

Antiapoptotic Cytokine IL-3 + SCF + FLT3L Influence on Proliferation of Gamma-Irradiated AC133⁺/CD34⁺ Progenitor Cells

(AC133⁺ cells / gamma irradiation / *ex vivo* expansion / cell cycle / apoptosis)

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Abstract. Recovery from radiation-induced bone marrow aplasia depends on appropriate cytokine support. The aim of our work was to find a cytokine combination allowing *in vitro* gamma-irradiated (2.5 Gy) CD34⁺/AC133⁺ haematopoietic stem cells to evade radiation-induced apoptosis and to enhance damage reparation, which should enable proliferation and *ex vivo* expansion of cells. Cells were isolated using separation in a Cobe separator followed by immunomagnetic selection by antibody against the AC133 antigen. Thus isolated cells were 80% AC133⁺/CD34⁺ and 10% of them expressed the CD33⁺ antigen. Ten thousand of AC133⁺ cells formed 1146 CFU-GM and 304 BFU-E. We proved a high expansion efficiency of cytokine combination SCF + IL-3 + FLT3L in comparison with the combination SCF + IL-3 + IL-11 in both, non-irradiated cells and cells irradiated with a dose of 2.5 Gy. The D₀ value for AC133⁺ cells was determined by the clonogenicity test. The D₀ value for CFU-GM was estimated to be 1.08 Gy and for BFU-E 0.95 Gy. The results of DNA analysis showed that the majority of isolated AC133⁺ cells were in G₀/G₁ phase of the cell cycle. We proved that the dose of 2.5 Gy induced massive apoptosis (80%) of these cells without progression through the cell cycle, which indicates interphase cell death. Under the influence of cytokine combination (SCF + IL-3 + FLT3L), the surviving 20% of cells entered the cell cycle and, similarly to non-irradiated control cells, on 7th day 35% of cells were in S phase.

The AC133 antibody provides an alternative to CD34 for selection and characterization of cells necessary for both, short- and long-term engraftment, in transplant situations and for studies of *ex vivo* expansion strategies. AC133 is one of a new panel of murine hybridoma lines producing monoclonal IgG antibodies to a novel stem cell glycoprotein antigen with the molecular weight of 120 kD (Miraglia et al., 1997). The AC133 antigen is selectively expressed on CD34⁺ haematopoietic stem and progenitor cells derived from human foetal liver and bone marrow, and blood (Yin et al., 1997). All of the noncommitted CD34⁺ cell population, as well as the majority of CD34⁺ cells committed to the granulocytic/monocytic pathway, are stained with AC133 antibody. In this report, we used the AC133⁺ population for an experimental study of *ex vivo* expansion of progenitors.

The choice of cytokine combination and culture system will largely determine the fate of the cells used to initiate the culture. Cytokine-mediated expansion has been proposed as a means of increasing the total number of cells as well as both committed and primitive haematopoietic progenitors. Nevertheless, most cytokine combinations have included SCF and IL-3 as an absolute requirement (Brandt et al., 1992). Used on its own, SCF has, at best, only a modest stimulatory activity. However, it has a profound synergistic effect when used in conjunction with other cytokines. FLT3L is a more recently cloned cytokine, which seems to share numerous properties with SCF. FLT3 gene expression is restricted to haematopoietic cells and tissues in human. The receptors for SCF and FLT3L are both members of the family of receptors with tyrosine kinase activity. These receptors transfer the signals to the nucleus using the ras-MAP kinase pathway, which could directly regulate cell survival and proliferation. FLT3L is able to accelerate cell cycling of IL3-dependent haematopoietic progenitors by shortening the G₁ phase of the cell cycle (Fichelson, 1998).

In our previous study we expanded AC133⁺ cells isolated from peripheral blood of patients suffering of breast cancer using the combination SCF + IL-3 + IL-

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Abbreviations: BFU-E – burst-forming unit erythroid progenitor, CFU-GM – colony-forming unit-granulocyte-macrophage, D₀ – dose that reduces the surviving fraction to 37%, FLT3L – FLT3 ligand, PBPC – peripheral blood progenitor cells, SCF – human recombinant stem cell factor.

11 and after seven days of long-term expansion the number of nucleated cells increased 20-fold (Vávrová et al., 1999). In this work we compared the expansion capabilities of two combinations of cytokines for AC133⁺ cell expansion: SCF + IL-3 + IL-11 and SCF + IL-3 + FLT3L, in the control group and group irradiated *in vitro* by a dose of 2.5 Gy.

Information about the response characteristic of clonogenic haematopoietic cells exposed to ionizing irradiation *in vitro* is important for prediction of the tolerance of the haematopoietic system to radiation exposure *in vivo*. The D₀ value of 1.34 Gy obtained for CFU-GM and BFU-E of CD34⁺ cells isolated from human umbilical cord blood (Kreja et al., 1993) was similar as the D₀ value for CFU-GM and BFU-E from adult bone marrow. In our work we established the D₀ value for AC133⁺ cells isolated from peripheral blood of healthy donors after mobilization by G-CSF.

The next important question is whether radiation damage can be repaired. Cells isolated after mobilization from peripheral blood are nearly all in G₀/G₁ phase of the cell cycle. From experiments carried with haematopoietic cell lines it is known that most of haematopoietic cells die by apoptosis after irradiation (Radford and Murphy, 1994; Vávrová et al., 2001). Apoptotic cell death was also described in haematopoietic cell cultures FDCP-1 and FDCP-mix after removal of growth factors from culture media (Cowling and Dexter, 1994). Drouet et al. (1999) described apoptotic cell death resulting from *in vitro* irradiation of CD34⁺ cells isolated from monkey bone marrow after doses 2.5–6.0 Gy. Our aim was to evaluate whether AC133⁺ cells isolated from peripheral blood of healthy donors die by apoptosis after irradiation with 2.5 Gy and whether in the presence of cytokine combination SCF + IL-3 + FLT3L part of these irradiated cells are able to repair damage and proliferate in cell culture *in vitro*. Apoptosis could also be labelled as a proliferation regulator. The presence of some growth factors could have a key role for reversion of the proliferation/apoptosis ratio of primitive haematopoietic cells in benefit to proliferation, which is necessary for haematopoietic cell regeneration of irradiated victims.

Material and Methods

Mobilization procedure and PBPC harvest

AC133⁺/CD34⁺ cells were obtained from peripheral blood of six healthy donors after mobilization by subcutaneous application of G-CSF (Neupogen, Roche, Basel, Switzerland) in a dose of 5 µg/kg/day for three days. Cells were collected on the 4th day after application by leukapheresis. The leukaphereses were performed using an automated COBE Spectra (Lakewood, CO) continuous blood flow cell separator with the following parameters: blood volume processed: 10 000–12 000 ml (median 11 000 ml) per procedure, inlet flow rate:

60–70 ml/min. We used the original COBE MNC version 5.1 software programme.

Reagents

Human recombinant IL-3 was purchased from Sigma-Aldrich chemie GmbH (Munich, Germany). Human recombinant IL-11 and human recombinant SCF were purchased from Stem Cell Technologies Inc. (Vancouver, Canada) and FLT3L from Genzyme diagnostics (Cambridge, MA). Iscove's modified Dulbecco's medium and foetal bovine serum were purchased from Sigma-Aldrich, foetal bovine serum (Sigma-Aldrich), complete methylcellulose medium for colony assays with 5637 CM conditioned medium were supplied from Institute of Haematology and Blood Transfusion (Prague, Czech Republic) and with erythropoietin (Eprex) from Janssen-Cilag AG (Baar, Switzerland). The MiniMACS separation system AC133⁺ cell isolation kits were supplied by Miltenyi Biotec (Bergisch Gladbach, Germany). Phycoerythrin-conjugated antiCD34 /QBend (IgG1)/, fluorescein isothiocyanate-conjugated antiCD33 /D3HL60.251(IgG1)/, antiCD34/581(IgG1)/ and antiCD15/80H51gM/ were purchased from Immuno-tech (Miami, FL). Phycoerythrin-conjugated antiAC133 was purchased from Miltenyi Biotec.

AC133⁺ cell purification and liquid culture

AC133⁺ cells were purified from fresh PBPC using the MiniMACS purification system. PBPC used for MACS separation of AC133⁺ cells were directly magnetically labelled using AC133 microbeads. AC133⁺ cell purity was determined by flow cytometric analysis.

The AC133⁺ cells were seeded at 1 x 10⁴ cell/ml in Iscove's modified Dulbecco's medium containing 10% foetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM/ml L-glutamine, 50 ng/ml hSCF, 50 ng/ml hIL-11 or 50 ng/ml FLT3L and 20 ng/ml hIL-3. All cultures were incubated in 5% CO₂ and 5% O₂ at 37°C. Aliquots of expanded cells were analysed by multiparameter flow cytometry over seven- and fourteen-day periods.

Gamma irradiation

AC133⁺ cells were suspended at a concentration of 5 x 10⁴ cells/ml in complete medium. Two ml of aliquots were given into the plate and irradiated at room temperature using a ⁶⁰Co gamma-ray source with a dose rate 0.66 Gy/min. After irradiation, plates were placed in a 37°C incubator in 5% CO₂ and 5% O₂ and aliquots of cells were removed at various times after irradiation for analysis. The cells were counted and cell viability was determined with the Trypan blue exclusion assay.