Clonogeneic assays for myeloid (CFU-GM) and erythroid (BFU-E) progenitors

Unseparated PBPC from the leukapheresis product, AC133$^+$ cells, irradiated AC133$^+$ cells, as well as expanded cells were grown in 0.9% methylcellulose as described by Coutinho et al. (1993). All semi-solid cultures were performed in duplicates and stimulated with 10% conditioned medium from the 5637 human bladder carcinoma cell line (Kaashoek et al., 1991) and 4 units/ml erythropoietin. CFU-GM and BFU-E colonies were counted after 14 days of incubation in 5% CO_2 and 5% O_2 at 37°C. Dose response curves of AC133$^+$ cells (from three persons) irradiated *in vitro* by an increasing dose of 0.5–5.0 Gy were used for D_0 value calculations.

Immunofluorescence staining and dual-colour flow-cytometric analysis

Enriched AC133$^+$ as well as expanded nucleated cells from suspension cultures were incubated with antiCD34 fluorescein (FITC)-conjugated or antiCD34 phycoerythrin (PE)-conjugated monoclonal antibodies and/or antiCD33-FITC, antiCD15-FITC and antiAC133-PE for 30 min. Flow-cytometric analysis was performed in a Coulter Epics XL flow cytometer. A minimum of 10 000 cells were collected for each 2-colour sample in a list mode file format. List mode data were analysed using Epics XL System II software colour eventing (Coulter Electronic, Hialeah, FL).

Flow-cytometric analysis of the cell cycle and DNA fragmentation

Following the incubation the cells were washed with cold PBS and fixed by 70% ethanol (minimally 30 min at 4°C). After centrifugation (200 *g*, 10 min at 4°C) and ethanol removal the cells were washed by ice-cold PBS, suspended in phosphate buffer and incubated 5 min at room temperature for extraction of low-molecular fragments of DNA. Then the cells were stained in 0.5 ml of Vindelov's solution (Vindelov, 1977), 1 h in a thermostat at 37°C and analysed by flow cytometry in a Coulter Electronic (Hialeah, FL) flow cytometer. A minimum of 10 000 cells analysed in each sample served to determine the percentages of cells in each phase of the cell cycle, using Multicycle AV software. Experiments were performed with AC133$^+$ cells from three persons, non-irradiated and irradiated by a dose of 2.5 Gy.

Statistical analysis

The results were statistically evaluated with Student's t-test. The values represent mean ± SD (standard deviation of the mean). Statistical significance of the difference of means in comparable sets is expressed in the text for $P < 0.05$.

Results

Characteristics of the sorted AC133$^+$ cells and changes in immunophenotype

The purity of fractions was controlled by flow-cytometric analysis using antiCD34 and AC133 monoclonal antibodies. CD34-FITC and AC133-PE expression on MiniMACS AC133$^+$ isolated cells is shown in Fig. 1. Table 1 shows that cells isolated by immunomagnetic separation were 78% AC133$^+$/CD34$^+$ and 77.8% CD34$^+$/CD33$^-$. Cell maturation in culture was assessed by monitoring the serial expression of AC133$^+$, CD34$^+$, CD33$^+$ and CD15$^+$ antigen. In Fig. 2, the decrease in number of primitive AC133$^+$/CD34$^+$ cells in culture after 14 days of expansion is apparent. On the contrary, expression of antigens showing differentiation of cells into granulocytes increased. After 14 days of expansion 86% of cells were CD33$^+$ and 39 % CD15$^+$. The absolute numbers of AC133$^+$ cells expressing various antigens immediately after isolation and after 7 and 14 days of cultivation are recorded in Table 2. It can be seen that after 14 days of cultivation with combination SCF + IL-3 + FLT3L

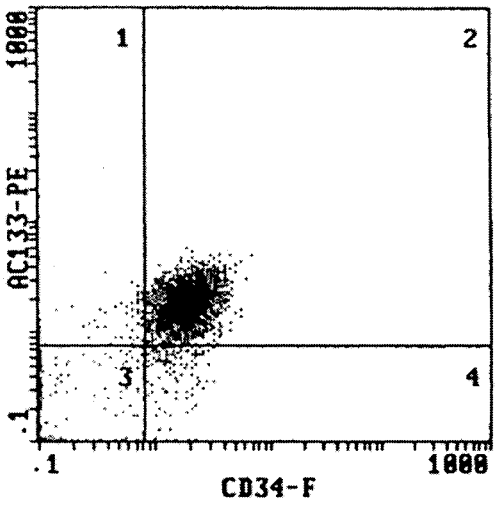

Fig. 1. AC133$^+$ haematopoietic stem and progenitor cells were positively selected from fresh PBPC using AC133 microbeads. The AC133$^+$ cell population was analysed using two-colour fluorescence AC133-PE versus CD34-FITC. A minimum of 10 000 cells were collected.

Table 1. Characteristic of AC133$^+$ cells

Expressing antigen	%
AC133$^+$ PE	81.8 ± 8.0
CD34$^+$ FITC	85.9 ± 9.3
CD33$^+$ FITC	10.4 ± 2.8
CD15$^+$ FITC	2.3 ± 2.3
AC133$^+$ PE/ CD34$^+$ FITC	78.0 ± 11.2
CD34$^+$ PE/ CD33$^-$ FITC	77.8 ± 11.6

The values represent mean of percentage ± SD (6 persons)

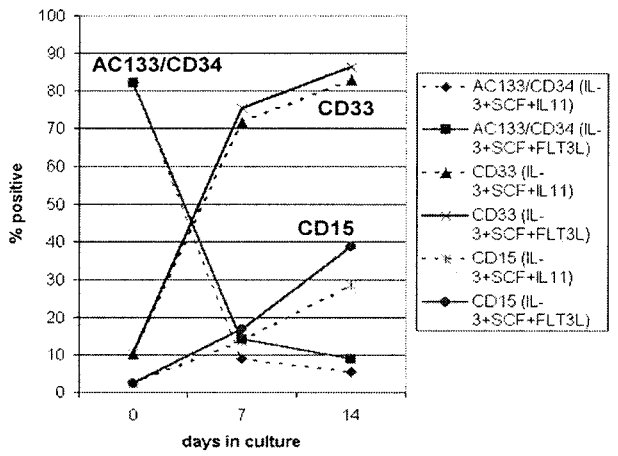

Fig. 2. The proportions of cells expressing CD34, AC133, CD33 or CD15 in the culture. The values represent the mean percentage (6 persons).

decrease of expansion ability was also observed when using the combination SCF + IL-3 + FLT3L; the number of nucleated cells increased after irradiation by 2.5 Gy 4.5-fold, which means a 27-fold decrease in comparison to non-irradiated cells.

Ten thousand of $AC133^+$ cells isolated by immunomagnetic selection formed 1146 (± 416) CFU-GM and 304 (± 70) BFU-E. Figure 5 shows the results of the clonogeneic assay for *in vitro* irradiated $AC133^+$ cells, which form both, CFU-GM and BFU-E colonies. The curve was obtained by irradiation of $AC133^+$ cells, from three healthy donors, by increasing the dose of radiation from 0.5 to 5.0 Gy. Each sample was processed twice, so the presented points represent the median of six measurements. Colonies were evaluated after 14 days of incubation in a thermostat with 5% CO_2 and 5% O_2. The curve shows the number of colonies as a percentage of the number of colonies formed by non-irradiated cells (100%).

Table 2. Absolute number of cells expressing lineage markers on fresh and cultured AC133 miniMACS-isolated cells

antigen	Days in culture 0	7	7	14	14
		SCF+IL–3 +IL-11	SCF+IL–3+FLT3L	SCF+IL–3 +IL-11	SCF+IL–3+FLT3L
$AC133^+$	0.8 ± 0.1	0.8 ± 0.4	2.2 ± 0.8	3.5 ±2.5	11.7 ± 7.0
$CD34^+$	0.9 ± 0.2	1.4 ± 0.7	2.3 ± 0.7	1.4 ± 1.0	2.7 ± 1.5
$AC133^+/CD34^+$	0.8 ± 0.1	0.6 ± 0.2	1.1 ± 0.3	1.0 ± 0.8	1.6 ± 0.6
$CD33^+$	0.1 ± 0.03	9.2 ± 3.5	13.0 ± 5.9	58.8 ± 8.6	142.5 ±35.5
$CD34^+/CD33^-$	0.8 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.2
$CD15^+$	0.02 ± 0.02	1.5 ± 0.7	2.8 ± 1.0	19.0 ±10	50.9 ±25.4

The values represent the cell numbers per ml x 10^4; mean ± SD (standard deviation of the mean) (6 persons).

there was no decrease in the absolute number of $CD34^+$ or $AC133^+$ cells, while a statistically significant increase of neutrophil granulocyte progenitors $CD33^+$ and $CD15^+$ was apparent.

Changes in number of nucleated cells and clonogeneity

Figure 3 shows the number of $AC133^+$ cells expanded *ex vivo* (1 x 10^4 cell/ml) in supplemented Iscove's modified Dulbecco's medium. A statistically significant increase in the number of nucleated cells could be seen in both cytokine combinations (IL-3 + SCF + IL-11 or FLT3L) after 7 and 14 days of cultivation. On the 14th day the number of nucleated cells increased in the group stimulated by the combination SCF + IL-3 + FLT3L 120-fold and in the group stimulated by the combination SCF + IL-3 + IL-11 55-fold; the difference between the groups was statistically significant.

As can be seen in Fig. 4, the expansion ability of 2.5 Gy-irradiated cells was very low. On the 14th day after the beginning of expansion the number of nucleated cells increased after incubation with the combination SCF + IL-3 + IL-11 only 3.2-fold, which means a 17-fold decrease in comparison to non-irradiated cells, where the number of cells after expansion increased 55-fold. A simi-lar

Using Microsoft-Excel software the curve was plotted, the slope of curve counted and the D_0 value established. The D_0 value for $AC133^+$ cells forming CFU-GM was **1.08 Gy** and for cells forming BFU-E **0.95 Gy**.

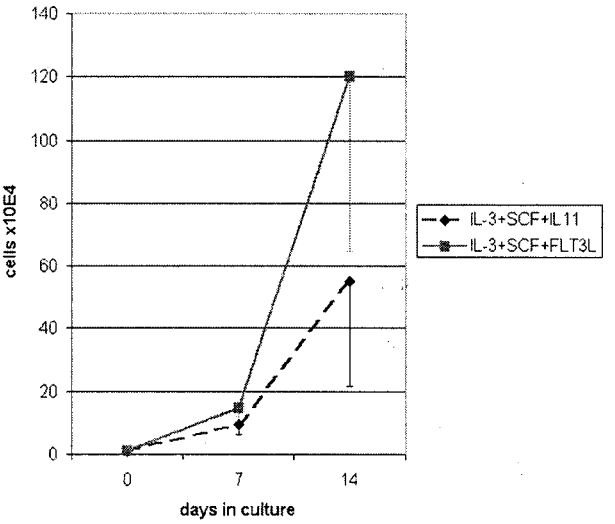

Fig. 3. Increase of the total number of cells in response to SCF, IL-3 and IL-11 or FLT3L after 14-day incubation in liquid culture. The values represent the cell number per ml x 10^4; mean ± SD (standard deviation of the mean), 6 persons.