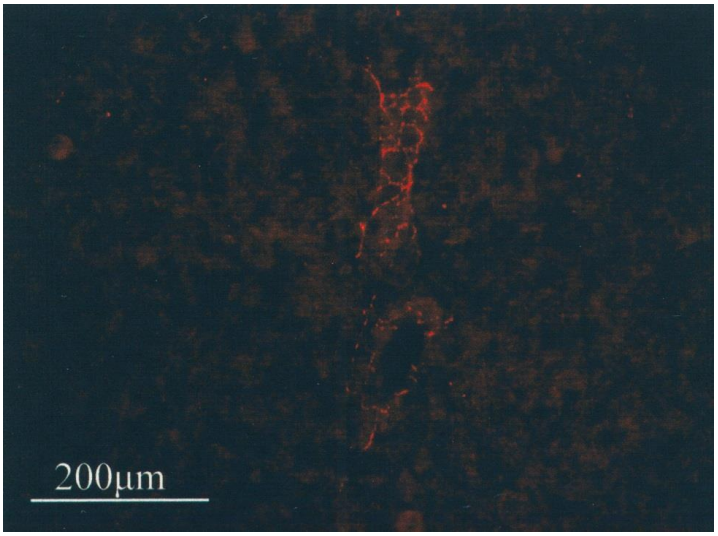

Fig. 5. Visible blood vessel densely entwined with nerves.

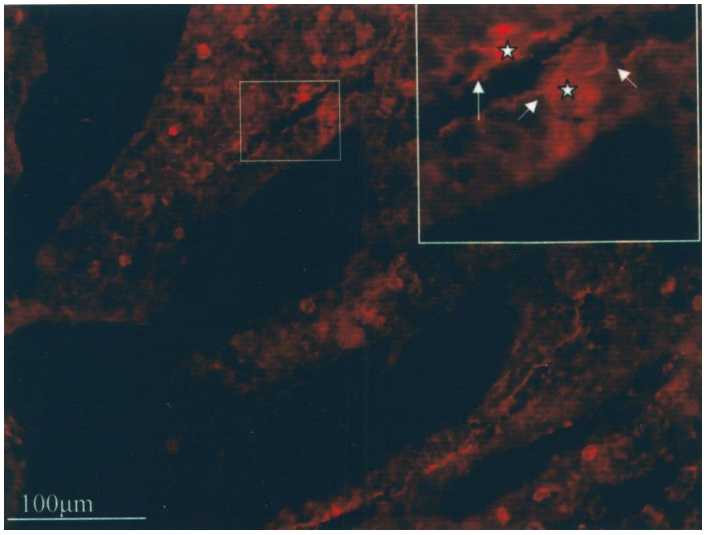

Fig. 6. Bone marrow of a rat. Immunohistochemical reaction using anti-CD42d and anti-PGP 9.5 antibodies. Visible 2 megakaryocytes (asterisk) located in close proximity to peripheral PGP 9.5-positive fibers (arrows).

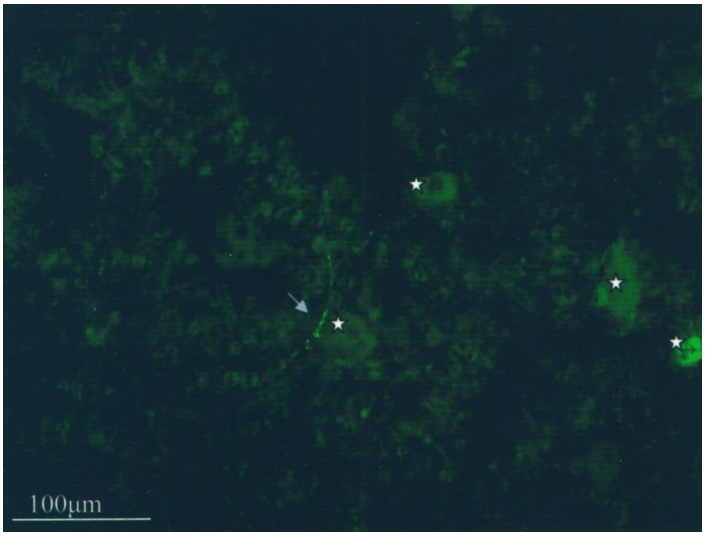

Fig. 7. Bone marrow of a rat. An immunohistochemical reaction detecting NPY.
Visible 4 megakaryocytes (asterisk), one of which is entwined by the NPY-positive nerve fiber (arrow).

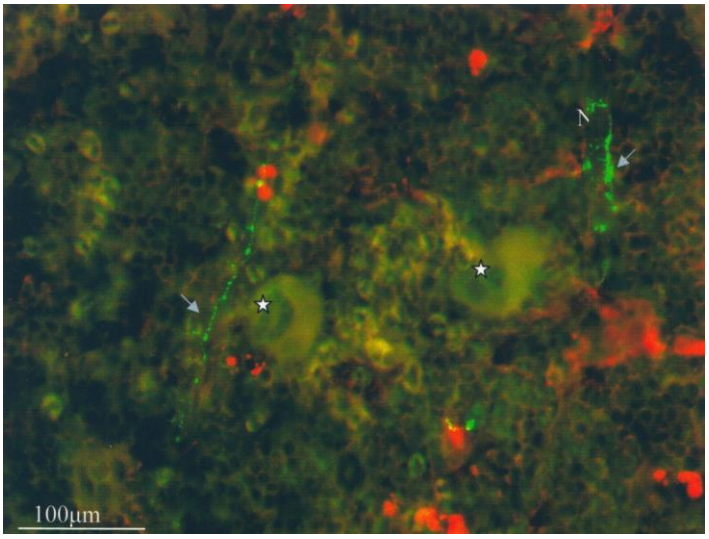

Fig. 8. Bone marrow of a rat. An immunohistochemical reaction using anti-CD42d and anti-NPY antibodies. Visible two mature megakaryocytes (asterisks), one of which is near the NPY-positive nerve fiber (arrow) and blood vessel (N) entwined with NPY-positive fiber.

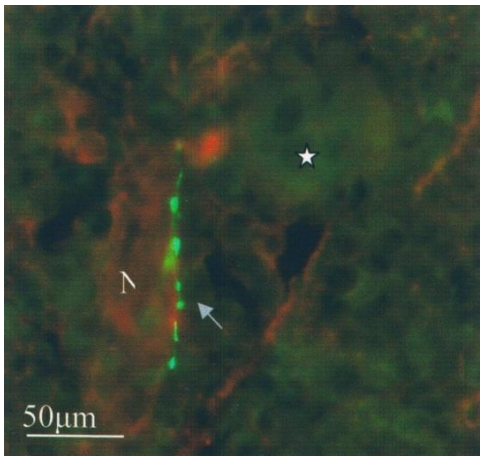

Fig. 9. Visible megakaryocyte (asterisk) located in close proximity to the NPY-positive fiber (arrow).

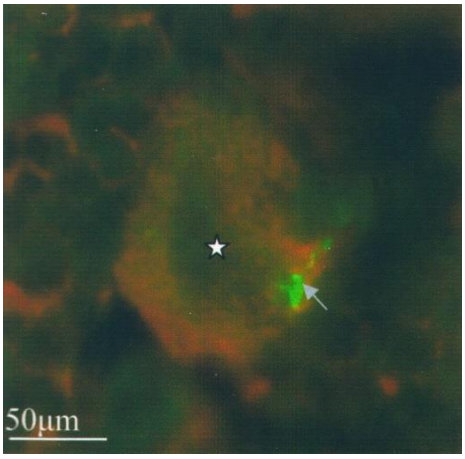

Fig. 10. Visible NPY-positive fiber (arrow) accompanying the blood vessel (N), located near the megakaryocyte (asterisk).

DISCUSSION

Research on the innervation of the BM has a long history and although the presence of nerves in the BM has been sufficiently documented, the importance of substances released by nerve endings with respect to the haematopoiesis process remains unclear. It is known that nerve fibers reach the bone with the right branch of the spinal nerve, supplying given region of the body. These nerves contain both sympathetic and afferent sensory fibers originating from the spinal ganglion [8,12].

Nowicki et al (2007) described that the nerves penetrate the medullary cavity together with the nutrient artery, branch out and follow the nutrient arterioles, entangling them in the form of a dense network [22]. More information about mice innervation of the BM was presented by Yamazaki and Allen (1990), who analyzed the electron microscope pictures and showed that the marrow nerve fibers contain both myelinated and non-myelinated axons that finish their course mainly between the tunica adventitia cells [5]. Some of them, although it was a smaller percentage, transfer to haematopoietic parenchyma and to the walls of medullary sinuses [23]. Since mouse BM has been well explored, we focused here on the distribution of the megakaryocytic cell line in myeloid environment of the femoral bone marrow of the rat.

Megakaryocytes in rodents are the least numerous and at the same time the largest of all BM cells and in the normal smear occur mainly in the vicinity of venous sinuses. Megakaryocytes develop in a myeloid environment rich in various growth factors, such as thrombopoietin and cytokines (e.g. interleukin), which affects their maturation, development, and plays an important role in signaling the start of platelet formation [16, 18, 20,24,25,26]. The influence of neurotransmitters, such as: dopamine, neuropeptide Y, serotonin, vasoactive intestinal peptide (VIP) on the development of Mks [27] was also demonstrated.

Our results seem to broaden the knowledge on the relationship between the distribution of Mks in the BM and the course of sympathetic and peripheral nerves. Previous studies on Mks and other haematopoietic cell lines have shown that their location is ordered and depends on the marrow vessel system [2,5,28]. However, there is not enough research on the distribution of nerve fibers in relation to the location of Mks, therefore it seemed advisable to carry out additional tests to check whether such dependence exists. The presence of megakaryocytes with a close vicinity of nerve fibers may suggest the influence of the released neurotransmitters on their location in the bone marrow.

Unfortunately, BM is an not easily obtainable organ for histological examination and its anatomical description is quite complicated. The main problem encountered by histologists is the difference in hardness between the BM and the bone, as well as the hardness within the bone itself [29]. In this study, the bones were subjected to the process of decalcification, which facilitated the process of slicing and enabled the preservation of the intact tissue structure.

After staining for the CD42d antigen, it was observed that the distribution of Mks was associated with the BM vessel system, as well as many Mks occurred in the parenchymatic space of the BM, in a way not directly related to the blood vessels. It was detected that red-labelled Mks occurred individually (Fig. 1), while their juvenile forms - megakaryoblasts occurred rather in clusters, mainly accumulating in the metaphysis of the bone (Fig. 2). The CD42d antigen is part of the CD42 complex which consists of 4 subunits: CD42b (GP Ib alpha), CD42c (GP Ib beta), which are linked through a disulfide bond and CD42a (GP IX) and CD 42d (GP V), non-covalently connected to the complex. CD 42d (GP V) is the major platelet membrane protein that is richly glycosylated and is cleaved into fragments during the activation of platelets induced by thrombin [30,31,32,33].

Immunohistochemical staining with the anti-PGP 9.5 antibody helped to show all types of nerve fibers. PGP 9.5 is the so-called protein gene product, an enzyme protein with a molecular mass of 9.5 kD belonging to the group of ubiquitin hydrolases. It occurs in all populations of nerve cells and in neuroendocrine cells. It is used as a marker of nerve endings. The development of bone innervation in rats [10,12,13] was described using an antibody against this antigen.

Our results showed that the majority of PGP 9.5-positive nerve fibers coursed along the blood vessels of the BM, running in their close proximity (Fig. 3, 4, 5). Unfortunately, the effects of double immunohistochemical staining using anti-CD42d and anti-PGP 9.5 antibodies were difficult to observe due to the fact that the same secondary anti-rabbit immunoglobulins antibodies conjugated with Cy3 fluorochrome were used. As a result, both labelled antigens showed red fluorescence, which made observation difficult. It was noticed that few Mks occurred near the PGP-positive fiber courses (Fig. 6). In this study, immunohistochemical staining with the use of anti-NPY antibody was also performed.

Neuropeptide Y (NPY) is an important neurotransmitter in the central and peripheral nervous system of mammals. It has been found in adrenergic fibers, detected immunohistochemically, especially in long and periosteal bones. High levels of NPY in rats have also been demonstrated in blood cells, bone marrow, and spleen. NPY expression in Mks has been shown, as confirmed by in situ hybridization [12]. The presence of NPY in Mks suggests that this cotransmitter is synthesized by them and then can be released by the platelets and act as a factor with significant angiospastic activity [3,27].

Our results despict the presence of NPY in Mks (Fig.7) as well as the relationship between the location of Mks and nerve endings containing NPY (Fig. 8). Some of the labelled Mks were in close proximity of the NPY-positive fibers (Fig 9,10).

The presence of NPY-positive fibers near Mks, which are a subpopulation of sympathetic fibers, may indicate the influence of the sympathetic nervous system on the development of this cell line.

Katayama et al. (2005) proved the influence of the sympathetic nervous system on haematopoietic cells, especially in the regulation of stem cell egress (HSCs) from myeloidal niches.

They suggest that granulocyte colony-stimulating factor (G-CSF) together with adrenergic signaling collaborate in inducing migration of HSCs from the bone marrow. To support this hypothesis, they conducted a number of research consisting of series of 6-hydroxydopamine (6-OHDA) injections into neonatal mice that caused damage to both dopaminergic and noradrenergic neurons due to the permeable blood-brain barrier in mice [34].

In order to distinguish these two types of neurons, mice with β-dopamine hydroxylase defficiency, an enzyme necessary for the conversion of dopamine into noradrenaline, were tested. A decrease in the number of HSCs that were to be mobilized in the enzyme deficient mice was observed. Thus, this study has proven that noradrenergic signaling regulates HSCs mobilizations. Subsequent studies involving the administration of adrenergic agonists, e.g. clenbuterol, and antagonists (propranolol) to mice, have shown their effect on the rate of mobilization of HSCs in mice. The results of these studies proved for the first time that the sympathetic nervous system regulates the attraction of stem cells to the medullary niches and can influence on their mobilizations and maturation [34].

However, Elefteriou (2018) in his study [35] explains that in the recent years, the research of the presence of autonomic nerves in the bone has passed from the usual histological observation to the mechanism in which neurons of the central nervous system communicate with cells of the bone microenvironment and regulate bone homeostasis. This is the reason for new preclinical and clinical studies aimed at determining the contribution of sympathetic, parasympathetic, and sensory nerves in the process of bone development or its remodeling.

The presence of Mks and other haematopoietic cells near the nerves speaks for the effects of substances released by nerve endings on these cells. There are speculations that the development of megakaryocytic line cells depends on the presence of glutamate, which may come from nerve endings. Thanks to the PCR technique, Genever et al. (1998) showed mRNA expression of NMDA type glutamate receptor on Mks surface, derived from smears of rat and human BM. They also detected glutamate and aspartate (GLAST) on the cells of the megakaryocytic line [36]. They demonstrated, by means of flow cytometer, that NMDA receptor activity was necessary for the proper differentiation of megakaryoblasts. Kalev-Zylinska et.al. (2014) showed that blocking this receptor using MK-801 antagonist caused megakaryoblasts' size and adhesion reduction [37].

The world literature does not have the evidence to show any relationship between BM innervation and the location of Mks, therefore it is difficult to discuss our results. Further research are needed to explain the effects of the nervous system on the development and proliferation of Mk precursors, as well as another blood cells.

CONCLUSIONS

1. Megakaryoblasts are located mainly in the metaphysis of the bone, while their mature forms - Mks - occur in connection with the bone marrow's vascular system.
2. Most nerve fibers run along the blood vessels of the BM, and only a few of them penetrate into their haematopoietic compartment by themselves.
3. A certain amount of Mks is located near the course of peripheral and sympathetic nerve fibers.
4. Neuropeptide Y occurs in Mks.
5. Neurotransmitters released from nerve endings affect the localisation and maturation of haematopoietic cells in the BM.

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