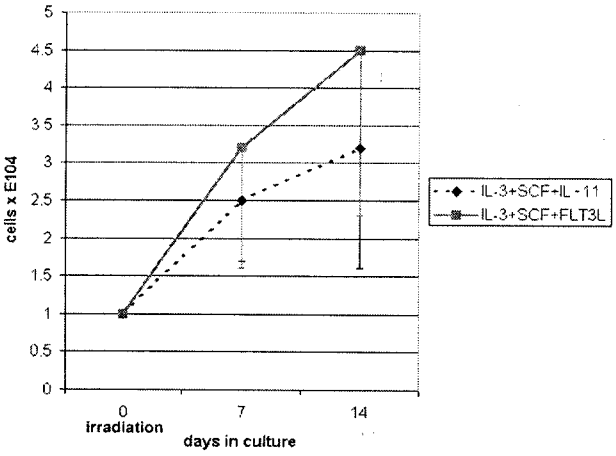

Fig. 4. Increase of the total number of 2.5 Gy-irradiated cells in response to SCF, IL-3 and IL-11 or FLT3L after 14-day incubation in liquid culture. The values represent the cell number per ml x 10^4; mean ± SD (standard deviation of the mean), 6 persons.

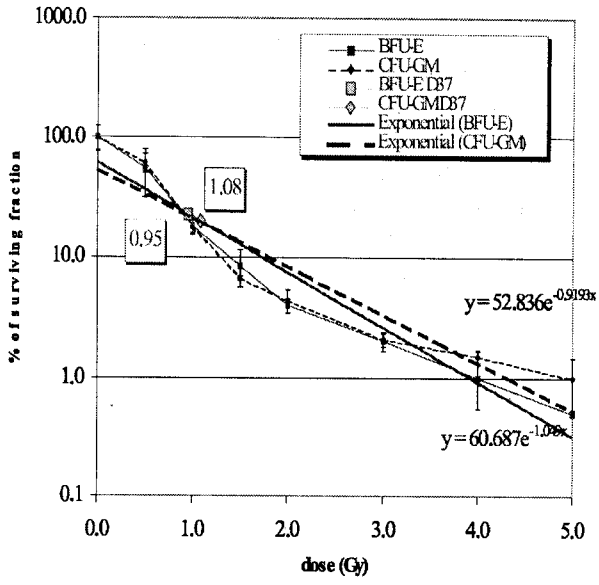

Fig. 5. Radiation response curves of AC133$^+$ haematopoietic progenitors obtained from human peripheral blood. Surviving fractions are presented as a mean value from three experiments (6 dishes). The D_0 value was determined for CFU-GM = 1.08 Gy and for BFU-E = 0.95 Gy.

Effect of cytokine combination SCF + IL–3 + FLT3L on proliferation of AC133$^+$ cells

Figure 6 shows cell cycle progression of AC133$^+$ cells expanded by combination SCF + IL-3 + FLT3L. It can

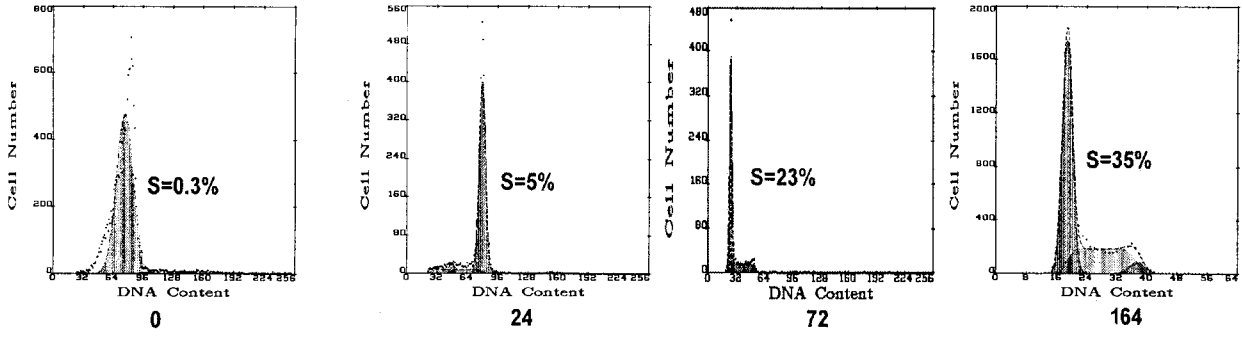

Fig. 6. Flow-cytometric analysis of DNA content and cell cycle of AC133$^+$ cells after immunomagnetic separation and *in vitro* incubation (24–164 h) with combination of cytokines SCF + IL-3 + FLT3L.

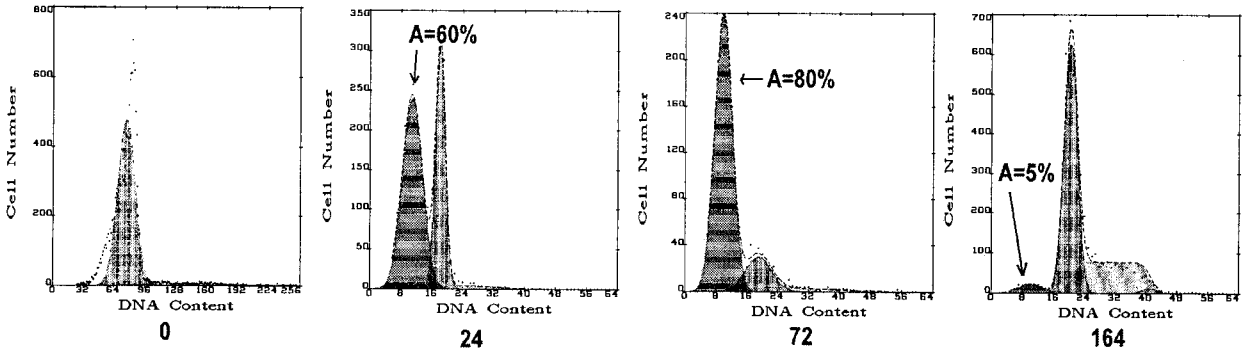

Fig. 7. Flow-cytometric analysis of DNA content and cell cycle of AC133$^+$ cells after immunomagnetic separation and irradiation with a dose of 2.5 Gy. Irradiated cells were cultivated (24–164 h) with combination of cytokines SCF + IL-3 + FLT3L.

be seen that after release of stem cells into peripheral blood by mobilization using G-CSF and their separation in a Cobe separator and miniMACS system nearly all cells were in resting G_0/G_1 phase (0.23% of cells were in S phase). Twenty-four hours after expansion the beginning 5% of cells appeared in S phase and on the 7th day of expansion 35% of cells were in S phase, which means that the cells proliferated.

Figure 7 shows cell cycle progression of cells irradiated with a dose of 2.5 Gy. It was observed that 24 h after irradiation 60% of cells had subdiploid DNA content, which represents apoptotic cells; 40% of cells were in G_0/G_1 phase. After 72 h the number of apoptotic cells increased to 80%, but cells in S phase were also detected (5%). On the seventh day after irradiation there was only a minimum of apoptotic cells (5%) and 36% of cells were in S phase, similarly to the control, non-irradiated group.

Discussion

In our previous study we proved the ability of $AC133^+$ cells to expand in the presence of cytokines SCF + IL-3 + IL-11 in patients with breast carcinoma. After seven days of expansion the number of cells increased 20-fold (Vávrová et al., 1999). In this study of healthy donors we proved 9.3-fold expansion after 7 days and 55-fold expansion after 14 days using cytokine combination SCF + IL-3 + IL-11. It is necessary to realize that different mobilization protocols were used for breast cancer patients (epirubicin and cyclophosphamide on day 1; 24 h after chemotherapy mobilization was started with subcutaneous administration of G-CSF for the next 13 days; Vávrová et al., 1999) and healthy donors (only subcutaneous application of G-CSF for three days). A higher expansion ability was observed with combination SCF + IL-3 + FLT3L – 15-fold after 7 days and 120-fold after 14 days. In our experiments CD33 antigen was expressed only on 10% of $AC133^+$ cells and CD15 antigen on 2% of cells. After 14 days of expansion the percentage of $CD33^+$ cells increased to 85% and the percentage of $CD15^+$ cells to 30% (IL-11) of cells or 40% (FLT3L) of cells, respectively. Our results indicate that during 14 days of cultivation the combination SCF + IL-3 + FLT3L induced a higher increase of proliferative rate of $AC133^+/CD34^+$ in comparison to the combination SCF + IL-3 + IL-11. The results showed no loss of the absolute amount of $AC133^+/CD34^+$ cells, but the amount of $CD34^+/CD33^-$ cells decreased. The increase of progenitors of the granulocyte lineage ($CD33^+$, $CD15^+$ and CFU-GM) was significantly pronounced in comparison to the combination with IL-11. Lyman and Jacobsen (1998) described an increase of $CD34^+$ and CFU-GM cells after addition of FLT3L to the medium with cytokines (SCF + IL-6 + G-CSF + IL-3) after 14 days of expansion of $CD34^+$ cells from umbilical blood.

Our results show that minimum of isolated $AC133^+$ cells was in S phase of the cell cycle (0.3%). However, in the presence of cytokine combination SCF + IL-3 + FLT3L these cells entered the cell cycle and on seventh day of expansion 35% of cells were in S phase. Chute et al. (1999) described that cultivation in the presence of cytokine combination SCF + IL-3 + IL-6 + GM-CSF and endothelial cells induced transition of cells into S phase. Fifty-one percent of $CD34^+/CD38^+$ cells were in S phase after 7 days and 17% of $CD34^+/CD38^-$ cells were also in S phase. This showed that the primitive $CD34^+/CD38^-$ subpopulation could enter the cell cycle under environmental influence and be expanded *ex vivo*. Brant et al. (1999) proved in experiments with baboons that it was possible to use expanded $CD34^+$ cells for autologous transplantation of lethally irradiated baboons and this treatment ensured survival of the animals.

The results showed that cells of a human leukaemic cell line were more radioresistant (D_0 = 2.2 Gy) (Vávrová et al., 2001) than $AC133^+$ cells isolated from peripheral blood of healthy donors after mobilization, where D_0 for CFU-GM was 1.08 Gy and for BFU-E 0.95 Gy. Our results are in good accord with other authors (Baird et al., 1989; Kreja et al., 1993). When using the combination SCF + IL-3 + FLT3L for expansion of 2.5 Gy-irradiated $AC133^+$ the cells expanded 4.5-fold after 14 days, contrary to non-irradiated cells, which expanded 120-fold. The ability of expanded cells to form CFU-GM increased only 2-fold (unpublished). Because the expanded cells were not used for transplantation *in vivo*, based on our results we are not able to determine if there was an increase of primitive stem cells, which are capable of providing long-term haematopoiesis or can guarantee survival of a myeloablated host.

While 23% of non-irradiated $AC133^+$ cells isolated from peripheral blood of healthy donors after mobilization enter S phase of the mitotic cycle during 72 h after *ex vivo* expansion and apoptosis is not observed, *in vitro* irradiation of these cells with a dose of 2.5 Gy induces apoptosis in 80% of cells 72 h after irradiation. However, in the presence of cytokine combination SCF + IL-3 + FLT3L, the surviving 20% of cells are able to divide and on the 7th day after irradiation 35% of cells are in S phase of the cell cycle. Drouet et al. (1999) proved that when the $CD34^+$ cells are irradiated *in vitro* with doses 2.5–6.0 Gy and incubated in the medium without cytokines, 97% of cells die by apoptosis during 24 h, and after 48 h all cells lose their functionality completely. When these authors incubated cells with cytokine combination SCF + IL-3 + FLT3L+ thrombopoietin, 15% of cells were saved from apoptosis after 2.5-Gy irradiation and 12% of cells after 4-Gy irradiation. In accordance with our results after 7 days of *ex vivo* expansion of 2.5 Gy-irradiated cells, the authors observed 5.6-fold increase of the nucleated cell number. The results suggest that early use of antiapoptotic cytokines is important for the expansion of irradiated haematopoietic progenitors, and it is possible to partially avoid induction of apoptosis by ionizing radiation in haematopoietic stem cells. Surviving of nuclear accident victims suffering from radiation-induced bone marrow aplasia could therefore

be significantly influenced by timely application of anti-apoptotic cytokines.

References

Baird, M. C., Hendry, J. H., Testa, N. G. (1989) The radiosensitivity of human haemopoietic progenitor cells. *Int. J. Radiat. Biol.* **56**, 617-621.

Brandt, J., Briddell, R. A., Srour, E., Leemhuis, T. B., Hoffman, R. (1992) Role of c-kit ligand in the expansion of human hematopoietic progenitor cells. *Blood* **79**, 634-641.

Brandt, J., Bartholomew, A. M., Fortman, J. D., Nelson, M. C., Bruno, E., Chen, L. M., Turian, J. V., Davis, T. A., Chute, J. P., Hoffman, R.(1999) *Ex vivo* expansion of autologous bone marrow CD34$^+$ cells with porcine microvascular endothelial cells results in a graft capable of rescuing lethally irradiated baboons. *Blood* **94**, 106-113.

Chute, J. P., Saini, A. A., Kampen, R. L., Wells, M. R., Davis, T. A (1999) A comparative study of the cell cycle status and primitive cell adhesion molecule profile of human CD34$^+$ cells cultured in stroma-free versus porcine microvascular cell cultures. *Exp. Hematol.* **27**, 370-379.

Coutinho, L. H., Gilleece, M. H., DeWynter, E. A., Will, A., Testa, N. G. (1993) Clonal and long-term cultures using human bone marrow. In: *Haemopoiesis. A Practical Approach*, eds. Testa, N. G., Molineux, G., pp.75-106, Oxford University Press, Oxford.

Cowling, G. J., Dexter, T. M. (1994) Apoptosis in the haemopoietic system. *Phil. Trans. R. Soc. Lond.* **345**, 257-263.

Drouet, M., Mathieu, J., Grenier, N., Multon, E., Sotto, J., Herodin, F. G. (1999) The reduction of in vitro radiation-induced Fas-related apoptosis in CD34$^+$ progenitor cells by SCF, FLT3 ligand, TPO, and IL-3 in combination resulted in CD34$^+$ cell proliferation and differentiation. *Stem Cells* **17**, 273-285.

Fichelson, S. (1998) The FLT3/FLK2 ligand: structure, functions and prospects. *Eur. Cytokine Netw.* **9**, 7-22.

Kaashoek, J. G. J., Mout, R., Falkenburg, J. H. F., Willemze, R., Fibbe, W. E., Landegent, J. E. (1991) Cytokine production by the bladder carcinoma cell line 5637: rapid analysis of mRNA expression levels using a cDNA-PCR procedure. *Lymphokine Cytokine Res.* **10**, 231-235.

Kreja, L., Thoma, C., Selig, C., Lamping, C., Ziegler, B. L., Nothdurft, W. (1993) The effect of recombinant human stem cell factor and basic fibroblast growth factor on the in vitro radiosensitivity of CD34$^+$ hematopoietic progenitors from human umbilical cord blood. *Exp. Hematol.* **21**, 1436-1441.

Lyman, S. D., Jakobsen, S. E. (1998) c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood* **91**, 1101-1134.

Miraglia, S., Godfrey, W., Yin, A. H., Atkins, K., Warnke, R., Holden, J. T., Bray, R. A., Waller, E. K., Buck, D. W. A (1997) Novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* **90**, 5013-5021.

Radford, I. R., Murphy, T. K. (1994) Radiation response of mouse lymphoid and myeloid cell lines. Part 3. Different signals can lead to apoptosis and may influence sensitivity to killing by DNA double-strand breakage. *Int. J. Radiat. Biol.* **65**, 229-239.

Vávrová, J., Filip, S., Vokurková, D., Bláha, M., Vaňásek, J., Jebavý, L. (1999) Ex vivo CD 34$^+$/AC 133$^+$ – selected autologous peripheral blood progenitor cells (PBPC) in high-risk breast cancer patients receiving intensive chemotherapy. *Hematol. Cell Ther.* **41**, 105-112.

Vávrová, J., Mareková, M., Vokurková, D. (2001) Radiation-induced apoptosis and cell cycle progression in TP53-deficient human leukemia cell line HL-60. Neoplasma 48, 26-33.

Vindelov, L. (1977) Microfluorometric analysis of nuclear DNA in cells from tumors and cell suspension. *Virchows Arch. Cell. Pathol.* **173**, 227-242.

Yin, A., Miraglia, S., Zanjani, D., Almeida-Porada, G. (1997) AC 133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* **90**, 5002-5012.