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ORIGINAL ARTICLE

The effect of stem cell mobilisation with granulocyte colony-stimulating factor on the morphology of the haematopoietic organs in mice

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The cellular mobilisation of mice with granulocyte colony-stimulating factor (G-CSF) results in an egress of haematopoietic stem/progenitor cells from the bone marrow and an increase in their level in the peripheral blood. While the mobilisation process with different agents is widely studied, little is known about the morphology of the murine haematopoietic organs during the mobilisation. The purpose of this study was to examine the morphology of the bone marrow, spleen and liver in mice mobilised with G-CSF. To address this issue mice were injected subcutaneously with G-CSF for 6 consecutive days. Morphological analysis revealed an increase in the number of mature neutrophils close to the wall of sinusoids in the bone marrow as well as hypertrophy of the red pulp in the spleen. At the same time no morphological changes were noticed in the livers of G-CSF-mobilised mice. In conclusion, G-CSF induces discrete ultrastructural changes in the bone marrow, which intensify the transendothelial traverse of haematopoietic stem and progenitor cells from it. The changes in the spleen are related to repopulation of this organ by mobilised early haematopoietic cells circulating in the peripheral blood. We also noticed that the process of migration of haematopoietic cells from the bone marrow into the peripheral blood began on day 2 and was most pronounced on day 4 after stimulation with G-CSF.

Key words: bone marrow ultrastructure, granulocyte colony--stimulating factor, liver morphology, spleen morphology

INTRODUCTION

Haematopoietic stem and progenitor cells (HSPC) reside mainly in the bone marrow, where they find an optimal environment for proliferation and differentiation [16]. A small number of bone marrow-derived cells circulate in the peripheral blood to keep in balance the stem cell pool in tissue niches located in different organs [18]. The number of these cells may be increased in peripheral blood after pharmacological mobilisation. Mobilisation of HSPC from the bone marrow into the peripheral blood is currently used to obtain HSPC for autologous and allogeneic transplantation [10]. Mobilisation of HSPC from the bone marrow can be induced in patients by

administration of growth factors (e.g. G-CSF), chemokines (e.g. GRO-β), chemotherapeutics (e.g. cyclophosphamide) [31], CXCR4 antagonist (AMD3100) or as combination of these. The agent most commonly used in clinical practice is granulocyte-colony stimulating factor (G-CSF). The multiple mechanisms that regulate HSPC mobilisation from the bone marrow into the peripheral blood are still not completely understood. Generally they involve modulation of chemokines and adhesion molecules and release of proteolytic enzymes [27, 32].

Although G-CSF is widely used in clinical practice, little is known about the morphology of bone marrow and other haematopoietic organs after exposure to this mobilising agent. In our previous study we described morphological changes in the bone marrow after mobilisation by cyclophosphamide [11] and after the combined administration of cyclophosphamide and G-CSF [31]. We also studied morphological changes in the bone marrow in mice after mobilisation by G-CSF [3]. The aim of the current study was to evaluate the ultrastructure and kinetics of morphological changes in the spleen and liver, in addition to those in the bone marrow, in mice mobilised by G-CSF.

MATERIAL AND METHODS

Mice

The experiment was performed on pathogen-free 5-week-old mature female inbred BALB/c mice (Polish Academy of Sciences, Wroclaw, Poland). The animals were maintained under standard laboratory conditions in a 12 h/12 h light-dark cycle at 21°C. Approval from the Local Ethical Committee was obtained. The animals were randomly divided into a control and three experimental groups. The mice of the experimental groups were injected subcutaneously with 250 μg/kg bw G-CSF (Neupogen, F. Hoffmann, La Roche, Switzerland) on each day of the experiment. The mice of the control group were injected with a phosphate-buffered saline (PBS) in the same volume as G-CSF in experimental mice. The mice of experimental group I were injected with G-CSF for 2 days, experimental group II for 4 days and experimental group III for 6 days. The mice of the control and experimental groups were sacrificed by lethal anaesthesia with 90 mg/kg bw Thiopental (Biochemie GmbH, Kundl, Austria) after 2, 4 and 6 days of the experiment. Fragments of spleen and liver were collected and fixed with Carnoy's solution [2]. For morphological studies the specimens were stained with haematoxylin and eosin and the specimens of liver with the periodic acid Schiff method [2]. Specimens of spleen and liver for immunostaining were fixed in 4% formaldehyde freshly prepared from paraformaldehyde and embedded in paraffin.

Transmission electron microscopy

For electron microscopic studies the bone marrows of the control and experimental mice was flushed from the femurs using a syringe and Iscove's medium. The bone marrows were fixed in 0.25 M glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 2 h at 4°C, post-fixed in 0.04 M OsO_4, and dehydrated in ethyl alcohol (30–96%) and 100% acetone, subsequently embedded in a Spurr low-viscosity embedding kit (Polysciences, Inc. Warrington, PA) [29]. Ultrathin sections were prepared with a Reichert OmU2 ultramicrotome (Leica Aktiengesellschaft, Vienna, Austria), contrasted with uranyl acetate and lead citrate [24] and analysed in a JEM-1200 EX transmission electron microscope (JEOL Ltd Tokyo, Japan) at 80 kV.

Immunohistochemical staining

For identification of early haematopoietic cells in specimens of bone marrow, spleen and liver immunohistochemical reactions were performed using the following antibodies:
— rabbit polyclonal anti-Thy-1 (H-110) (Santa Cruz Biotechnology, Inc., USA) — the antibody reacts with Thy-1 of mouse, rat and human origin;
— rabbit polyclonal anti-c-Kit (C-19) (Santa Cruz Biotechnology, Inc., USA) — the antibody reacts with c-Kit p145 (CD117) of mouse, rat and human origin.

For identification of apoptotic cells in the tissues examined polyclonal rabbit anti-human/mouse active caspase 3 (R&D System, UK) antibody was used.

In brief, specimens were cut into 5-μm sections, mounted on poly-L-lysine-coated microscope specimens, deparaffinised, rinsed with graded alcohol and washed with water. To reduce non-specific staining the specimens were immersed in 3% hydrogen peroxide for 30 min at room temperature to quench endogenous peroxidase activity. After being washed in Tris-buffered saline (TBS) twice for 5 min, the sections were submerged in 10 nM citric buffer, pH 6.0, and microwaved at high power twice for 5 min each time. The specimens were then left to cool to room temperature in the buffer solution (20 min). The specimens were again washed in TBS twice for 5 min and non-specific binding was blocked by incubation with

3% normal goat (for polyclonal antibodies) serum for 30 min at room temperature followed by incubation at room temperature with primary antibodies anti-Thy-1 antibody (1:500) or anti-c-Kit-R antibody (1:200) and anti-caspase 3 (0.2 μg/mL) for the time recommended in the manufacturer's protocol. The specimens were subsequently washed twice with TBS for 5 min and incubated with biotinylated anti-rabbit//mouse/goat immunoglobulins (LSAB+AP Link, Universal, DAKO, Carpinteria, USA). The sections were washed in TBS and incubated for 30 min with streptavidin conjugated to alkaline phosphatase (AP) (DAKO, Carpinteria, USA). The sites indicating antibody binding were visualised using Fuchsin+chromogen (DAKO LSAB®+SYSTEM, AP, Carpinteria, USA). The sections were washed in TBS and counterstained with Mayer haematoxylin. Control specimens were incubated without the primary antibodies.

Flow cytometry

Six hours after G-CSF injection on days 2, 4, and 6 peripheral blood was obtained from the vena cava (with a 25-gauge needle and 1-mL syringe containing 250 U heparin) and enriched for light-density mononuclear cells as described [9].

To determine the number of c-Kit-R$^+$ cells flow cytometry analysis was performed. In brief, 50 μL whole blood was stained with rat anti-mouse c-Kit-R fluorescein isothiocyanate conjugated monoclonal antibody (final concentration 1 μg/mL; BD Biosciences Pharmingen, San Diego, CA, USA). Samples stained with appropriate isotype controls (BD Biosciences Pharmingen, USA) were examined in parallel. After a 20-min incubation on ice 2 mL FACS Lysing solution (BD Biosciences Immunocytometry Systems, USA) was added to lyse the erythrocytes. Afterwards cells were washed twice in PBS, resuspended in 0.3 mL PBS and analysed by FACScan using CellQuest v.3.1 software (BD Biosciences Immunocytometry Systems, USA). Typically, 20 000 events were acquired and the percentage of c-Kit-R$^+$ cells was determined for a whole leukocyte population.

Cell cultures

2×10^5 peripheral blood mononuclear cells were plated in methylcellulose cultures and stimulated to form CFU-GM colonies. The growth factors employed for colony stimulation were recombinant human IL-3 (20 U/mL) (Genetic Institute, USA) plus murine GM-CSF (5 ng/mL). The *in vitro* colonies were counted with an inverted microscope on day 11. Cultures were performed in quadruplicate.

Statistical analysis

Arithmetic mean and standard deviation (mean ± SD) data were determined using the STATGRAPHICS v.5.0. package (Manugistisc, Inc., Rockville, MD, USA). Statistical significance was defined as $p < 0.05$. Data were analysed using Student's *t*-test for unpaired samples.

RESULTS

The ultrastructural analysis of the bone marrow

Sinusoids composed of a single layer of endothelial cells with normal structure were observed in the bone marrow of the control mice (Fig. 1A, B, insert). After day 2 of mobilisation with G-CSF mainly neutrophils with segmented nuclei (Fig. 1C) were present close to the wall of capillary (Fig. 1D). The cells showed a nucleoplasmic differentiation into light and dark areas. The dark nucleoplasm developed into an irregular but very distinct band inside the nuclear membrane and extended into the nucleus in irregular clumps. The endoplasmic reticulum was in the form of very minute profiles. The specific granules were, in general, small. The initiation of the migration process of neutrophil across the wall of the endothelium was visible (Fig. 1E). By days 4 and 6 the remaining ultrastructure of the bone marrow of mice mobilised by G-CSF was similar to that of the animals in group I.

Immunolocalisation of early haematopoietic cells in the bone marrow

Immunoexpression was visible in the cytoplasm of early haematopoietic cells of the bone marrow cells. No positive reaction was detectable in the nuclei of the cells. There were no differences in the localisation of the immunopositive cells in specimens stained with c-Kit-R or Thy-1 antibodies. Few cells in the bone marrow of the control and experimental mice displayed immunostaining.

Spleen morphology during mobilisation with G-CSF

Control mice. The splenic pulp of the control mice was composed of lymphoid nodules, which formed part of the white pulp that appeared within the red pulp. The white pulp consisted of lymphatic tissue, which ensheathed the extra-trabecular arteries and the lymphoid nodules with the central artery. The white pulp and red pulp were separated by a marginal zone. The red pulp was composed of elongated splenic cords separated by splenic sinusoids.
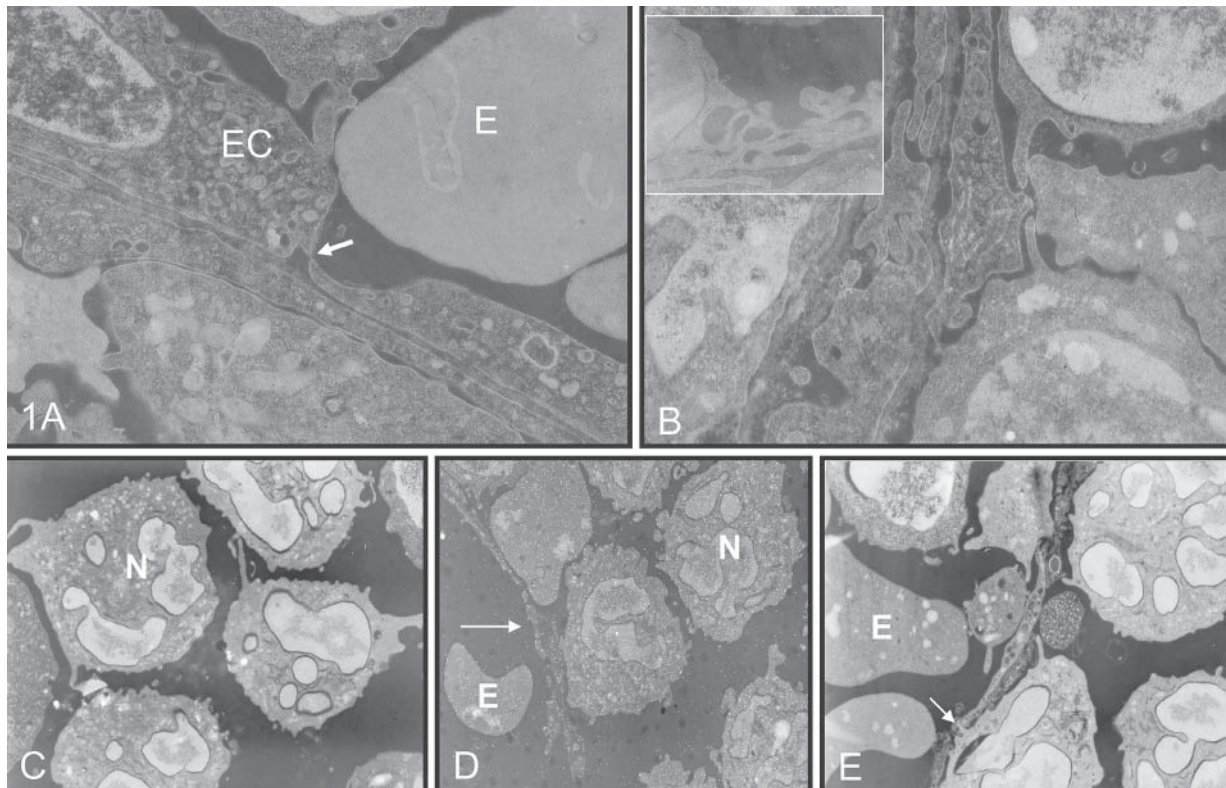
Figure 1. Ultrastructure of the bone marrow of the control group of mice (**A, B, insert**) and of mice on the second day after mobilisation with G-CSF (**C, D, E**). Endothelial cells with pinocytotic vesicles in the cytoplasm are present (**A**). Processes of endothelial cell cytoplasm (**B**) in the perinuclear region (**B, insert**). Neutrophils with mature segmented nuclei (**C, D**) are located close to the sinusoid wall (arrow) (**D**). Neutrophil with the extension of cell is visible at the outset of the migration process (**E**). E — erytrocyte; EC — endothelial cell; N — neutrophil; TEM: A. × 15000; B. × 15000; C. × 6000; D. × 5000; E. × 7500; insert. × 40000.
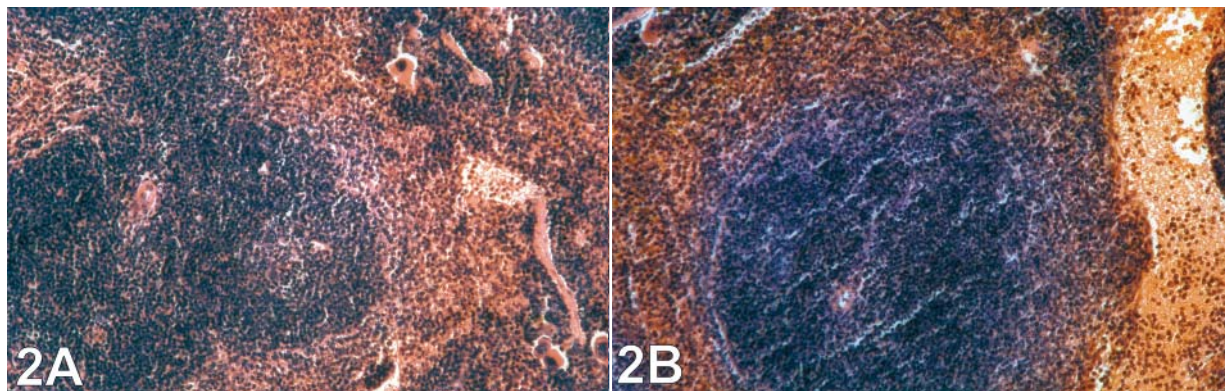

Figure 2. The spleen of the control group of mice (**A**) and of mice on the fourth day after G-CSF mobilisation (**B**). White pulp and red pulp with trabecular vessels and megakaryocytes (**A**) and the domination of red pulp over the white pulp. The lumen of the trabecular vessels is extremely dilated (**B**). H-E. A, B. × 330.

Megakaryocytes with an irregularly lobulated nucleus were visible in the red pulp (Fig. 2A).

Group I — 2 days after treatment with G-CSF. The spleen of these mice displayed an increase in the area occupied by the red pulp and a decrease in the size of the lymphoid nodules. A marginal zone surrounding the white pulp was not clearly visible and lymphocyte infiltration into the red pulp was observed. In the lumina of the parenchymal arteries numerous leukocytes were observed. Megakaryocytes frequently formed small groups of cells.

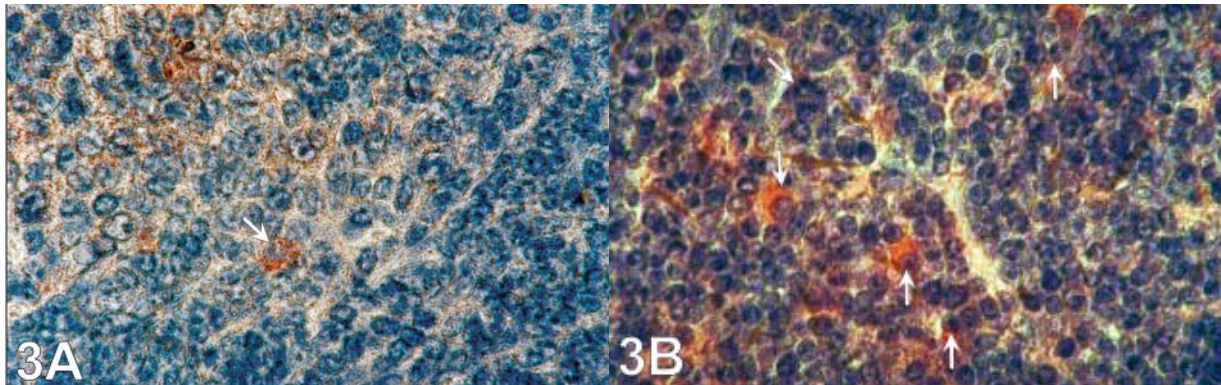**Group II — 4 days after treatment with G-CSF.** The spleen of the mice presented the red pulp

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Figure 3. Immunolocalisation of haematopoietic stem cells in the spleen. C-Kit-R-positive cells in the spleen of the control group of mice (**A**) and of mice on the fourth day after G-CSF mobilisation (**B**) (arrows). A, B. × 1340.

extended over the white pulp as observed in the first group. The lumina of the trabecular vessels were filled with an increased number of blood cells. Single megakaryocytes were visible in the red pulp (Fig. 2B).

Group III — 6 days after treatment with G-CSF. In the spleen of this group of mice the red pulp dominated over the white pulp. However, at the same time an increase in white pulp was also observed as compared to the spleen of mice in the first and second groups. A marginal zone of white pulp was partly retained.

Immunolocalisation of early haematopoietic cells in the spleen

For identification of early haematopoietic cells in the spleen of the control and G-CSF-mobilised mice the same antibodies were used as for the bone marrow specimens. The immunostaining was observed in the cytoplasm of the cells. In the spleen of the control mice individual Thy-1- or c-Kit-R-positive cells were localised among the lymphocytes in the white pulp (Fig. 3A). Most of the cells with Thy-1 or c-Kit-R immunoexpression (from 3 to 5) were observed in the spleen of mice during day 4 of mobilisation (Fig. 3B).

Liver morphology during the mobilisation of mice with G-CSF

Control mice. The typical liver lobules were not markedly separated from each other by the connective tissue. At the angles of the hexagonal lobules there were portal spaces with portal triads and scant connective tissue. Hepatocytes were grouped in anastomosing plates. The cytoplasm of hepatocytes stained with haematoxylin and eosin was eosinophilic and contained one or two nuclei with granular chromatin. The plates of cells were directed from the periphery of the lobule to its centre, where the centrolobular vein was located. The spaces between the plates of hepatocytes contained sinusoids. In the lumina of the sinusoids the nuclei of Kupffer cells were visible.

Groups I, II and III — 2, 4 and 6 days after treatment with G-CSF. There were no morphological changes in the livers of mobilised mice.

Immunolocalisation of early haematopoietic cells in the liver

In the control animals the c-Kit-R- or Thy-1-positive cells were observed. These were located in the portal spaces and within the typical liver lobules, where two types of cell were visible. In the portal spaces the cells were small. Within the lobule the first type of cell located between the hepatocytes was small, while the other looked like the hepatic cells (Fig. 4A, B).

There were no differences in the numbers of c-Kit-R- or Thy-1-positive cells in the livers of mice after days 2 and 4 of mobilisation with G-CSF (Fig. 4C, D).

Immunohistochemical visualisation of apoptotic cells in the bone marrow, spleen and liver

The immunohistochemical product of reaction, visualisation of the activity of caspase 3, was detectable in the cytoplasm of some cells. There were no differences between the numbers of apoptotic cells in the examined organs of the control and experimental mice.

The percentage of c-Kit-R-positive cells circulating in the peripheral blood

We found the most significant increase in circulating c-Kit-R$^+$ cells analysed by flow cytometry in the animals after 4 days of mobilisation ($p < 0.01$) (Fig. 5).
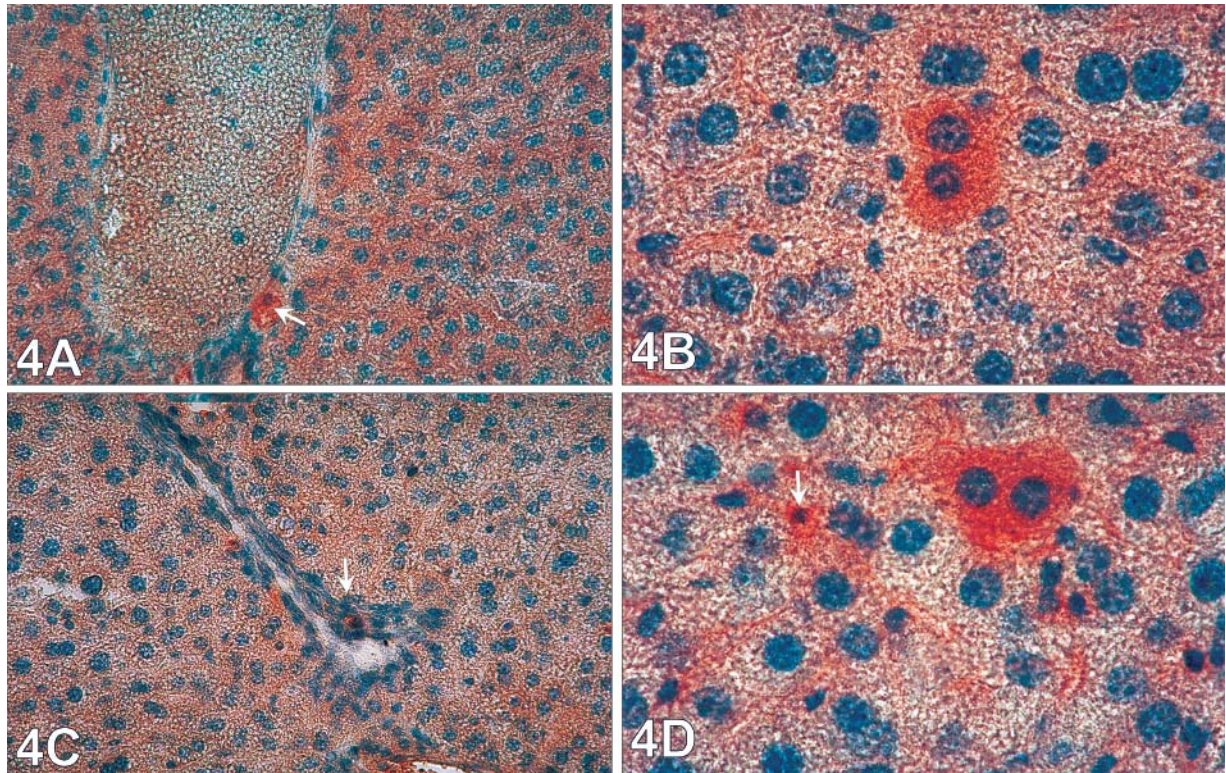
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Figure 4. Immunolocalisation of haematopoietic stem cells in the liver. c-Kit-R-positive cells in the portal space (arrow) (**A**) and in the hepatic lobule of the liver of control mice (**B**). The cells with immunoexpression of c-Kit-R in the portal space (arrow) (**C**) and hepatic lobule of the liver on the second day after G-CSF mobilisation of mice (arrow) (**D**). A, C. × 670; B, D. × 1340.

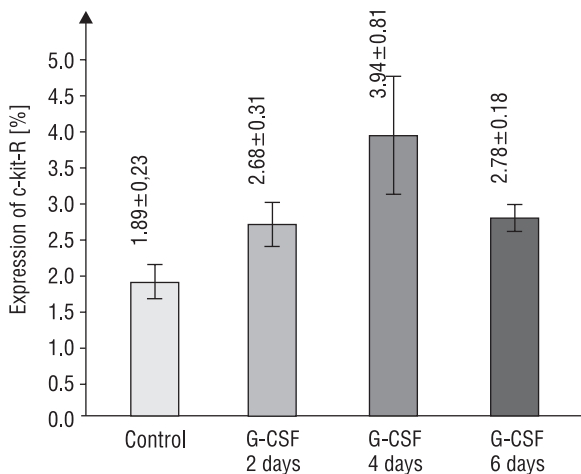**Figure 5.** Percentage of circulating c-Kit-R-positive cells of control and experimental mice.

Figure 6. The number of circulating clonogenic CFU-GM of control and experimental mice. The number of circulating clonogenic CFU-GM is shown as a percentage of the colonies formed by clonogenic cells of control mice.

The number of myeloid progenitors in the peripheral blood

We noticed that the number of circulating clonogeneic CFU-GM was significantly higher in the peripheral blood of mice mobilised with G-CSF than in that of the control group. There was a marked 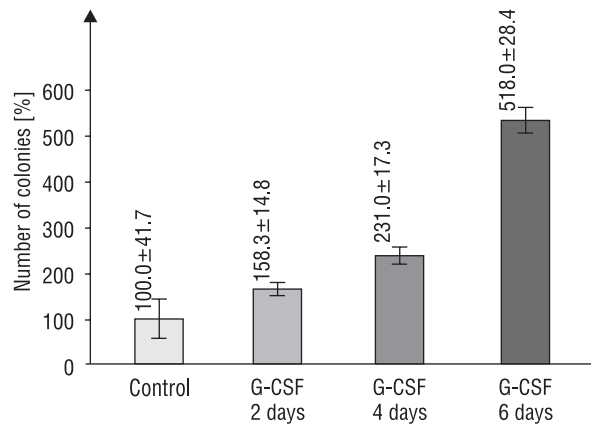increase in circulating myeloid CFU-GM progenitors on successive days of the experiment, reaching a maximum on day 6 ($p < 0.01$) (Fig. 6).

DISCUSSION

In our previous study [3] we observed changes in the bone marrow morphology of mice mobilised with G-CSF. The first effect of G-CSF stimulation, on the

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second day, was an increase in cellularity of the bone marrow. This was connected with the physiological property of the G-CSF as a main regulator of the proliferation, differentiation and survival of neutrophils [8]. The morphological pictures of the bone marrow on the fourth day of G-CSF mobilisation indicated an increased egress of early haematopoietic cells from the bone marrow. The process of migration of the haematopoietic cells was still in progress on the sixth day. However, the morphology of the bone marrow after 6 days of stimulation was similar to that of the control mice. In the lumina of sinusoids only single leukocytes were visible [3].

Our data demonstrate that the morphology of the bone marrow of mice stimulated with cyclophosphamide (CY) alone or CY in combination with G-CSF is different from that of mice mobilised with G-CSF alone. In the experimental animals mobilised with CY destructive changes in the bone marrow were observed on the second day with an increase in the area occupied by strongly dilated sinusoids and a significant reduction in the haematopoietic compartment. The HSPC migration was enhanced on the sixth day after CY administration [11]. During mobilisation with CY plus G-CSF the destructive effect of CY was ameliorated to a certain extent by the pro-proliferative activity of G-CSF, and the migration of early haematopoietic cells began on the fourth day of mobilisation [31].

In the current study we noticed that the changes in bone marrow morphology appeared earlier than in animals mobilised with CY or CY + G-CSF. Ultrastructural examination of the bone marrow on the second day demonstrated an increase in the number of neutrophils. They were located near the walls of sinusoids in the bone marrow and the passage of some cells through migrating pores of endothelial cells was noticed. Moreover, at the same time an increase in the distances between the endothelial cells in the bone marrow vessels was already visible. These changes facilitated the egress of cells from the bone marrow.

Flow cytometry analysis demonstrated the highest percentage of early haematopoietic c-Kit-R+ (CD117) cells in the peripheral blood on the fourth day of mobilisation with G-CSF. A similar effect was observed during G-CSF plus CY-induced mobilisation [31]. However, the percentage of c-Kit-R+ cells was significantly lower when G-CSF alone was used. The egress of early haematopoietic c-Kit-R+ (CD117) cells into the peripheral blood corresponded with an increased number of circulating clonogeneic CFU-GM,

although the number of CFU-GM colonies gradually increased and peaked on day 6. The egress of these cells into the peripheral blood was triggered by the mobilisation process induced by G-CSF [25]. It is possible that the difference between the peak of c-Kit-R+ cells and CFU-GM in the peripheral blood can be explained by c-Kit-R cleavage by proteolytic enzymes released during mobilisation.

The early haematopoietic cells released from the bone marrow circulate with the peripheral blood where the level of SDF-1 is elevated [7] and are able to repopulate the peripheral organs [21]. The spleen is one such organ and is rapidly repopulated by circulating cells, playing a significant role in embryonic haematopoiesis in mice and humans [34]. While in adult humans the spleen is a lymphoid organ, in mice it remains an active alternative haematopoietic organ throughout adulthood [34]. This could indicate that the normal murine spleen could be the site at which haematopoietic stem cells reside and a significant percentage of these have indeed been shown to migrate to the spleen and colonise it after transplantation [20].

In our studies we also observed morphological changes in the organisation of the spleen pulps of mice mobilised with G-CSF. The morphology of the spleen on the second day of the experiment was unchanged and was similar to the morphology of the spleen of the control mice. In contrast, on the fourth day of mobilisation with G-CSF the red pulp appeared extended over the white pulp, which could be connected with intensive splenic myelopoiesis. On the sixth day of murine mobilisation we observed that the red pulp was still developed. Similar changes in spleen morphology were noticed during mobilisation with CY plus G-CSF, where hyperplasia and the domination of the red pulp were considerable [31]. On the basis of the morphological observation we concluded that the greatest influx of early haematopoietic cells into the spleen occurs on day 4 of mobilisation with G-CSF. At this time the largest number of Thy-1- or c-Kit-R-positive cells was identified in the red and white pulp of the spleen. The same study of mice mobilised with CY plus G-CSF revealed an increase in immunopositive cells on the sixth day [31].

Injected haematopoietic stem cells have been shown to localise in the spleen of formerly irradiated mice [14, 20]. In these experiments the haematopoietic stem and progenitor cells repopulated the spleen in the largest numbers 20–48 h after transplantation. The suggested mechanism of repopula-

tion of the spleen by haematopoietic stem and progenitor cells is similar to that associated with the migration of haematopoietic stem and progenitor cells from the bone marrow and their homing during mobilisation with G-CSF. A change in adhesive molecule expression and a modulation of CXCR4 expression on the haematopoietic stem cell surface, as well as chemotaxis toward an increasing concentration of SDF-1, are all involved in this [15, 33].

The foetal liver of mice is a major site of embryonic blood cell production from, approximately, embryonic day 12 to birth [36]. In adults the liver is an auxiliary organ where between 10% and 15% of the total pool of erythropoietin is synthesised [12, 28]. In our experiments no morphological changes in the liver were observed after G-CSF treatment. With respect to the presence of haematopoietic stem cells, we were able to identify two types of cell with c-Kit-R- or Thy-1-immunostaining. It is known that reconstitution of the liver may result from three cell types, depending on the nature and anatomical site of injury, namely hepatocytes, liver stem cells (oval cells) and extrahepatic stem cells [1, 19, 26]. In normal mice the liver is the site at which the bipotential stem cells, termed "oval cells", reside [5]. These cells will produce hepatocytes and bile epithelial cells belonging to the adult liver. They are expected to be activated to proliferate when liver damage is extensive or chronic [35]. Liver stem cells express markers also characteristic for haematopoietic stem cells such as CD34, c-Kit-R and Thy-1 [4]. The presence of these markers might suggest their common origin. Moreover, oval cells express CXCR4, the only known receptor for SDF-1alpha. *In vitro* chemotaxis assays have demonstrated that the oval cells migrate along a SDF-1alpha gradient [6]. The study suggested that the SDF-1alpha/CXCR4 axis is the mechanism by which oval cells can be activated and which regulates the recruitment of bone marrow stem cells to the damaged liver [6, 13, 22, 23].

The immunolocalisation studies revealed small c-Kit-R- or Thy-1-positive cells in the portal space usually occupied by liver oval cells. Because the oval cells and haematopoietic stem cells express the same cell surface markers, it is difficult to explain the origin of immunopositive cells. Nevertheless, the presence of small immunostainable c-Kit-R+ or Thy-1+ cells among the hepatocytes indicated that they could be recruited from the bone marrow.

We observed that there were no differences in the number of apoptotic cells in the examined organs of experimental and control mice. Similarly, Suzuki et al. [30]

reported that G-CSF prolongs the survival of mature neutrophils by means of inhibition of caspase activity. It was has also been reported that G-CSF retarded the apoptosis of mature human neutrophils [17].

In conclusion, we revealed that G-CSF caused detectable ultrastructural changes in the bone marrow and spleen, which facilitated the transendothelial traverse of haematopoietic stem and progenitor cells from the bone marrow and repopulation of spleen in G-CSF-mobilised mice. Our findings showed that treatment with G-CSF does not significantly affect the morphology of the major haematopoietic organs in mice. The process of migration of haematopoietic cells from the bone marrow into the peripheral blood began on day 2 and was most pronounced on day 4 after stimulation with G-CSF.

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