Megakaryocytes and platelets in experimentally induced renovascular hypertension (2K1C) in rats

Megakariocyty i płytki w doświadczalnym nadciśnieniu naczyniowo-nerkowym (2K1C) u szczurów

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Summary

Introduction: Hypertension is one of the most frequently occurring diseases worldwide. Approximately 10% of the population with hypertension reveals the secondary type of the disease. The aim of the study was to evaluate the megakaryocyte-platelet system in the course of renovascular hypertension.

Material/Methods: An experimental model of hypertension in rats according to Goldblatt was used in the study. The experimental material (blood, bone marrow) was collected in the 4th, 8th, and 16th weeks of the study. Bone marrow megakaryocytes (MKs) were evaluated using immunohistochemical and morphometric methods. Blood platelets were analyzed based on their count (PLT) and mean volume (MPV). Plasma thrombopoietin (TPO) concentration was also assessed.

Results: The investigation showed increased numbers of MKs 16 weeks after partial unilateral ligation of the renal artery. Statistically significant increase in platelet count, platelet mass, and the number of MK naked nuclei (NKs) as well as elevation of the circular deviation of the nuclei (CDN) of MKs accompanied the changes. MPV and TPO concentration did not change during the experiment. There was significant positive correlation between the increase in blood pressure and the numbers of MKs and NKs. The number of MKs correlated positively with PLT and CDN. Although TPO plasma level did not change significantly, there was marked negative correlation between plasma TPO concentration and PLT.

Conclusions: Although features of intensified platelet turnover were not observed, on the basis of the study it can be assumed that the megakaryocytic system undergoes changes in the course of renovascular hypertension. This can contribute to blood platelet production and the development of possible hypertension complications.

Key words: megakaryocytes • blood platelets • renovascular hypertension • rats

Streszczenie

Wstęp: Nadciśnienie tętnicze należy do najczęściej występujących chorób na świecie. U około 10% populacji osób z nadciśnieniem tętniczym ma ono charakter wtórny. Celem badań była ocena układu megakaryocyty-płytkowego w przebiegu nadciśnienia naczyniowo-nerkowego.

INTRODUCTION

Hypertension is one of the most common diseases worldwide. Along with diabetes mellitus, lipid balance disturbances, smoking, and obesity, it is a significant risk factor of atherosclerosis, cardiovascular diseases such as coronary heart disease, brain stroke, peripheral artery disease, and their direct complications (heart failure, renal failure, encephalopathy). Renovascular hypertension is the most frequent cause of secondary hypertension with potentially removable cause [9]. The complications of hypertension are related to, among others, a hypercoagulative state and a tendency for the formation of thrombi [24,33]. Blood platelets play an important role in these events, in addition to their role in coagulation and fibrinolysis [4,33].

Blood platelets are un-nucleated structures of discoid shape produced as a result of megakaryocyte (MK) cytoplasm fragmentation [17]. Platelets’ morphological and functional properties are conditioned during MK development [54]. The influence of various factors on megakaryocyte proliferation is reflected in the function and amount of circulating platelets [54]. Platelets and megakaryocytes create the system of a regulative circle, described as the megakaryocyte-platelet hemostatic axis (MPHA) [37,38,54]. The system is regulated mainly by thrombopoietin (TPO) [11,42]. TPO is a key cytokine which controls the stages leading eventually to the formation of fully mature MKs and enables the formation of the suitable number of platelets [10,11]. The influence of TPO on megakaryocyte proliferation is reflected by the elevated number of MKs and their progenitors in the bone marrow and spleen, MK size, nuclear ploidy (chromosomal DNA content), cytoplasm maturation, intensification of the expression of cellular differentiation markers, and the increase in pro-platelet formation by MKs [11,16,48]. MKs’ size and their ploidy correlate directly with the circulating platelet mass, which is the product of the platelet number and mean platelet volume (MPV) [2,35,55]. It is assumed that MKs with high ploidy are the source of large hyper-reactive platelets and the increase in platelet volume is the effect of changes in MK cytoplasm fragmentation [54]. The size of platelets and their functional potential are determined in MKs during thrombopoiesis. Platelet size in the circulation does not undergo significant changes [2,35,55]. The physiological mechanisms that regulate platelet volume are not yet known. However, it is known that disturbances concerning both MK and platelet ionic canals are the background of these mechanisms [2]. Changes in platelet mass, involving an imbalance between the number of platelets and their volume, are observed under certain pathological conditions. Thus it seems that platelet number and volume undergo independent regulation [2,55].
In cases of atherosclerotic renal artery narrowing [2,3], diabetes mellitus [52], unstable angina, and myocardial infarction [46,60], normal or increased numbers of large platelets were observed. Patients after myocardial infarction showed increased MPV, which is a predictor of a subsequent ischemic incident. MPV did not correlate with other known vascular diseases risk factors. This suggests that MPV is an independent risk factor of a next acute coronary syndrome [2,54]. On the other hand, in the course of acute ischemic brain stroke, MPV increase was accompanied by a drop in blood platelet number [2,35]. MPV elevation was also observed in patients with hypertension, but the platelet number was normal or lowered [33,38]. It is now known that large hyper-reactive platelets contribute to the development of atherosclerosis [54]. The occurrence of such platelets in prothrombotic conditions is explained as a consequence of the elevated use of corpuscles and their accelerated production [54]. In atherosclerosis-induced diseases, both large and hyper-reactive platelet numbers as well as MK ploidy increase were observed [5,54,55,60]. Similar changes were also presented in diabetes [5] and primary hypertension [38].

So far, the evaluation of the megakaryocyte-platelet system in the course of renovascular hypertension, one of the secondary types of hypertension, has not been performed. In the present study a morphometric evaluation of bone marrow megakaryocytes (count, cell and nuclear size, nuclear-cytoplasmic ratio, and the circular deviation of the cells and their nuclei) was performed. We also analyzed blood platelets based on their count and mean volume. Moreover, the assessment of plasma TPO concentration was taken into consideration. In order to analyze the dynamics of possible changes in the megakaryocyte-platelet system, the study material was collected 4, 8, and 16 weeks after surgical narrowing of the renal artery. Blood pressure measurements were also conducted at the same intervals.

**Materials and Methods**

**Animals and induction of renovascular hypertension**

Male Wistar rats (180–220 g) were used in the experiment. A two-kidney one-clip model of hypertension was induced by partial standardized clipping of the left renal artery under pentobarbital anesthesia (40 mg/kg, i.p.). The animals were divided into six groups (Table 1). The rats were then left untouched for the next 4, 8, or 16 weeks. A sham-operated groups of rats (animals that received the same surgical intervention except for the clipping of the artery) served as the controls. During the operation all the animals obtained lincomycin to prevent infection.

**Blood pressure measurement**

Systolic and mean blood pressure were measured in conscious rats by a tail-cuff method (Harvard Rat Tail Pressure Monitor System) according to the method described by R. Zatz [58].

**Hematological and biochemical analyses**

The blood samples for these analyses were obtained from the living animals, from the study and control groups at 4, 8, and 16 weeks of the experiment. Venous blood was taken without stasis from the right ventricle of the beating heart into the vacutainers containing EDTA for platelet counts (PLT) and sodium citrate (4:1) for MPV. Samples of the blood for estimation of TPO plasma levels were also obtained. During this procedure the animals were under pentobarbital anesthesia (40 mg/kg, i.p.) and the thoracic cavity was opened. PLT and MPV were measured by an autocounter. Moreover, platelet mass was calculated according to the formula PLT x MPV [2] for each sample. The blood samples for TPO level estimation were centrifuged immediately and the plasma was stored in several aliquots at -85°C until assayed. Thrombopoietin was assayed by sandwich-type ELISA (Santa Cruz Biotechnology, Inc.; TPO (N-19): sc-1298).

**Table 1. Division of rats into groups in relation to the time of material collection after the surgical procedure**

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>4th</td>
<td>E1</td>
<td>n=9</td>
</tr>
<tr>
<td>8th</td>
<td>E2</td>
<td>n=10</td>
</tr>
<tr>
<td>16th</td>
<td>E3</td>
<td>n=10</td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>n=6</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>n=7</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>n=7</td>
</tr>
</tbody>
</table>

E – study groups (rats with narrowed renal artery): E1 – rats sacrificed after 4 weeks, E2 – rats sacrificed after 8 weeks, E3 – rats sacrificed after 16 weeks; C – control groups (rats operated on without ligation of the renal artery, so-called sham-operated rats): C1 – rats sacrificed after 4 weeks, C2 – rats sacrificed after 8 weeks, C3 – rats sacrificed after 16 weeks; n – number of rats in a group.

**Analysis of bone marrow megakaryocytes**

**Experimental material**

After the rats were sacrificed, the femur and sternum were removed, fixed in 10% buffered formalin, decalcified, and embedded in paraffin.

**Staining procedure**

Two 5-μm-thick longitudinally oriented bone marrow sections were removed from each sample of both the femur and sternum. One section each from the femur and sternum were stained with hematoxylin-eosin (HE) for complex morphometric analysis of MKs. Other sections (one from the femur and one from the sternum) were prepared for immunohistochemistry. The monoclonal antibody CD61 (clone Y2/51, catalog no. M 0753, DAKO) was used to identify glycoprotein IIIa (GPIIIa). The aim of this examination was to identify, for numerical analysis not only large mature MKs, but also the small young MKs that may be mistaken for other cells.

**Morphometry**

Following HE staining and immunostaining, morphometric evaluation was performed using an Olympus BX41 microscope with a digital camera connected to a computer in which a standard morphometric program (Micro Image IncD UDF Packed Writing Software for Windows, OLYMPUS) was installed. To estimate the number of MKs and naked nuclei (NKS), i.e. megakaryocytes after the loss
of cytoplasm transformed to thrombocytes, the immuno-
histochemical sections were analyzed. Three randomly 
selected fields in each section were chosen at a magnification 
240× (only areas containing well-preserved hematopoietic tissue were accepted). Only nuclear MKs were included 
(NKs formed a separate group) [50].

For morphological analysis, HE-stained sections were 
used. In each section, MKs were identified as cells with 
diameters ≥20 μm and visible nuclei [23]. The following 
parameters were evaluated in the study: the area of the 
cell (AC), the area of the nucleus (AN), the nuclear-cyto-
plasmatic ratio (N/C), and the circular deviation (CD) of 
both MKs (CDC) and their nuclei (CDN). The circular 
development is defined as CD=4πA/C² (C – circumference, 
A – surface area), giving the value of 1.0 for a circular 
shape and a lower factor for irregular outline [50]. The 
ratio of nuclear material to cytoplasm (N/C) is high in 
MKs without granulation (less mature MKs) and tends to decrease as granulation increases (more mature MKs) 
[41]. The N/C ratio was calculated from the quotient of 
AN and AC. Three randomly selected fields in each section 
were chosen at a magnification of 480× (only areas 
containing well-preserved hematopoietic tissue were 
accepted) and suitable MKs and their nuclei were me-
asured. Then the median values of the parameters were 
calculated.

We obtained permission from the local ethics committee to 
perform the investigations on the animals (no. 1004/36).

Statistical analysis

The Statistica PL program was used for the statistical analy-
sis of the results. Minimum, maximum, mean, and standard 
development (SD) were determined for the particular parame-
ters. The evaluation of distribution normality was perfor-
mved using the Shapiro-Wilk test. The results underwent 
analysis of variance ANOVA. The significance of differ-
ces between groups were estimated with the Tukey-Kramer 
test and correlation assessment was conducted using the 
linear correlation test according to Pearson.

Results

Systolic blood pressure (SBP), mean blood pressure 
(MBP)

SBP values in all the study groups (E1: 135.8, E2: 150.5, 
E3: 157.0 mmHg) were statistically significantly higher 
than in the control groups (C1: 116.2, C2: 124.7, C3: 127.3 
mmHg, p<0.01). The same was true for MBP (E1: 100.4, 
E2: 117.0, E3: 119.8 mmHg) compared with the controls 
(C1: 81.3, C2: 101.7, C3: 102.1 mmHg). SBP values corre-
alated positively with both MK number (p<0.05, r=0.337) 
and the number of NKs (p<0.05, r=0.371).

Megakaryocytes and blood platelets

The first changes in the megakaryocyte-platelet system 
were observed in the 8th week after partial renal artery 
ligation; however, they were statistically insignificant. 
They concerned decreases in MK and NK counts in the 
bone marrow and PLT in the blood compared with the 
control group as well as with the study group evaluated in 
the 4th week. The megakaryocyte-platelet system showed 
significant changes in the 16th week of the study. At 
that time, E3 presented elevated MK and NK numbers in 
the bone marrow with accompanying increased 
PLT, platelet mass, and CDN compared with the control 
group and the study groups assessed earlier (E1, E2; 
Table 2, Figure 1).

MK number correlated positively with both PLT (p<0.05, 

r=0.477) and platelet mass (p<0.01, r=0.557). Moreover, 
positive correlation was found between the number of MKs 
and CDN (p<0.05, r=0.467). There was also positive corre-
lation between PLT and platelet mass (p<0.001, r=0.922) 
and PLT and NK (p<0.05, r=0.434). On the other hand, 
we did not find changes in MKs size (AC) and MPV, MK 
nuclear size (AN), or the values of the nuclear-cytomero-
plasmatic ratio (N/C) and circular deviation of MKs (CDC) at 
the particular stages of the study.

TPO plasma level

In the study rats, plasma TPO concentration, although it did 
not show any significant changes, oscillated in accord-
ance with the regulating mechanism determined by pla-
telet mass, i.e. in the 8th week an increasing tendency of 
TPO concentration was found in comparison with the 
values obtained in the controls and the first study group (E1), 
while in the 16th week a decreasing tendency of TPO le-
vel was observed (Table 2). Moreover, negative corre-
lation was observed between plasma TPO concentration 
and PLT (p<0.05, r=-0.4305) and platelet mass (p<0.01, 
r=-0.5306).

Discussion

Hypertension is a disease in the course of which mor-
phological and functional changes of various organs oc-
cur. This happens due to both the unfavorable influence 
of increased blood pressure on vascular walls and patho-
genic factors. Hypertensive complications are connected, 
among others, with hypercoagulability and a tendency for 
thrombus formation. Besides the coagulative system and 
fibrinolysis, blood platelets play an important role in these 
events. Both experimental and clinical studies on hyper-
tension have paid much attention to the evaluation of blo-
od platelets. Only a few of them concerned MKs [1,38]. 
However, the cells were evaluated under conditions of 
primary hypertension and not renovascular hypertension, 
which is a secondary hypertension. As opposed to prima-
ary hypertension, the causes of which have not yet been 
fully determined, the etiology of secondary hypertension 
is well established.

The pathogenesis of renovascular hypertension is complex. 
The hemodynamically significant narrowing of a renal ar-
tery or arteries leads to kidney hypoperfusion and an in-
crease in plasma renin-angiotensin system (RAS) activity. 
Afterwards, the activation of tissue RAS, overstimulation 
of the sympathetic nervous system (SNS), and increased 
aldosteron (ALDO) and vasopressin (VSP) synthesis and 
release take place. These factors contribute to the mainte-
nance of increased blood pressure, mainly in the chronic 
phase of renovascular hypertension. The duration of the
particular phases of renovascular hypertension is variable. In rat studies it was observed that the acute phase developed from 2 to 4 weeks and the chronic phase, 9 weeks after partial unilateral ligation of the renal artery [26,32].

In the present study, the first changes in the megakaryocyte-platelet system were observed only 16 weeks after renal artery ligation, i.e. during the time of the chronic phase of renovascular hypertension in rats. The significant elevation of MK number in the bone marrow was found with a simultaneous, slightly decreased concentration of the main factor stimulating megakaryocytopoiesis, i.e. TPO. It is possible that ALDO and/or VSP, factors included in the pathogenesis of renovascular hypertension, had their effect on bone marrow. The results of some studies pointed to the potentially stimulating influence of these factors on megakaryocytic cell-line growth [7,19,27,28,47]. Some studies revealed a stimulating influence of SNS on hematopoietic progenitor cells, including a megakaryocytic cell line [20,25,34]. Increased RAS activation could possibly occur in bone marrow, which contributed to the effect. In some studies concerning the issue, Haznedaroglu’s hypothesis on tissue RAS functioning in bone marrow was confirmed [18]. It was shown that angiotensin II, through AT1 receptors, stimulated the proliferation of the progenitor cells of various hemopoietic cell lines, including megakaryocytic cells [44].

Table 2. Megakaryocyte and platelet parameters and plasma thrombopoietin concentration in the study and control groups of rats.

<table>
<thead>
<tr>
<th>Group Parameter</th>
<th>C 1 (x±SD)</th>
<th>E 1 (x±SD)</th>
<th>C 2 (x±SD)</th>
<th>E 2 (x±SD)</th>
<th>C 3 (x±SD)</th>
<th>E 3 (x±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MKs count in field of vision (mag. 240×)</td>
<td>6.890±1.58</td>
<td>6.980±1.8</td>
<td>8.190±2.01</td>
<td>6.950±2.34</td>
<td>6.990±0.81</td>
<td>10.030±2.04</td>
</tr>
<tr>
<td>2 NK number in field of vision (mag. 240×)</td>
<td>1.540±0.25</td>
<td>1.820±0.72</td>
<td>2.290±0.99</td>
<td>1.710±1.01</td>
<td>1.570±1.02</td>
<td>3.050±0.960</td>
</tr>
<tr>
<td>3 Area of MKs (AC) [μm²]</td>
<td>319.0±27</td>
<td>335.8±30.1</td>
<td>303.0±25.9</td>
<td>323.3±28</td>
<td>327.0±41.1</td>
<td>321.3±23.3</td>
</tr>
<tr>
<td>4 Area of nucleus (AN) [μm²]</td>
<td>101.3±8.2</td>
<td>105.6±8.7</td>
<td>95.4±6.6</td>
<td>96.0±11.7</td>
<td>103.5±12.8</td>
<td>102.8±10</td>
</tr>
<tr>
<td>5 Nuclear-cytoplasmic ratio (N/C)</td>
<td>0.328±0.051</td>
<td>0.321±0.027</td>
<td>0.319±0.025</td>
<td>0.305±0.039</td>
<td>0.320±0.028</td>
<td>0.322±0.024</td>
</tr>
<tr>
<td>6 Circular deviation of MKs (CDC)</td>
<td>0.711±0.029</td>
<td>0.746±0.035</td>
<td>0.739±0.04</td>
<td>0.760±0.03</td>
<td>0.756±0.039</td>
<td>0.752±0.024</td>
</tr>
<tr>
<td>7 Circular deviation of MKs nuclei (CDN)</td>
<td>0.455±0.062</td>
<td>0.446±0.032</td>
<td>0.440±0.046</td>
<td>0.436±0.063</td>
<td>0.437±0.05</td>
<td>0.511±0.026</td>
</tr>
<tr>
<td>8 Platelet count (PLT) [10³/μl]</td>
<td>881±119</td>
<td>819±45</td>
<td>803±136</td>
<td>799±140</td>
<td>787±114</td>
<td>988±39</td>
</tr>
<tr>
<td>9 Mean platelet volume (MPV) [fl]</td>
<td>5.43±0.32</td>
<td>5.30±0.25</td>
<td>5.50±0.09</td>
<td>5.38±0.21</td>
<td>5.56±0.45</td>
<td>5.59±0.55</td>
</tr>
<tr>
<td>10 Platelet mass [10³/μL x fl]</td>
<td>4814±860</td>
<td>4338±317</td>
<td>4421±768</td>
<td>4308±825</td>
<td>4374±730</td>
<td>5529±693</td>
</tr>
<tr>
<td>11 Thrombopoietin plasma level (pg/ml)</td>
<td>20.60±1.67</td>
<td>20.71±1.7</td>
<td>21.50±2.81</td>
<td>22.25±2.6</td>
<td>19.50±1.38</td>
<td>18.75±2.87</td>
</tr>
</tbody>
</table>

Symbols of the groups were described in the Table 1. MKs – megakaryocytes; NK – naked nuclei; N/C – nuclear-cytoplasmic ratio. Data presented as mean. Statistic significance level p<0.05 was determined:

- k - in comparison with the control group;
- 1 - in comparison with the 1st examined group;
- 2 - in comparison with the 2nd examined group.

In the present study, the first changes in the megakaryocyte-platelet system were observed only 16 weeks after renal artery ligation, i.e. during the time of the chronic phase of renovascular hypertension in rats. The significant elevation of MK number in the bone marrow was found with a simultaneous, slightly decreased concentration of the main factor stimulating megakaryocytopoiesis, i.e. TPO. It is possible that ALDO and/or VSP, factors included in the pathogenesis of renovascular hypertension, had their effect on bone marrow. The results of some studies pointed to the potentially stimulating influence of these factors on megakaryocytic cell-line growth [7,19,27,28,47]. Some studies revealed a stimulating influence of SNS on hematopoietic progenitor cells, including a megakaryocytic cell line [20,25,34]. Increased RAS activation could possibly occur in bone marrow, which contributed to the effect. In some studies concerning the issue, Haznedaroglu’s hypothesis on tissue RAS functioning in bone marrow was confirmed [18]. It was shown that angiotensin II, through AT1 receptors, stimulated the proliferation of the progenitor cells of various hemopoietic cell lines, including megakaryocytic cells [44].

It should be stressed that hypertension is a factor contributing to the development of atherosclerosis. In previous studies it was observed that in the course of diseases with...
The increase in NK number (remnants of MKs which released pro-platelets and platelets into the circulation) showed that the main site of platelet production could be the bone marrow vascular sinuses. Positive correlation between NK count and PLT was shown. Of the evaluated morphological parameters of MKs in rats with renovascular hypertension, CDN was significantly elevated. This shows that the MK nuclei had a more regular circumference, which is a feature of less mature MKs compared with MKs of more advanced maturation with multilobular nuclei of irregular circumference [23,41]. Young MKs are also characterized by a high value of N/C. However, mean N/C and MK size did not change in the course of the experiment. It should be considered that, according to Levine [23], the nuclear circumference of MKs that are in the last stage of maturation can become more regular than those in intermediate stages of maturation. The positive correlation between MK number and CDN found in the study can indicate that a marked part of the MK pool consisted of both young cells and MKs in the last stage of maturation, in which the formation and release of cells take place. The increased MK count and PLT in the last stage of the experiment could be a compensative reaction to earlier changes in the megakaryocyte-platelet system, the decreasing tendency of PLT observed in the 8th week of the study. Humoral and neurogenic factors included in the pathogenesis of the chronic phase of renovascular hypertension could participate in the condition.

On the other hand, it should be remembered that the changes in the megakaryocyte-platelet system observed in the 16th week (the last stage of the experiment) could be connected with the longer duration of hypertension and its higher values. This could contribute to the induction of atherosclerotic changes in vessels. Morshita et al., examining the abdominal part of the aorta of rats with hypertension induced by the Goldblatt method (2K1C), observed the presence of hypertrophy and thickening of the vessel wall and an elevation of wall-to-lumen ratio [31]. It can be assumed that in animals with experimental renovascular hypertension examined to evaluate the megakaryocyte-platelet system, the increase in platelet release was the reaction to so-called corpuscle use. Such factors as the elevation of shear stress, endothelial dysfunction/damage, RAS activation, and the influence of vasopressin could contribute as well [15,21,29,36,43,49,51,57]. In the study rats the process was not intensified, as MPV increase and MK size elevation (which reflects their ploidy) were not observed in the study.

On the basis of this study, although the features of intensified platelet turnover could not be observed it can be assumed that in the course of renovascular hypertension, the megakaryocyte-platelet system undergoes changes. This can contribute to blood platelet production and the development of possible hypertension complications. Previous studies on a similar experimental model showed intensified platelet aggregation induced using ADP compared with rats of a control group [8,30]. The evaluation of advancement stage of atherosclerotic changes and humoral factors related to their occurrence in rats with experimental renovascular hypertension need to be explained.


