Megakaryocyte formation in vitro to expand and explore
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Citation for published version (APA):
Chapter 3

A combination of Megakaryocyte Growth and Development Factor and interleukin-1 is sufficient to culture large numbers of megakaryocytic progenitors and megakaryocytes for transfusion purposes.

British Journal of Haematology, 1999, 106:553-563
A combination of Megakaryocyte Growth and Development Factor and interleukin-1 is sufficient to culture large numbers of megakaryocytic progenitors and megakaryocytes for transfusion purposes.

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Abstract

Chemotherapy-induced thrombocytopenia is a major risk factor in cancer treatment. The transfusion of autologous ex vivo expanded megakaryocytes could be a new therapy to shorten the period of thrombocytopenia. Therefore, we investigated in a liquid culture system the effect of various cytokine combinations composed of pegylated megakaryocyte growth and development factor (PEG-rHuMGDF), interleukin-1 (IL-1), IL-3, IL-6, IL-11 and stem cell factor (SCF) on the proliferation and differentiation of CD34⁺ cells, in order to define the most optimal and minimal cytokine combination for megakaryocyte expansion.

Besides PEG-rHuMGDF, IL-1 was found to be important for optimal megakaryocyte expansion. Depletion of either SCF, IL-6 or IL-11 did not exert a large effect, but the absence of IL-1 strongly diminished the number of megakaryocytic cells. Addition of IL-3 to the combination PEG-rHuMGDF, IL-1, IL-6, IL-11 and SCF significantly reduced the number of megakaryocyte progenitors (CD34⁺CD41⁺ cells) and the number of CFU-Meg. Furthermore, we found a strong correlation between the number of CD34⁺CD41⁺ cells and the number of CFU-Meg obtained after eight days culture.

Our study shows that optimal ex vivo expansion of megakaryocytes is achieved by the combination of PEG-rHuMGDF and IL-1. The numbers of megakaryocytes and megakaryocyte progenitors (CD34⁺CD41⁺) obtained in our liquid culture system with the growth factor combination PEG-rHuMGDF and IL-1 are suitable for transfusion purposes.
Chapter 3

Introduction

Patients treated with intensive high-dose chemotherapy usually develop profound thrombocytopenia. Although hematopoietic recovery is hastened by stem cell transplantation, generally a considerable number of platelet transfusions are needed. However, recurrent platelet transfusions carry the risk of alloantibody formation and subsequent refractoriness to platelet transfusions. New potential therapies to shorten the period of thrombocytopenia would seem to be provided by recombinant thrombopoietin (Tpo), which was cloned a few years ago [1-5]. Tpo has been shown to be the major regulator in the process of differentiation, proliferation and maturation of stem cells into megakaryocytes. Moreover, Tpo appears to be essential for optimal megakaryocyte expansion ex vivo [6-10]. To date, clinical trials have shown that administration of recombinant Tpo does not result in a satisfactory reduction in the duration of chemotherapy-induced thrombocytopenia [11-14].

In addition to the nowadays routinely used stem cell transplantation, reinfusion of ex vivo expanded autologous megakaryocyte progenitors may contribute to enhanced platelet recovery in patients with chemotherapy-induced thrombocytopenia. Peripheral blood stem cell transplants contain variable amounts of megakaryocyte-committed cells. In the past we and others have shown that the number of CD34⁺CD41⁺ cells or CFU-Meg in a stem cell transplant is positively related with the time of platelet recovery [15-18]. Bertolini et al. [19] reported that administration of autologous ex vivo expanded megakaryocyte progenitors in combination with autologous stem cell transplantation was well tolerated and that it may reduce the need of platelet transfusions if high enough numbers of megakaryocytic cells are reinfused. Therefore, it would seem worthwhile to investigate whether addition of megakaryocytic cells to a peripheral blood stem cell transplant will accelerate platelet recovery in vivo.

However, the cytokine cocktail and culture time necessary to expand ex vivo high numbers of megakaryocyte progenitors from CD34⁺ cells has not yet been defined. A broad range of cytokines, i.e. Tpo (pegylated Megakaryocyte Growth and Development Factor (PEGrHuMGDF), a truncated, pegylated mpl ligand related to Tpo), stem cell factor (SCF), interleukin 1 (IL-1), IL-3, IL-6, IL-11, erythropoietin, granulocyte macrophage-colony stimulating factor (GM-CSF), flt3-ligand, macrophage inflammatory protein-1α and G-CSF, have been tested, and different cytokine cocktails have been shown to be able to stimulate megakaryocytopoiesis in vitro [6-8,19-24]. However, inclusion of large numbers of cytokines in an ex vivo expansion protocol will be too expensive for routine clinical use. Moreover, it is difficult to obtain all cytokines at clinical grade. Therefore, we performed a detailed analysis of various cytokine combinations composed of
Megakaryocyte expansion with MGDF and IL-1

pegylated MGDF, IL-1, IL-3, IL-6, IL-11 and SCF to define the most optimal combination including as few cytokines as possible.

IL-6 and IL-11 both synergizes with other growth factors, like IL-3 and IL-1, in the induction of megakaryocyte differentiation, and they mainly have an effect in the late stages of megakaryocyte development [25-27]. IL-1 also has been found to enhance megakaryocyte proliferation [24,26]. IL-3 increases the absolute number of megakaryocytes in liquid culture [7,8,28] and expands the number of megakaryocyte colonies [21,22,29]. SCF has been shown to promote colony formation of hematopoietic stem cells in combination with other growth factors (IL-3, IL-6, G-CSF, GM-CSF) [30,31].

Our study shows that the cytokine combination of only PEG-rHuMGDF and IL-1 was sufficient to culture large amount of megakaryocytes and megakaryocytic progenitors. Furthermore, we found that the number of CFU-Meg after culture of CD34+ cells was correlated to the number of CD34+CD41+ cells. Addition of IL-3 to the culture had a negative effect on the number of CFU-Meg and CD34+CD41+ cells.

Material and Methods

Cells
Peripheral blood stem cells were obtained (with approval of the medical ethical committee and after informed consent) from leukapheresis material of patients (five with mamma carcinoma, one with Kahler disease, one with Hodgkin’s disease, one with non Hodgkin’s lymphoma, one with ovarium carcinoma) treated with chemotherapy and G-CSF (5-10 μg/kg/day subcutaneously; Filgastrim, Amgen, CA, USA). Two healthy donors for allogeneic transplantation were only treated with G-CSF (2 x 5 μg/kg/day).

Cytokines
Cytokines used in the experiments were: PEG-rHuMGDF (100 ng/ml; a generous gift from Amgen, Thousand Oaks, CA, USA), SCF (20 ng/ml; Peprotech, Rocky Hill, NJ, USA), IL-1 (IL-1β, 10 ng/ml; Peprotech), IL-3 (10 ng/ml; R&D, Abingdon, UK), IL-6 (25 ng/ml; CLB, Amsterdam, The Netherlands), IL-11 (25 ng/ml; Intergen company, Purchase, NY, USA).

Flow cytometry and monoclonal antibodies
After eight days of culture, the cells were harvested and immediately fixed with 1% (w/v) paraformaldehyde for 10 minutes on ice. The cells were spun for 10 minutes (180 g) with the brake on half maximum, then resuspended in PBS containing 0.2% bovine serum albumin (BSA) and used for FACS analysis. The
cells were incubated with fluorescein isothiocyanate (FITC)- or Phycoerythrin (PE)-labelled monoclonal antibodies for 30 minutes at 4°C. Isotype matched mouse IgG subtypes served as controls. After 30 minutes of incubation, the cells were washed in PBS/0.2% BSA. After washing, the cells were resuspended in an appropriate volume of PBS/0.2% BSA and analysed by FACScan (Becton and Dickinson (B&D), San Jose, CA, USA).

The following FITC-conjugated monoclonal antibodies (moabs) were used: IgG1 isotype control (CLB-203: CLB), CD13 (myeloid; CLB-mon-gran/2, Q20, CLB), CD15 (myeloid; CLB-gran/2,B4; CLB), CD19 (B cells; CLB-172; CLB), CD36 (erythroid/megacytic/megakaryocytoid; CLB-703; CLB), CD41 (megakaryocytic; CLB-48) and CD42b (megakaryocytic; CLB-704; CLB). PE-conjugated moabs that were used: IgG1 isotype control (X40; B&D), CD3 (T cells; SK7; B&D), CD11c (myelomonocytic; S-HCL-3; B&D), CD14 (monocytic; CLB-mon/1,8G3; CLB), CD33 (myeloid; P67.6; B&D), CD34 (stem cells; 581; Immunotech, Marseille, France), CD66 (granulocytic; CLB-139; CLB) and glycophorin A (erythroid; AME1; Dako, Glostrup, Denmark).

Cell purification and culture

Leukapheresis material was usually stored overnight at 4°C in PBS containing 2% (m/v) human serum albumin (HAS) (CLB) and 10% (m/v) trisodiumcitrate.

Mononuclear cells from leukapheresis material were isolated by density gradient centrifugation over Ficoll (1.077 g/cm³; Pharmacia Biotech, Uppsala, Sweden). CD34+ cells were isolated from mononuclear cells by magnetic cell sorting (VarioMACS system; Miltenyi Biotec, Gladbach, Germany) according to the manufacturer’s instruction. The purity was determined by FACS analysis.

CD34+ cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% heparinized human AB plasma from a healthy donor, 1 mM sodium pyruvate (Gibco, Paisley, Scotland), 1x MEM vitamins (Gibco), 1x MEM non-essential amino acids (Gibco), 0.2% human serum albumin (m/v) (CLB), 0.02 mg/ml L-asparaginase (Gibco), 0.01 mM monothioglycerol (Sigma, St.Louis, MO, USA), glutamine and penicillin/streptomycin [32].

Two x 10⁵ CD34+ cells/ml were seeded in a total volume of 1.5 ml in a 6-well plate (Costar, Cambridge, MA, USA). The cells were cultured for eight days at 37°C, 5% CO₂, with the indicated combinations of growth factors, without additional feeding of growth factors or medium. After eight days of culture, the cells were analysed for surface marker expression by FACS analysis. Viable cells were determined with trypan blue exclusion. The proliferation factor was determined as the absolute increase in cell number (absolute number of viable cells present at day eight of culture divided by the number of viable cells seeded at day 0).
CFU-Meg

CFU-Meg assays were performed at day zero and after eight days of culture with PB-derived CD34^ cells. CFU-Meg was performed with the Megacult-C kit (StemCell Technologies, Vancouver, BC, Canada) according to manufacturer's instructions. Briefly, cells were cultured in a collagen-based serum-free medium containing Tpo, IL-3 and IL-6. After 10 to 12 days of incubation at 37°C, 5% CO_2, cultures were dehydrated and fixed with methanol/aceton. Megakaryocytic colonies were stained with CD41 by means of an alkaline phosphatase detection system. The cells were counterstained with Evans Blue, causing the nuclei of cells to turn blue regardless of lineage. Positive colonies were scored according to size, i.e. small (3 -10 cells/colony), medium (11 - 40 cells/colony), large (> 40 cells/colony) and mixed (non-megakaryocytes and megakaryocytes within the same colony).

Absolute number of colonies were calculated by multiplying their frequency (per seeded cell) with the number of viable cells put in culture (at day 0) or the total number of viable grown cells after eight days of culture. Viability was determined by trypan blue exclusion.

Ploidy

Megakaryocyte ploidy was measured by flow cytometry [8]. The cultured cells were fixed with 1% paraformaldehyde and were subsequently labelled with CD41-FITC-conjugated moab. Thereafter, the cells were incubated for 1 hour at 4°C with propidium iodide (50 µg/ml) to stain the DNA, in a medium containing RNAse (100 µg/ml) and 0.1% (v/v) Tween 20.

Electron microscopy

CD34^ cells were cultured for eight days in the presence of PEG-rHuMGDF and IL-1. After eight days of culture, cells were fixed with 2.5% glutaraldehyde (v/v) in 0.1 M cacodylate buffer (pH 7.2) for one hour and postfixed in 1% (w/v) osmium tetroxide in the same buffer for 1 hour, block stained with uranyl acetate, dehydrated and embedded in LX-112. Thin sections were stained with uranyl acetate and lead citrate and examined with a CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Statistical analysis

Statistical analysis was performed using SPSS for windows, release 7.5 (SPSS inc.). Independent t-test was used to determine statistical differences. p < 0.05 was considered significant.
Results

Number of megakaryocytic cells obtained after culture of CD34+ cells

The purity of the isolated CD34+ cells was high, viz. 99.4% ± 0.9 (mean ± SD, n = 11). The CD34+ cells were cultured for eight days, because there was a strong increase in the number of megakaryocytic cells up to day eight of culture without the need to refresh the medium (data not shown).

After eight days culture of CD34+ cells we determined the proliferation factor (Table 1) and measured the expression of the platelet marker CD41 (glycoprotein IIb) by flow cytometry to analyse the number of megakaryocytic cells obtained. In Table 1, the percentage of CD41+ cells is depicted and figure 1 shows the absolute number of megakaryocytic (CD41+) cells obtained per seeded CD34+ cell. In all cultures supplemented with combinations of PEG-rHuMGDF, IL-1, IL-6, IL-11 and SCF the percentage of CD41+ cells was about 55%. Addition of IL-3, either to

<table>
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<th>CD34</th>
<th>CD34/CD41</th>
<th>CD41</th>
<th>CD15</th>
<th>CD14/CD36</th>
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<td>1-6-11-S</td>
<td>3.0 ± 1.5</td>
<td>30 ± 1.5</td>
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<td>16 ± 2.2</td>
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<td>6-11-S-M</td>
<td>4.7 ± 1.5</td>
<td>37 ± 3.9</td>
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<tr>
<td>6-11-M</td>
<td>2.7 ± 1.5</td>
<td>47 ± 2.6</td>
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<td>48 ± 9.2</td>
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<td>11-S-M</td>
<td>4.4 ± 2.5</td>
<td>38 ± 8.1</td>
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<td>3 ± 1.7</td>
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<tr>
<td>6-S-M</td>
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<td>40 ± 6.4</td>
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<td>4 ± 0.3</td>
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<td>46 ± 3.2</td>
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<td>38 ± 2.2</td>
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<td>5 ± 0.9</td>
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<tr>
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<td>18 ± 2.0</td>
<td>6 ± 0.8</td>
<td>34 ± 3.3</td>
<td>10 ± 1.8</td>
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Immunophenotyping of cultured CD34+ cells by flow cytometry analysis. CD34+ cells were cultured for eight days in the presence of the indicated cytokine combinations. After eight days of culture the proliferation factor (absolute increase in cell number) was determined and lineage specific mAbs were used to identify cells present in culture. The percentage positive cells is depicted. The percentage CD34+CD41+ are a subfraction of the CD41+ and CD34+ cells. The mean ± SEM of at least three independent experiments is given. 1 = IL-1, 3 = IL-3, 6 = IL-6, 11 = IL-11, S = SCF, M = PEG-rHuMGDF.
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Figure 1. Absolute number of CD41+ cells obtained per seeded CD34+ cell.

Absolute number of CD41+ cells obtained per seeded CD34+ cell in CD34+ cultures. CD34+ cells were cultured for eight days in the presence of the indicated cytokine combinations. The percentage of CD41+ cells was determined with flowcytometry. The mean of at least three independent experiments per cytokine combination + SEM is given. 1 = IL-1, 3 = IL-3, 6 = IL-6, 11 = IL-11, S = SCF, M = PEG-rHuMGDF.

PEG-rHuMGDF or to the mixture of IL-1, IL-6, IL-11, SCF and PEG-rHuMGDF decreased the percentage of CD41+ cells to approximately 33% (Table 1). In the absence of PEG-rHuMGDF only a few percent of the cells was found to express CD41.

With respect to the absolute numbers of CD41+ megakaryocytic cells, more differences were found. As shown in figure 1, in addition to PEG-rHuMGDF, the presence of IL-1 was needed to obtain high numbers of CD41+ cells. The addition of other growth factors, such as IL-6, IL-11 and SCF, to the combination of PEG-rHuMGDF and IL-1 did not yield significantly more megakaryocytes (Fig. 1). In the absence of IL-1, SCF had a positive effect on the number of CD41+ cells: the cytokine combination PEG-rHuMGDF, IL-6, IL-11 and SCF gave rise to twice the number of CD41+ cells compared with this combination lacking SCF (p < 0.05, Fig 1).
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Figure 2. Absolute number of CD34$^+$ cells obtained per seeded CD34$^+$ cell.

Absolute number of CD34$^+$ cells obtained per seeded CD34$^+$ cell in CD34$^+$ cultures. CD34$^+$ cells were cultured for eight days in the presence of the indicated cytokine combinations. The percentage of CD34$^+$ cells was determined with flow cytometry. The mean of at least three independent experiments per cytokine combination + SEM is given. 1 = IL-1, 3 = IL-3, 6 = IL-6, 11 = IL-11, S = SCF, M = PEG-rHuMGDF.

Presence of CD34$^+$ cells and CD34$^+$CD41$^+$ cells after culture of CD34$^+$ cells

Besides the screening for CD41 we tested for the expression of CD34 and determined the number of CD34$^+$CD41$^+$ cells (results shown in Table 1). The percentage of cells that remained CD34$^+$ after eight days of culture, was comparable (~ 40%) for all growth factor combinations composed of PEG-rHuMGDF, IL-1, IL-6, IL-11 and SCF; Table 1). In cultures with IL-3 added, only 20% of the cells still expressed CD34 after eight days of culture. Moreover, the absolute number of CD34$^+$ cells was lower in cultures with IL-3 added (Fig. 2).

The negative effect of IL-3 on megakaryocytic progenitor cells (CD34$^+$CD41$^+$ cells) was even more pronounced: Upon addition of IL-3 to the combination of PEG-rHuMGDF, IL-1, IL-6, IL-11 and SCF, the percentage of cells that coexpressed CD34 and CD41 declined significantly from 23% to 8% (p < 0.001) (Table 1). This was also reflected in the significant decrease in absolute numbers of CD34$^+$CD41$^+$ cells (p < 0.05) (Fig. 3).
Figure 3. Absolute number of CD34^+CD41^+ cells obtained per seeded CD34^+ cell. Absolute number of CD34^+CD41^+ cells obtained per seeded CD34^+ cell in CD34^+ cultures. CD34^+ cells were cultured for eight days in the presence of the indicated cytokine combinations. The percentage of CD34^+CD41^+ cells was determined with flow cytometry. The mean of at least three independent experiments per cytokine combination ± SEM is given. 1 = IL-1, 3 = IL-3, 6 = IL-6, 11 = IL-11, S = SCF, M = PEG-rHuMGDF.

Fig. 2 and 3 show that in combination with PEG-rHuMGDF, IL-1 was necessary to obtain and maintain high numbers of CD34^+CD41^+ cells and CD34^+ cells, respectively. The addition of IL-6, IL-11 or SCF to the combination of PEG-rHuMGDF and IL-1 did not result in a significant increase in CD34^+ or CD34^+CD41^+ cell numbers. With PEG-rHuMGDF and IL-3, significantly lower numbers of both CD34^+ cells and CD34^+CD41^+ cells were obtained compared with the combination of PEG-rHuMGDF and IL-1 (p < 0.05) (Fig. 2 and 3).

Clonogenic capacity of cultured CD34^+ cells

The number of CFU-GM in a stem cell transplant is related with the time of neutrophil recovery [33]. There is also some evidence that the number of CFU-Meg might be related with the time to platelet recovery [16-18]. Therefore, we determined in a CFU-Meg assay the clonogenic capacity of purified CD34^+ cells at day zero and of the cells obtained after eight days of culture in our liquid culture system.
CD34+ cells were analysed in a CFU-Meg colony assay at the day of purification and after eight days of liquid culture in the presence of the indicated cytokine combinations. The absolute number of CFU-Meg colonies obtained per initially in liquid culture seeded CD34+ cell is depicted. CFU-Meg colonies were scored according to size, i.e. small (3–10 cells/colony), medium (11–40 cells/colony), large (> 40 cells/colony) and mixed (non-megakaryocytes and megakaryocytes within the same colony). The mean ± SEM of at least three independent experiments is shown. 1 = IL-1, 3 = IL-3, 6 = IL-6, 11 = IL-11, S = SCF, M = PEG-rHuMGDF.

Between the various growth factor combinations composed of PEG-rHuMGDF, IL-1, IL-6, IL-11 and SCF no significant differences were found in the absolute number of CFU-Meg (Fig. 4). After eight days of culture with combinations of these growth factors, the absolute number of CFU-Meg expressed per initially in liquid culture seeded CD34+ cell was 11- to 15-fold increased. Cells cultured with PEG-rHuMGDF and IL-3 showed only a 6-fold increase in CFU-Meg number (p < 0.05) (Fig. 4).

The CFU-Meg colonies formed by the purified CD34+ cells were generally large colonies (> 40 cells/colony), therefore representing more immature CFU-Meg (Fig. 4). CFU-Meg found within the eight days-cultured cells were mostly small and medium sized (3 to 10 cells and 11–40 cells per colony respectively), indicating that the CD34+ cell committed to the megakaryocytic lineage differentiated during culture into more mature megakaryocytic progenitors. However, the number of CFU-Meg forming large colonies was also 1.5 to 2-fold increased in all cultures with IL-1 present (Fig. 4). Thus, during culture we observed a slight increase in the number of immature CFU-Meg and a large expansion of more mature CFU-Meg.
Correlation between the number of cultured CD34^+CD41^+ cells and CFU-Meg

In the population of cells obtained after culture with PEG-rHuMGDF and IL-3, a decrease in both the number of CD34^+CD41^+ cells and the number of CFU-Meg was observed (Fig. 2 and Fig. 4). These two parameters were indeed related, because we observed a highly significant correlation between the number of CD34^+CD41^+ cells and the number of CFU-Meg (r = 0.72; p < 0.0001) (Fig. 5).

Differentiation and ploidy of cultured megakaryocytes

The morphology of the cultured CD34^+ cells was determined by electron microscopy to establish whether the CD41^+ cells displayed morphological features of megakaryocytes. After eight days of culture, cells representing different stages of megakaryocyte maturation were found. Figure 6 shows two megakaryocytes with abundant granules (Fig. 6, large arrows) and a developed demarcation membrane system (Fig. 6, thin arrows).

Furthermore, the ploidy of CD41^+ cells obtained after eight days of culture was measured to evaluate the degree of polyploidisation of these cells. With all tested growth factor combinations 2N, 4N, 8N, 16N and some 32N CD41^+ cells were obtained (data not shown).

Phenotypic characterisation of the non-megakaryocytic cultured cells

To identify, apart from CD41^+ cells, the other cell types formed upon culturing CD34^+ cells in the presence of various combinations of PEG-rHuMGDF, IL-1, IL-3, IL-6, IL-11 and SCF, we determined by flow cytometry the expression of a panel of cell-lineage specific markers (Table 1). No expression of glycophorin A,
Figure 6. Immunelectron microscopy of megakaryocytes after eight days of culture.
CD34+ cells were cultured for eight days in the presence of PEG-rHuMGDF and IL-1. Megakaryocytes are shown with in a) a megakaryocyte at small magnification and in b) a part of another megakaryocyte at higher magnification. Demarcation membranes (thin arrows) and abundant granules (thick arrows) were present. Nucleus (n). Bars = 1000 nm.

CD3 or CD19 was detected, indicating that no erythroid or lymphoid cells expand from CD34+ cells under the culture conditions used. Other cell types that were present after eight days of culturing were myeloid cells (CD15+) and monocytes (CD14+CD36+) (Table 1).

Cultures containing IL-3 either in combination with PEG-rHuMGDF or with PEG-rHuMGDF, IL-1, IL-6, IL-11 and SCF resulted in a two-fold increase in the percentage of CD33+ cells and in a three- to four-fold increase in the percentage of CD11c+ cells (both markers for myeloid cells) as compared with cultures containing PEG-rHuMGDF, IL-1, IL-6, IL-11 and SCF (data not shown).

Discussion

In this report the effect of various growth factor combinations, composed of PEG-rHuMGDF and different combinations of IL-1, IL-3, IL-6, IL-11 and SCF on proliferation and differentiation into the megakaryocytic lineage of CD34+ cells is described. All cytokine combinations that included PEG-rHuMGDF induced formation of a high percentage of CD41+ cells carrying morphological features of megakaryocytes and showing polyploidisation. A combination of PEG-rHuMGDF with IL-1 was found to be sufficient to culture high numbers of megakaryocytic cells. Addition of IL-6, IL-11 or SCF to PEG-rHuMGDF and IL-1 did not result in significantly higher numbers of megakaryocytes and megakaryocytic progenitors. In accordance with previous reports [19,20], our culture conditions did not lead to
formation of erythroid or lymphoid cells. Besides megakaryocytes, megakaryocytic progenitors and CD34+/lineage marker negative cells (CD14−, CD15−, CD41+), some myeloid cells (CD15+) cells and monocytes (CD14+CD36+) were observed.

In cultures supplemented with IL-1, IL-6, IL-11 and SCF only modest proliferation and some megakaryocyte formation was observed. Addition of PEG-rHuMGDF to this combination resulted in a four-fold increase in proliferation and a thirty-two-fold increase in megakaryocyte number. In contrast, no proliferation but only megakaryocyte differentiation was observed in cultures in the presence of PEG-rHuMGDF alone (data not shown). Thus, as described by others, PEG-rHuMGDF must be combined with other growth factors to achieve proliferation of megakaryocytes [6-8,20,21,34-36]. In our culture system, IL-1 was found to induce highest proliferation in combination with PEG-rHuMGDF. Piacibello et al. [37] also described an effective two cytokine combination composed of Tpo and Flt3-ligand. With this combination cord blood progenitors cells could be maintained in liquid culture for more than six months.

Several lines of evidence suggest a role for IL-1 in platelet production. In vivo administration of IL-1β to humans, treated with chemotherapy [38-41] and to mice [42,43] resulted not only in an improved neutrophil recovery but also in thrombocytosis. In patients with rheumatoid arthritis with marked thrombocytosis, increased plasma IL-1β levels were found [44]. The effects of IL-1 on platelet production could be partly indirect by its stimulating role in formation of other cytokines. IL-1-induced production of IL-6 has been described both in humans [45,46] and in mice [42,43]. In our culture system IL-6 production was observed if cells were cultured with PEG-rHuMGDF and IL-1 (data not shown), this is in agreement with other papers in which IL-6 production by megakaryocytes was described [46,47]. However, in our culture system, presence of IL-6 in combination with PEG-rHuMGDF and IL-11 was not sufficient to culture the high numbers of megakaryocytic cells obtained with IL-1. Moreover, addition of a blocking IL-6 antibody during culture did not inhibit megakaryocyte formation (data not shown). Therefore induction of IL-6 production does not seem to explain all effects of IL-1.

Both IL-6 and IL-11 are cytokines affecting the late stages of megakaryocyte maturation, and play a role in polyploidization [25,27]. We did not observe an effect of these or other cytokines on the extent of polyploidization of the CD41+ cell population. However, CD34+ cells were cultured for only eight days, which might be too short to show an effect of IL-6 or IL-11.

IL-3 was found to have a large proliferative potential. Addition of IL-3 to PEG-rHuMGDF combined with IL-1, IL-6, IL-11 and SCF resulted in a 1.6-fold increase in cell numbers. However, the enhanced proliferation of the cells did not yield higher numbers of megakaryocytes, but myeloid cell formation was induced
instead. The absolute number of CD41⁺ cells was not reduced in the presence of IL-3; however, the number of megakaryocyte progenitors (CD34⁺CD41⁺ cells) and CFU-Meg was strongly diminished. The loss of CD34 expression on CD34⁺ cells grown in the presence of IL-3 was also described by Dolzhanskiy et al. [29] and by Koizumo et al., who reported loss of CD34 expression after eight days culture of CD34⁺ cells in the presence of Tpo, IL-3 and SCF [48].

We obtained a 12.5-fold increase in CFU-Meg numbers after eight days of liquid culture in the presence of PEG-rHuMGDF and IL-1. This is comparable to the increase in CFU-Meg numbers that Bertolini et al. [19] and Ratajczak et al. [49] described after seven days of liquid culture in serum-free medium. Furthermore, we observed a strong correlation between the number of CFU-Meg and CD34⁺CD41⁺ cells; 1.8 CD34⁺CD41⁺ cells correlates with the presence of one CFU-Meg. In line with our finding was the observation of Feng et al. [16] who described a correlation between the number of CFU-Meg and the number of CD34⁺CD41⁺ cells in peripheral blood stem cell transplants. Also a correlation of the number of CFU-Meg [16-18] or the number of CD34⁺CD41⁺ cells [15,16] in a stem cell transplant with the time to platelet recovery has been described. Therefore the number of CD34⁺CD41⁺ cells reflects the number of CFU-Meg in a stem cell transplant or ex vivo expansion product and may predict the potential of the graft in support of platelet production. Furthermore, this finding supports the conclusion that flow cytometry is an easy and reliable test for the determination of the quality of the stem cell graft with regard to the platelet recovery capacity, as we have described before [15].

The procedure of megakaryocyte expansion may be added to the autologous stem cell transplantation protocol as part of high-dose chemotherapy regimes. Bertolini et al. have already administered ex vivo expanded autologous cells, containing a variable number of megakaryocytic cells (CD61⁺) cells, together with unmanipulated PBPC to ten patients treated with chemotherapy [19]. Subsequently, eight patients needed a single allogeneic platelet transfusion while two patients receiving 20 and 5.2 x 10⁵ CD61⁺ cells/kg bodyweight respectively, did not require any platelet transfusion, compared to a mean platelet transfusion need of 1.2 in historic controls [19]. In our culture system with PEG-rHuMGDF and IL-1, 3.6 x 10⁵ CD34⁺ cells/kg bodyweight would be sufficient to generate 20 x 10⁵ CD41⁺ cells/kg bodyweight.

Because of the described correlation between the number of CD34⁺CD41⁺ and CFU-Meg in a stem cell transplant with the time to platelet recovery, it is also important to consider the numbers of CD34⁺CD41⁺ and CFU-Meg obtained in our ex vivo expanded cells. Derecksen et al. [15] have shown that at a threshold of 3.4 x 10⁵ infused CD34⁺CD41⁺ cells/kg, rapid platelet recovery was observed. An input of 3.6 x 10⁵ PB CD34⁺ cells/kg bodyweight in our ex vivo expansion culture
Mesakmrocyte expansion with MGDF and IL-1 system will generate $9.2 \times 10^5$ CD34$^+$CD41$^+$ cells/kg bodyweight, which is approximately three times more than Dercksen et al. described as being the threshold for a rapid platelet recovery [15]. On average, $1 \times 10^5$ CFU-Meg/kg are present in a normal transplant [16-18]. Thus within our cultured cells five times more CFU-Meg will be present, which may implicate a more rapid platelet recovery.

On average, $60 \times 10^5$ CD34$^+$ cells per kg bodyweight are reinfused [15]. Thus, with only a very small fraction (6%) of the stem cell transplant a megakaryocyte transfusion product can be generated, that may be capable of restoring platelet levels.

From the data obtained sofar we conclude that we have developed a culture system useful to expand *ex vivo* megakaryocytes from purified peripheral blood CD34$^+$ cells. A combination of PEG-rHuMGDF and IL-1 is sufficient to generate high numbers of megakaryocytic cells and megakaryocytic progenitors for transfusion purposes. Clinical trials have to prove whether extension of a peripheral blood stem cell transplant with *ex vivo* expanded autologous megakaryocyte cells can prevent the occurrence of severe thrombocytopenia after high-dose chemotherapy.

Acknowledgements

We thank Dr Ineke Slaper-Cortenbach and Prof Dr Dirk Roos for their critical reading of the manuscript. Furthermore, we acknowledge the people of the stem cell laboratory at the CLB for providing us with the leukapheresis samples.

References


