Introduction

Hematopoietic stem cells (HSCs) in mice are defined as having self-renewal ability, and can repair all blood cell lineages. The transplantation of HSCs proved a protection effect on bone marrow and the peripheral blood of lethally irradiated mice. A population of cells that may be equivalent to HSCs in mice are Thy-1.1lo Lin-Sca-1+ cells. It has been demonstrated by classical studies that transplantation of these cells to lethally irradiated mice has a significant radioprotective effect, such as Thy-1.1lo Lin- Sca-1+ cells in C57BL/Ka Thy-1.1 of bone marrow (13, 14).

The c-kit receptor is expressed in hematopoietic stem cells and progenitor cells but not in lymphopoietic differentiating cells. Sequential analysis of hematopoietic renewal showed that myeloid and B-lymphoid lines developed sooner than T-lymphoid lines (11). Proto-oncogene c-Kit was displayed on 4q11–q12 chromosome encoding transmembrane tyrosin-kinase receptor (CD117) (1). Cell receptors with tyrosin-kinase activity are significant regulators of cell proliferation, differentiation and survival. They also have a significant role in the development and progression of many human tumors (8). Transplantation of some of the marrow phenotypes may not be successful. It was found that Lin' c-kit' cells were present in 0.08 % of nuclear bone marrow cells. These cells, however, did not form spleen colonies (CFU-S) by day 8. The formation of spleen colonies was observed as late as on day 12, when 80 % of CFU-S were found. (10). Another example may be of bone marrow cells in a mouse population with phenotypic characteristic of resting HSCs but without renewing hematopoiesis in irradiated mice. These cells express high levels of Sca-1, H-2K and CD38 and low levels of Thy-1.1 but did not express CD34 or lineage markers CD3, CD4, CD5, CD8, NK1.1, I-A, B220, Ig(MGA), CD40, Mac-1, Gr-1 and Ter119. The phenotypically characterized cells of Sca-1+, Thy-1.1low (cell surface markers) and their insufficient expression of c-kit do not respond adequately to hematopoietic growth factors in vitro. They do not form spleen colonies and have no ability to reconstitute hematopoiesis in irradiated mice (12).

Transgenic mice express beta-galactosidase (lacZ) or green fluorescent protein (GFP). These markers seem suitable for analysis of hematopoiesis repair after stem cell transplantation. A method with inflamed cells carrying the beta-galactosidase gene was also used in the follow-up of he-
matopoiesis recovery in lethally irradiated mice given hematopoietic stem cells. In this experiment, detection of CFUs colonies showed the presence of these cells in the spleen on the 12th post-transplant day and after 3–6 post-transplant weeks laZ expression was detected in hematopoietic tissues in all recipients (6). In the other experiment, lethally irradiated mice were transplanted with primitive hematopoietic marrow stem cell fraction Sca-1+ c-kit+ Lin- from ROSA26 mice. The kinetics of hematopoietic reconstitution was analysed in the bone marrow and spleen after transplantation. Activity of laZ gene was also detected in donor-derived myeloid (Mac-1+), B-lymphoid (B220+) and T-lymphoid (Thy-1+) cells in the bone marrow and spleen after stem cell transplantation. Kinetics of hematopoietic reconstruction shows that, for example, early erythroid cells (TER119lowCD71med) were discovered in the bone marrow and spleen 2 days post-transplantation and after proerythroblast appearance (TER119+ CD71brigh), and this massive erythropoiesis and myelopoiesis was observed in the spleen 2–4 weeks post-transplantation (2).

The issues associated with hematopoietic rescue and in vivo homing of transplanted cells are very interesting but answers elucidating the repair processes of damaged tissues and organs are yet to be found. In our experiments we studied the regeneration of hematopoiesis in lethally irradiated mice after transplantation of separated CD117+ marrow cells and the relationship between kinetics and in vivo homing in resident CD117+ cells and transplanted CD117+ cells. The interval for analysis was chosen to cover the monitoring period of hematopoietic repair (days 8, 12 and 30 post irradiation). We also presumed that important processes associated not only with hematopoiesis but with repair of other damaged tissues might occur during the reparation period (3).

**Fig. 1:** Flow cytometric analysis of the surface-marker expression of transplanted bone marrow cells. FACS assays CD117+ (lacZ+) sorted bone marrow cells from ROSA26 mice (donor of bone marrow cells) B6;129S-Gt (ROSA)26Sor before transplantation. A: Expression of CD117+/c-kit and CD45R/B220 on whole bone marrow cells; B: Expression of CD117+/c-kit and CD45R/B220 on separated bone marrow cell lin cells was separated using Lineage Cell Depletion Kit (Miltenyi Biotec). CD117+ cells were isolated by CD117 MicroBeads (Miltenyi Biotec). 72% cells were CD117+/CD45R/B220-.

### Methods

#### Animals

ROSA26 mice (donor of bone marrow cells), B6;129S-Gt (ROSA)26Sor were obtained from the Jackson Laboratories, Bar Harbor, Maine, USA; cells of the ROSA mice are tagged with lacZ gene and express E. coli β-galactosidase (β-gal), which allows unambiguous identification of transplanted cells. F2 hybrid mice (B6129SF2/J) purchased from Jackson Laboratories were used as recipients of bone marrow transplanted cells. Animals used for experiments were housed in groups of six in a temperature- and humidity-controlled colony room that was maintained on a 12-hour light/dark cycle. Food and water were available ad libitum throughout the experiment. All procedures were approved by the Ethical Committee supervising experimental procedures performed on animals at the Faculty of Medicine Hradec Králové, Charles University in Prague.

#### Isolation of CD117+ from bone marrow cells

Bone marrow cells were obtained from the femur of 8–10 week old ROSA26 mice and suspended in Hanks balanced salt solution (HBSS), suspended with 2% fetal calf serum (FCS) and 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid) – as a staining medium. Lymphocytic fraction was isolated from marrow cell suspension by separation on Histopaque 1077 (Sigma). 3x10^7 nuclear cells (MNC) were subjected to negative immunomagnetic sorting using Lineage Cell Depletion Kit (Miltenyi Biotec) for removal of differentiated cells. These cells were further subjected to positive immunomagnetic sorting with CD117 MicroBeads (Miltenyi Biotec). Cells from the bone marrow were double-stained with rat anti-mouse CD45R/B220–FITC antibody (clone RA3–6B2, SouthernBiotech) and CD117/c-Kit-PE (clone 2B8, SouthernBiotech). Prior to separation most of the cells were CD117+/CD45R/B220- (86.48 %), CD117+/CD45R/B220- (4.21 %) and CD117+/CD45R/B220+ (3.23%). Following both magnetic separations, we obtained suspension with 72 % of CD117+/CD45R/B220- stem cells. In the suspension practically no differentiated cells were present (Fig. 1).

#### Irradiations and reconstructions

Recipient animals (F2 hybrid mice, see above) were exposed to 9 Gy of radiation from a 60Co source (Chisotron-Chirana) at a dose rate of 1.3 Gy/min. Suspension of whole bone marrow cells (5x10^6 /mouse) or isolated CD117+ cells (5x10^6/mouse) of donors (ROSA26 mice, see above) was transplanted by i.v. injection into irradiated recipient animals 3 hours after irradiation. Six recipient mice were used in each tested group.

#### Clonogenic assays for myeloid CFU-GM progenitors

Cells from bone marrow and spleen were harvested in mice on days 8, 12, and 30 following lethal irradiation.
Granulocyte-macrophage colony forming units (CFU-GM) were usually prepared by plating cells in FCS-containing (30 %) methylcellulose IMDM (Iscove’s Modified Dulbecco’s Medium) medium (2.7 %) supplemented with 10 % bovine serum albumin and 2 % (2 ml of conditioned medium in 100 ml) conditioned medium containing IL-3 (interleukin 3) and GM-CSF (The Institute of Hematology and Blood Transfusion Prague). Only aggregates with more than 40 cells were considered colonies and were scored using a microscope 12–14 days after incubation at 37 °C in 5 % CO₂ and 5 % O₂ humidified atmosphere.

**Statistical analysis**

Data have been expressed as a mean ±SD (standard deviation of the mean). The statistical significance was determined by application of Student’s t-test to learn the mean differences between the groups. The differences were considered significant at p ≤0.001.

**Results**

Hematopoietic rescue after transplantation of CD117⁺ bone marrow cells. Hematopoietic rescue was noted through culture of CFU-GM (lacZ⁺) in the bone marrow (Fig. 2) and spleen in lethally whole-body irradiated mice exposed to LD 9 Gy. Mice in the control group were transplanted with bone marrow (5x10⁶), CFU-GM cultures were performed and colonies from bone marrow and spleen were calculated for 10⁵ MNC on days 8, 12 and 30 post-transplant (Tab. 1). In the control group 252±33.8 (x10⁵ MNC) CFU-GM in the bone marrow and 138±27.2 (x10⁵ MNC) CFU-GM in the spleen were found on day 8. 271±46.8 (x10⁵ MNC) CFU-GM in the bone marrow and 124±21.7 (x10⁵ MNC) CFU-GM in spleen were present on day 12. By day 30 317±34.5 (x10⁵ MNC) CFU-GM in the bone marrow and 43.5±17.4 (x10⁵ MNC) CFU-GM in the spleen were present. In the group of mice transplanted with sorted CD117⁺ bone marrow cells, the number of CFU-GM colonies was 115±12 (x10⁵ MNC) in the bone marrow and 67.5±14.7 (x10⁵ MNC) in the spleen on day 8 post-transplantation. On day 12, there were 207±120.7 (x10⁵ MNC) CFU-GM colonies in the bone marrow and in 13±26.8 (x10⁵ MNC) CFU-GM in the spleen. On day 30, the bone marrow showed 178.5±24.1 (x10⁵ MNC) CFU-GM and spleen had 83.6±17.8 (x10⁵ MNC) CFU-GM colonies. Significantly higher number of CFU-GM was noted on day 8 post-transplantation in the control group. This difference was statistically significant 252±33.8 (x10⁵ MNC) in the bone marrow vs. 115±12 (x10⁵ MNC) CFU-GM (p < 0.001) and in the spleen 138±27.2 (x10⁵ MNC) vs. 67.5±14.7 (x10⁵ MNC) CFU-GM (p=0.0031). When the number of colonies at CFU-GM was compared in the control group and the group with transplanted HSCs, no statistically significant difference was found in either the bone marrow or spleen on day 12 after irradiation, but this difference was at the significance limit on day 30. The results showed there was no increase of CFU-GM up to the value of the non-irradiated group in the bone marrow of either group. CFU-GM was 918.7±47.82 in the group of non-irradiated mice. There was significant increase of CFU-GM in the spleen of non-irradiated mice – 29.8±19.23 CFU-GM for 10⁵ spleen cells.

**Tab. 1:** Hematopoietic recovery after transplantation CD117⁺ (HSCs) sorted bone marrow cells in lethal irradiated mice.

<table>
<thead>
<tr>
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<th>Day 8</th>
<th>Day 12</th>
<th>Day 30</th>
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<tbody>
<tr>
<td></td>
<td>Control (HSCs)</td>
<td>Control (HSCs)</td>
<td>Control (HSCs)</td>
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<tr>
<td>Bone marrow (CFU-GMx10⁵ MNC)</td>
<td>252±33.8</td>
<td>115±12*</td>
<td>271±46.8</td>
</tr>
<tr>
<td>Spleen (CFU-GMx10⁵ MNC)</td>
<td>138±27.2</td>
<td>67.5±14.7*</td>
<td>124±21.7</td>
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The data reported of mean±SD (standard deviation of the mean) colonies of CFU-GM (granulocyte-macrophage colony-forming unit cells).

* Differences in the hematopoietic recovery of the total number colony CFU-GMx10⁵ MNC (mononuclear cells) is significant (p < 0.01).
Discussion

A high dose of ionizing irradiation causes damage to hematopoiesis. It means that the process of bone marrow repair and hematopoiesis reconstitution is also associated with more extensive repairation processes and with issues such as homing of transplanted cells, much discussed at present (15, 5). Our experiments were aimed at hematopoietic rescue of transplanted CD117<sup>+</sup> (lacZ<sup>+</sup>) cells sorted from bone marrow after whole-body (WB) lethally irradiated mice. Experiments on lethally irradiated mice transplanted with CD117<sup>+</sup>B220<sup>−</sup> sorted from bone marrow showed renewal of B-cells (B220<sup>+</sup> cells) on day 8 post-transplantation. This increase stopped by day 10, but the number of cells remained at the same level for 8 weeks. B-cell production in the bone marrow of recipients occurred 8 weeks post transplantation, and reparation of these cells after application was faster than application of CD117<sup>+</sup>CD71<sup>+</sup>. This difference was detrimental to transplanted CD117<sup>+</sup>CD71<sup>+</sup> cells for 2–4 days (7). A much discussed issue is, for example, what the myeloid developmental potential is of populations of hematopoietic progenitor cells B220<sup>−</sup>CD117<sup>+</sup>CD19<sup>−</sup> (4). That was the reason why we used the population of CD117<sup>+</sup>B220<sup>−</sup> cells in order to strengthen the myeloid developmental potential. When we evaluated the hematopoiesis repair in our experiments, we got similar results to that on day 8 after transplantation of CD117<sup>+</sup>B220<sup>−</sup> (lacZ<sup>+</sup>) and, when compared with the control group, there was a statistically significant difference in the number of CFU-GM in both the bone marrow (p<0.01) and spleen (p = 0.003). Reconstitution of hematopoiesis in irradiated mice transplanted with sorted HSCs cells started on day 12. In that time the numbers of CFU-GM in the bone marrow and spleen were similar to the controls. Further on, the repair of hematopoiesis stagnated and on day 30 the number of CFU-GM was lower, but this difference was at the limit of statistical significance. Based on a classical study discussing what divides multipotent hematopoietic progenitor lineage into three subpopulations and describing a population coming from a lineage of stem cells that had the long-term self-renewing property (9), we can presume certain significant differences in hematopoietic repair and also in vivo homing of these transplanted cells and their possible association to niche (17). We already know that the number of HSCs in the bone marrow in mammals is approximately 1/100000. These cells have the capacity of hematopoietic and immunologic renewal when applied to recipients after induced myeloablation. Recent research is focused on increasing the purity of HSCs isolated from bone marrow. Cells Thy-1<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>−</sup>c-kit<sup>+</sup> from the marrow of young mice are highly enriched with HSCs (1 of 5 cells brings long-term multilineage renewal after transplantation to irradiated mice), the same cell population in cytokine-mobilized mice brings less effective (1 of 78 to 185 cells) long-term multilineage renewal (16). So the question is what real potential does the subpopulation of CD117<sup>+</sup>B220<sup>−</sup> bone marrow cells used in our experiments have.

Our results demonstrate that sufficient hematopoietic repair occurs after transplantation of CD117<sup>+</sup>B220<sup>−</sup> (lacZ<sup>+</sup>) in lethally irradiated mice, and the difference in CFU-GM numbers in the bone marrow and spleen found on day 8 post-transplant has no influence on the survival of lethally irradiated mice (30 days follow-up). It is not clear what the proportion of resident and transplanted cells in the reparation process is. Nevertheless, our experiments showed that hematopoiesis repair may be achieved with transplantation of sorted CD117<sup>+</sup>B220<sup>−</sup> and the differences, which were observed, had no influence on survival of lethally whole-body irradiated mice.

The result showed that extramedullar hematopoiesis in the spleen is very important for hematopoiesis repair for lethally irradiated mice.

Acknowledgement

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References

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