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GP130 and c-kit signalling, initiated by the sIL-6R/IL-6 complex, is insufficient to expand the primitive adult bone marrow CD34+CD38- pre-CFU cell

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Abstract It has previously been shown that gp130 and c-kit signalling synergize for the ex vivo expansion of human cord blood (CB) CD34+ haematopoietic progenitor cells. We were interested in evaluating this synergy within an ontogenetically different haematopoietic tissue [i.e. adult bone marrow (BM)] and on a more primitive progenitor subset (i.e. CD34+CD38− cells), which are highly enriched for pre-colony forming unit (CFU) cells. These cells were plated out in a primary liquid culture supplemented with either interleukin (IL)-6+stem cell factor (SCF), IL-6+SCF+soluble IL-6 receptor (sIL-6R), IL-6+SCF+sIL-6R+IL-3+IL-1 or SCF+IL-3+IL-6+IL-1. Cell counting after liquid culture revealed an absolute expansion of 2.2-, 4.1-, 89.5- and 65.7-fold compared with initial cell input for the four-cytokine combinations, respectively. The secondary read-out assay revealed that this cell expansion in the liquid culture also resulted in CFU generation, with absolute cloning efficiencies of 0.002, 0.024, 12.13 and 7.73 (per cell initially present) for the respective cytokine combinations. These results indicate that gp130 and c-kit signalling alone (i.e. using IL-6+SCF+sIL-6R), in terms of both cell number and CFU generation, insufficiently stimulate primitive adult BM CD34+CD38− haematopoietic cells in order to reach a CFU generation comparable with that obtained after multifactor stimulation. Adding sIL-6R to the multifactor stimulation and compared with this multifactor stimulation, a 1.7-fold synergy in terms of cell expansion and a 3.0-fold synergy in terms of CFU generation are obtained. The sIL-6R/IL-6 complex thus has a narrower spectrum of action on primitive adult BM CD34+CD38− cells than on CB CD34+ cells.

Keywords Haematopoiesis · sIL-6R/IL-6 complex · CD34+CD38− · Pre-CFU, gp130

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Introduction

The interleukin (IL)-6R system consists of an 80-kDa IL-6-binding molecule termed IL-6Rα and a signal transducing β chain gp130 that is shared by other receptor complexes such as ciliary neurotrophic factor (CNTF), leukaemia-inhibitory factor (LIF), oncostatin M (OSM), IL-11 and cardiotrophin-1 (CT-1). Both chains belong to the cytokine receptor superfamily. Binding of ligand initiates the formation of a hexameric receptor complex (two molecules each of IL-6, IL-6Rα and gp130). Dimerization of gp130 herein induces intracellular signalling. The restricted presence of the ligand-specific receptor in combination with the ubiquitous expression of a common signal transducer for multiple cytokine receptors explains the functional pleiotropy and redundancy of IL-6- and IL-6-related cytokines (for recent reviews see [4, 9, 16, 22]).

Because the cytoplasmic domain of IL-6Rα is not required for signal transduction, it may be inferred that a complex of IL-6 and a soluble form of IL-6Rα (i.e. soluble IL-6 receptor, sIL-6R) will act on cell types that only express gp130 without expressing the α chain. IL-6 and sIL-6R may be sufficient to stimulate gp130 and haematopoiesis (for review, see [14]). There is evidence suggesting that this is the case for cells within the human CD34+ cord blood (CB) haematopoietic progenitor cell compartment: signalling through gp130 by sIL-

6R/IL-6, in synergy with c-kit signalling induced by stem cell factor (SCF) (i.e. 6S6r), results in expansion of the colony forming units (CFU) within this cellular compartment as compared with induction by IL-6 and SCF alone (i.e. 6S) [13, 19].

Because these results were obtained starting from CD34+ umbilical CB progenitors, of which only a very small subset are very primitive in nature, we wanted to further explore this effect on adult bone marrow (BM) progenitors highly enriched for primitive cells (i.e. CD34+CD38− cells). In contrast to CD34+ cells, these cells are too primitive to give rise to a CFU in a classical semisolid assay and are termed pre-CFU. This type of cell has previously been shown to only respond to a multifactor combination of early acting cytokines [18]. We followed the effect of sIL-6R on the generation of CFUs out of these CD34+CD38− (pre-CFU) cells within a 14-day liquid culture system, using a classical semisolid methylcellulose clonogenic assay as read-out. The results obtained using 6S or 6S6r were compared with those obtained using a previously established multifactor stimulatory cytokine cocktail (IL-6+SCF+IL-3+IL-1; i.e. 6S31) and with the cytokine cocktail with the addition of sIL-6R (i.e. 6S6r31).

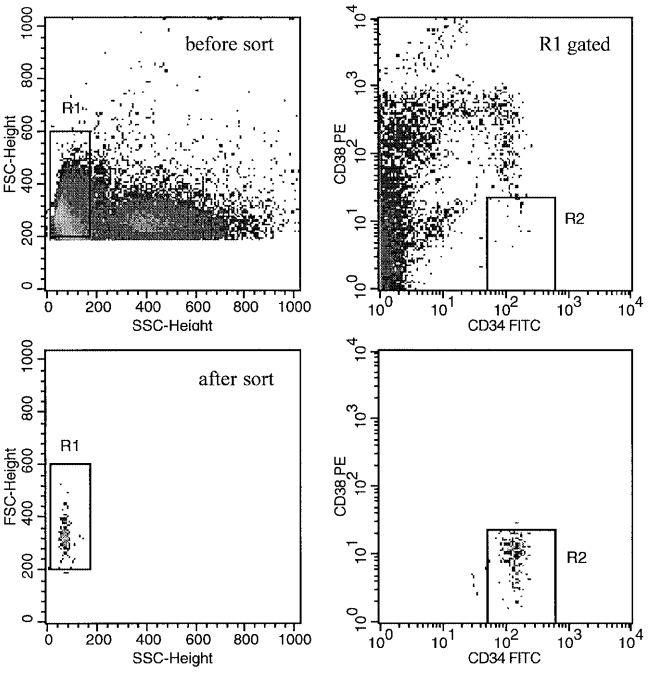

Fig. 1 Representative example of an adult bone marrow (BM) CD34+CD38− cell sort experiment. The CD34+CD38− sorted events satisfy gate settings R1 and R2 as depicted in the top (before sort) histograms. *Bottom histograms* represent a re-run of the sorted cells

Materials and methods

Adult BM cells

BM samples were aspirated by sternal puncture from haematologically normal patients undergoing cardiac surgery. Cells were collected in sterile tubes containing Iscove's modified Dulbecco's medium (IMDM)/10% foetal calf serum (FCS) and heparin (100 U/ml; Novo Nordisk, Bagsvaerd, Denmark). The mononuclear cells (MNC) were then isolated by density gradient centrifugation over lymphocyte separation medium (ICN Biomedicals, Costa Mesa, Calif., USA). Samples were taken after informed consent was obtained in accordance with the guidelines of the Medical Ethics Committees of the Antwerp University Hospital.

Monoclonal antibodies

The supernatant of the 43A1 hybridoma [immunoglobulin G3 (IgG_3), kindly donated by Dr. H.J. Bühring, University of Tübingen, Germany] was used as a source of anti-CD34 antibody [1]. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (RAM) immunoglobulins [$F(ab')_2$ fragments] were purchased from Dako (Glostrup, Denmark). Phycoerythrin (PE)-conjugated anti-CD38 antibodies, as well as isotype-matched control antibodies, were purchased from Becton Dickinson (Erembodegem, Belgium). Mouse gammaglobulins were purchased from Jackson lmmunoresearch Laboratories (West Baltimore Pike, Pa., USA).

Cell sorting

Freshly prepared adult BM MNC cells were resuspended in IMDM at 10^7 cells/ml and incubated with 43A1 supernatant in a 1/10 dilution for 20 min at 4 °C, washed twice in IMDM supplemented with 10% FCS and incubated with RAM-FITC (1/50 dilution) for 20 min at 4 °C. After washing twice in IMDM/10% FCS, the cells were incubated with mouse gammaglobulins for 10 min and incubated with anti-CD38-PE for 20 min at 4 °C. After washing twice in IMDM/10% FCS, the cells were sorted on an FACStarPlus Cell Sorter (Becton Dickinson, Erembodegem, Belgium) equipped with an air-cooled argon ion laser (ILT model 5500A; Ion Laser Technology, Salt Lake City, Utah, USA), tuned to 488 nm at 40 mW. Cells were retained as CD34+CD38− cells if the following criteria were met: a low to medium forward scatter and a low side scatter; highly positive green (CD34) fluorescence; and an orange (CD38) fluorescence signal lower than the mean fluorescence of cells labelled with an irrelevant isotope-matched control antibody+2 standard deviations (SDs). Cells with an orange fluorescence above this threshold were retained as CD34+CD38+ cells. Average purity of sorted cells was 98% (range 96–100%). A representative example is depicted in Fig. 1.

Primary pre-CFU (liquid) and secondary (semisolid) CFU read-out assays

Pre-CFU assays [12, 17] were started by making primary liquid cultures in duplicate in 96-well flat-bottomed plates in IMDM supplemented with 10% FCS, 1% bovine serum albumin (BSA) and different combinations of the following products: 100 U/ml IL-1; 5000 U/ml IL-6; 20 U/ml IL-3; 50 ng/ml SCF; and 1.3 μg/ml sIL-6R. The indicated concentrations of IL-1, IL-3, IL-6 and SCF were shown to be optimal for CFU- out of pre-CFU generation in previous experiments [5, 6, 10, 18]. Pre-CFU cultures were initiated with 500 cells/well in 200 μl culture medium. After 14 days of culture at 37 °C in 7.5% O_2 and 5% CO_2 in a fully humidified incubator, the number of cells in each well was counted using a flow cytometric cell counting program on a Coulter Epics XL-MCL (Coulter, Hialeah, Fla., USA) flow cytometer. The cells were then harvested, washed three times in IMDM/10% FCS and plated in duplicate at 500 cells/well (1000 μl) in secondary methylcellulose cultures (0.9%) supplemented with 20% FCS, 1% BSA, 10% 5637 CM (conditioned medium of the 5637 bladder carcinoma cell line, producing granulocyte colony-stimulating factor (G-

CSF) and granulocyte-macrophage (GM)-CSF [7]), 500 U/ml IL-6, 20 U/ml IL-3, 3.2 U/ml erythropoietin (epo) and 10^{-5} M 2-mercaptoethanol. These were shown to be optimal concentrations for colony formation in preliminary experiments. The cultures were microscopically scored for colony formation after 14 days of culture at 37 °C in 7.5% O_2 and 5% CO_2 in a fully humidified incubator.

Cytokines

Recombinant human (rh) IL-1 (specific activity $>5\times10^7$ U/mg), rh IL-6 (specific activity $>1\times10^8$ U/mg) and rh SCF (specific activity $>1\times10^5$ U/mg) were obtained from Boehringer Mannheim GmbH (Penzberg, Germany). sIL-6R (130 μg/ml) was a kind gift from Eurogenetics (Tessenderlo, Belgium) and rh IL-3 (biological activity 14×10^3 U/ml) was a gift from Dr. S.C. Clark (Genetics Institute, Cambridge, Mass., USA). Epo (specific activity, 1×10^5 U/mg) was purchased from Cilag (Brussels, Belgium).

Statistics

Intra-BM comparisons (for a total of 10 samples) were validated using Student's *t*-test for paired samples. Figure results are expressed as mean ± standard error of the mean (SEM). A P value <0.05 was considered statistically significant.

Results

Absolute expansion, in terms of cell number, during the primary liquid culture (see Fig. 2, insert) was 65.7-, 89.5-, 4.1- and 2.2-fold compared with the initial number of inoculated cells depending on the cytokine combinations used, being the multifactor cocktail (6S31), 6S6r31, 6S6r or 6S, respectively. The latter two combinations differed significantly from the first ($P<0.05$) in that there was a significantly lower expansion. However, the second combination exhibited a significantly higher expansion ($P<0.025$/number of samples (n) = 10).

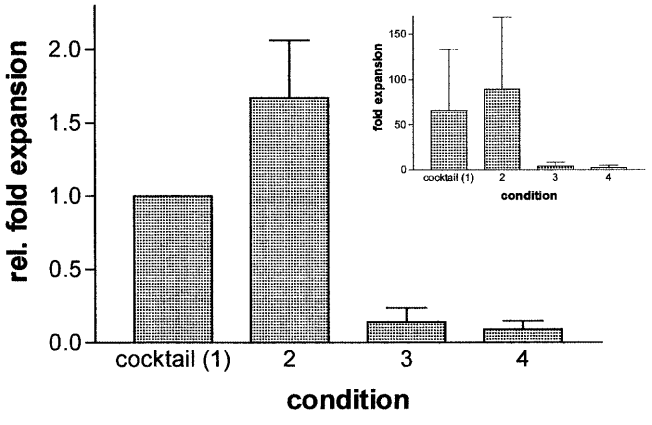

Fig. 2 Relative (compared with cocktail condition 1) and absolute (*insert*) cellular expansion of adult bone marrow (BM) CD34 + CD38– cells after a 14-day primary liquid culture in either SCF + IL-3 + IL-6 + IL-1 (condition 1), SCF + IL-3 + IL-6 + sIL-6R + IL-1 (condition 2), SCF + IL-6 + sIL-6R (condition 3) or SCF + IL-6 (condition 4), $n = 10$

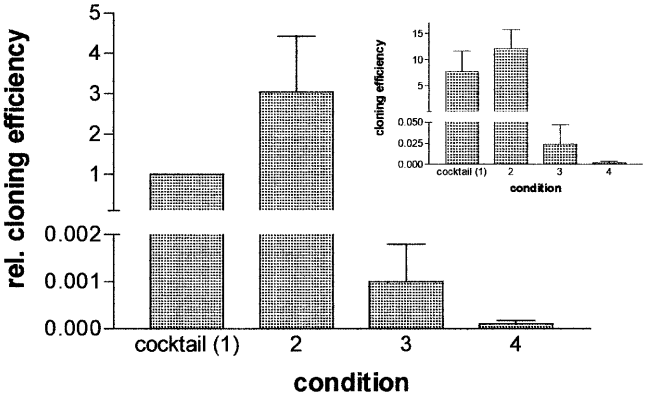

Fig. 3 Relative (compared with cocktail condition 1) and absolute (*insert*) overall cloning efficiency (per initial adult bone marrow (BM) CD34 + CD38– cell) after a 14-day liquid culture in either SCF + IL-3 + IL-6 + IL-1 (condition 1), SCF + IL-3 + IL-6 + sIL-6R + IL-1 (condition 2), SCF + IL-6 + sIL-6R (condition 3) or SCF + IL-6 (condition 4), $n = 9$. Notice the segmented (double scale) *Y*-axis

Compared with our classical multifactor cocktail for growth of CD34 + CD38 − cells (6S31), the three other combinations (cocktail + sIL-6R, i.e. 6S6r31, 6S6r or 6S) demonstrated a 1.67-, 0.14- and 0.09-fold relative expansion, respectively (see Fig. 2). The addition of sIL-6R to the cocktail resulted in a moderate increase of expansion, although this was not statistically significant ($P<0.1/n=10$). The two-cytokine combination 6S was insufficient for expansion (reduction of cell number with $P<0.0005/n=10$) and the addition of s-IL-6R did not ameliorate this (sustained reduction with $P<0.0005/n=10$).

Expansion in terms of generated CFU per initially inoculated (pre-CFU) cell (see Fig. 3, insert) was 7.73-, 12.13-, 0.024- and 0.002-fold when using our classical multifactor 6S31 cocktail, the cocktail + sIL-6R, 6S6r and 6S, respectively. The latter two combinations differed significantly from the first ($P<0.05/n=9$) in that there was a significantly lower CFU generation, whereas the second combination exhibited a significantly higher CFU generation ($P<0.01/n=9$).

Compared with the classical multifactor cocktail 6S31, the three other combinations (the cocktail + sIL-6R, 6S6r and 6S) demonstrated a 3.0-, 0.0009- and 0.0001-fold relative CFU generation, respectively (see Fig. 3). The addition of sIL-6R to 6S31 resulted in a moderate increase of CFU generation, although this was not statistically significant ($P<0.1/n=9$). The two-cytokine combination (IL-6 + SCF) was insufficient for CFU generation (almost complete absence, $P<0.0005/n=9$) and the addition of s-IL-6R did not ameliorate this (sustained reduction with $P<0.0005/n=9$).

Discussion

From our results, it appears that the synergy of the c-kit and sIL6R/IL-6 complex for ex vivo expansion (in

terms of cell and CFU generation) of primitive adult BM CD34+CD38− haematopoietic progenitor cells is less pronounced than that reported for expansion of umbilical CB CD34+ cells.

Sui et al. [19] initially observed a 61-fold expansion of CFU (final overall cloning efficiency 20.9) during a 14-day suspension culture of CD34+ CB cells stimulated by sIL-6R+IL-6+SCF. The same group has extended these observations by describing that these stimulatory effects especially occur within the CD34+/IL-6R− CB subset (which should be enriched in more primitive cells, although this subset still comprises 50–70% of the CB CD34+ cells) [23]. They also described the fact that the same combination exerted a specific stimulatory effect on the growth of both epo-independent erythroid colonies [20] and megakaryocytic colonies [21]. In comparison with these results, which were due to combined gp130 and c-kit signalling, combined signalling through gp130 and Flt3 resulted in a similar but weaker effect: 5-fold expansion (both in cell numbers as in CFU number) after 14 days of suspension culture [2].

Little data from other groups are available and most conduct their research on an ontogenetically different source of cells without enrichment for primitive progenitors. For example, Fischer et al. [3] followed the effect of sIL-6R+IL-6 or their designer fusion protein on mobilized peripheral blood CD34+-enriched cells, whereas Kimura et al. [8] investigated the effect on IL-6R+ and IL-6R− subsets of CD34+-mobilized peripheral blood cells. It is interesting to note that neither group found substantial expansion by gp130/c-kit stimulation but only by multifactor (i.e. sIL-6R+IL-6+SCF+IL-3) stimulation. This indicates that the CB CD34+ compartment may be more susceptible to the action of the sIL-6R/IL-6 complex than ontogenetically different haematopoietic compartments, such as mobilized adult peripheral blood CD34+ cells or, as in our case, adult BM cells.

Moreover, the use of only CD34+-enriched cells may be insufficient to unmask the effects of the sIL-6R/IL-6 complex on primitive progenitors compared with those on the more committed progenitor cells (the majority of the CD34+ compartment). Our data clearly demonstrate that the most primitive compartment (i.e. CD34+CD38− in adult BM) needs other factors besides sIL6-R/IL-6 and SCF for significant cell and CFU generation to occur (i.e. at least the addition of IL-3) and that the addition of sIL-6R results in only a modest and non-statistically significant enhancement. This is in agreement with the results obtained by Zandstra et al. [27], who found that sIL-6R+IL-6 (in addition to Flt-3 Ligand (FL), SCF and IL-3) were not necessary for the enhanced expansion of LTC-IC out of a CD34+CD38− adult BM starting population and only exhibited synergy on CFU expansion. In contrast, their results for CB CD34+CD38– cells as well as the results of Kollet et al. [11], on the same starting population, showed a more pronounced synergistic effect of the sIL-6R/IL-6 combination (again in addition to multifactor stimulation) on the expansion of the earliest type of CB progenitors (SCID mouse repopulating cell, long-term culture-initiating cell, pre-CFU).

In conclusion, our results indicate that the combination of sIL-6R/IL-6/SCF is not effective in generating CFU or even in sustaining CFU starting from a primitive adult BM CD34+CD38− haematopoietic population: a *multifactor* [15, 18, 26] combination remains a prerequisite for adult BM CD34+CD38− stimulation.

These results warrant further evaluation of the sIL-6R/IL-6 complex for its potential use in haematopoietic stem cell expansion: the ontogenetic source and the (im)maturity of the starting populations should always be taken into account. The same immunophenotype (for instance CD34+CD38−) may differ in terms of maturity and important functional differences and cytokine requirements exist between early haematopoietic progenitor cells, depending on the ontogenetic age [24, 25, 27].

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