Cytokine Therapy With Gene-Transfected Cells: Single Injection of Irradiated Granulocyte-Macrophage Colony-Stimulating Factor–Transduced Cells Accelerates Hematopoietic Recovery After Cytotoxic Chemotherapy in Mice

By Felicia M. Rosenthal, Reinhard Früh, Reinhard Henschler, Hendrik Veelken, Peter Kulmburg, Andreas Mackensen, Bernd Gansbacher, Roland Mertelsmann, and Albrecht Lindemann

Development of cell-based delivery systems that can release therapeutic levels of hematopoietic growth factors into the systemic circulation would facilitate treatment of patients requiring cytokine therapy. In this study, we have investigated the potential of granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting, irradiated syngeneic murine cells to accelerate hematopoietic recovery after cytotoxic chemotherapy. As a model, CMS-5 fibrosarcoma cells, were transduced with a retroviral vector containing the murine GM-CSF cDNA. Transduced cells secreted 38 ng GM-CSF/10^6 cells in 24 hours. After irradiation, in vitro GM-CSF production initially increased up to fivefold and was measurable for about 2 weeks. One and 2 days after injection of irradiated, GM-CSF–secreting CMS-5 cells (N2/CMVM-

HEMATOPOIETIC GROWTH factors have been shown to accelerate bone marrow (BM) recovery after chemotherapy and radiation therapy in mice.1-7 In humans, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are widely used to shorten the phase of neutropenia after cytotoxic therapy and after BM transplantation.8-12 However, serum half-life of these cytokines is relatively short (eg, 35 minutes for GM-CSF after interperitoneal injection in mice) and continuous parenteral administration, eg, by the use of Alza miniosmotic pumps, or repetitive injections, has been used to maintain biologically effective serum concentrations.13-15 Methods to provide sustained cytokine serum levels for several days without the need for daily injections or surgical implantation of delivery devices would be desirable. Autologous or allogeneic cells, genetically modified to constitutively secrete the cytokine of interest, could fulfill these criteria. Several investigators have already described the successful introduction of genes, coding for enzymes like α1-antitrypsin or hormones like insulin, into murine fibroblasts or myoblasts, and the therapeutic application of these cells in animal deficiency models.16-24 Other groups have introduced cytokine genes into tumor cells to induce antitumor immune responses or into hematopoietic cells to study the controls modulating self-renewal and differentiation of hematopoietic cells.25-27

Neutropenia after cytotoxic therapy is only transient, and thus, requires cytokine substitution for a limited time period only. Tani et al28 have designed a subcutaneous diffusion chamber apparatus for cytokine-producing fibroblasts that allows regulation of cytokine secretion either by reimplantation of additional cells into the chamber or by removing cells through ethanol treatment of the chamber. Ideally, gene expression would be regulated at the transcriptional level, eg, by the use of inducible promoters. Here we propose the use of irradiated cytokine-gene–transduced cells that have lost the capability to proliferate in vivo, but have retained the ability to secrete biologically active levels of cytokines for several days to weeks. Moreover, irradiation would be a precaution when cells are injected in vivo, as malignant transformation of genetically engineered fibroblasts has been described after in vivo implantation in mice.28

In this report, we show acceleration of hematopoietic recovery after injection of irradiated GM-CSF–transduced murine fibrosarcoma cells in cyclophosphamide-treated mice.

MATERIAL AND METHODS

Retroviral vector design and conversion of vectors into viruses. Cloning of retroviral vectors N2/CMVM-CSF and DGAd/R/GM-CSF has been described previously.29 Retroviral vector constructs were converted to the corresponding virus by electroporating vector DNA into the helper-free, ecotropic packaging cell line (Sonderforschungsbereich 364). Address reprint requests to Felicia Rosenthal, MD, University Medical Center Freiburg, Department of Internal Medicine II, Hematology/Oncology, Hugstetter Strasse 35, 79106 Freiburg, Germany.

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infected cells were isolated by G418 selection, expanded to cell lines and used for further analysis.

Cytokine assay. Secretion of GM-CSF into supernatants by retrovirally infected tumor cells and GM-CSF serum levels were determined using an appropriate bioassay and confirmed by enzyme-linked immunosorbent assay (ELISA; Endogen, Boston, MA). Supernatant from semi confluent irradiated or unirradiated parental or cytokine-secreting CMS-5 cells was collected after 24 hours, and assayed for GM-CSF production after determining the number of viable cells. Irradiation was performed at room temperature with a 36C-irradiation source at a dose-rate of 3 Gy/min. Irradiated (35, 50, and 100 Gy) CMS-5 and N2/CVMG-CSF/CMS5 # 6 cells (10⁶) were plated in 25-mL tissue-culture flasks and the amount of GM-CSF in a 24-hour culture supernatant 1, 3, 6, 9, or 12 days after irradiation was determined. GM-CSF biologic activity was measured by testing the ability of GM-CSF-containing preparations to induce thymidine incorporation into DNA of GM-CSF-dependent murine 32DC13 cells as previously described.29

Treatment of mice. Female BALB/c mice were purchased from the Zentralnstitut fur Versuchstierzucht in Hannover, Germany and used at 9 to 11 weeks old. On day 0 and day 2, all mice received 150 mg/kg of cyclophosphamide interperitoneally. One group of mice did not receive further treatment, another group was injected subcutaneously with 100 ng of recombinant murine GM-CSF (mmGM-CSF; Boehringer Mannheim, Germany) twice daily on days 3 through 10. This dose of rmGM-CSF has previously been shown to accelerate hematopoietic recovery after chemotherapy in mice. The last group of mice was injected subcutaneously on day 3 with a total of 10³ irradiated N2/CVMG-CSF/CMS5 # 6 cells, split into two injection sites. From day 4, blood was drawn daily from lateral tail veins into EDTA-coated microvette tubes (Sarstedt, Heidelberg, Germany). After lysis of erythrocytes with Turk solution, absolute leukocyte counts were determined in a Neubauer Chamber. Differential counts were performed every other day. GM-CSF serum levels were determined on days 1 through 4 after injection of GM-CSF-producing cells.

Statistical analyses. Mean ± SE was calculated. Differences between groups were examined for statistical significance by a two-tailed, two-sample t-test. A P value ≤ .05 was considered to indicate statistical significance.

RESULTS

Infection of fibrosarcoma cells. The structures of the retroviral vectors used to transduce the murine fibrosarcoma cell line CMS-5 have been described previously.29 Virus-containing, cell-free supernatant of infected packaging cell clones was used to transduce CMS-5 cells. G418-resistant CMS-5 bulk-infected cells were screened for GM-CSF expression by analyzing GM-CSF release into the culture supernatant. Parental CMS-5 cells did not secrete any detectable GM-CSF in either assay. G418-resistant cytokine gene transduced cells differed in their ability to synthesize and secrete GM-CSF, depending on the retroviral vector construct and the virus-producer clone used to transduce target cells (Table 1). GM-CSF secretion of bulk-infected fibrosarcoma cells transduced with the GM-CSF vector ranged from 0 to 128 ng/10⁶ cells in 24 hours. GM-CSF production of cells was found to decrease after extended periods of culture without G418 selection. Bulk-infected N2/CVMG-CSF/CMS5 # 6 cells that were used for the in vivo studies described here, secreted 38 ng/10⁶ cells in 24 hours unless irradiated (see below).

GM-CSF secretion after irradiation of cytokine-gene-transduced fibrosarcoma cells. To prevent proliferation of cytokine producing cells in vivo, cells were irradiated before injection. To study how long cytokine secretion would continue after irradiation, N2/CVMG-CSF/CMS5 # 6 cells were irradiated with 35, 50, and 100 Gy, respectively, and plated at a density of 10⁶ cells/25-mL tissue-culture medium. GM-CSF levels in 24-hour culture supernatants were determined 1, 3, 6, 9, and 12 days after irradiation. Unirradiated N2/CVMG-CSF/CMS5 # 6 cells produced 38 ng/10⁶ cells in 24 hours (Fig 1, day -1 before irradiation). After irradiation, cytokine secretion initially increased threefold to fivefold depending on the dose of irradiation applied. GM-CSF levels decreased to levels similar to those observed before irradiation by day 12. As the number of viable cells decreased continuously, these data suggest that biologically active GM-CSF is also released by radiation-damaged cells for several days.

GM-CSF serum levels after injection of GM-CSF-secreting fibrosarcoma cells in BALB/c mice. To obtain information about the time course of GM-CSF serum concentrations, blood was drawn at different time points after subcutaneous injection of irradiated N2/CVMG-CSF/CMS5 # 6 cells or 3 and 10 hours after subcutaneous injection of rmGM-CSF. GM-CSF serum levels were determined in a bioassay and confirmed by ELISA. In Fig 2A, GM-CSF serum levels of mice injected subcutaneously with 100 ng rmGM-CSF are shown 3 and 10 hours after injection. Three hours after injection of rmGM-CSF, a serum level of 93 ± 11 pg/mL was measured, whereas at 10 hours, serum GM-CSF levels were below the limit of detection. Figure 2B shows GM-CSF serum levels 1 to 4 days after injection of 10³ irradiated N2/CVMG-CSF/CMS5 # 6 cells, which is 3 to 6 days after initiation of cytotoxic therapy. One and 2 days after injection of GM-CSF-secreting cells, GM-CSF serum levels of 405 ± 58 pg/mL and 183 ± 36 pg/mL were measured, respectively. On days 3 and 4, GM-CSF serum levels were 88 ± 24 pg/mL and 82 ± 26 pg/mL, respectively, comparable with the level detected 3 hours after subcutaneous injection of 100 ng rmGM-CSF. No GM-CSF was found in the serum of mice injected with the same number of parental, nontransduced CMS-5 cells.

Hematologic changes in cyclophosphamide-treated mice. After injection of GM-CSF-secreting fibrosarcoma cells or twice daily injections of recombinant GM-CSF. Nine- to 11-week-old female BALB/c mice were injected intraperito-
Fig 1. GM-CSF production by N2/CMVGM-CSF/CMS5 #6 CMS-5 cells and cell survival after irradiation. One million cells were irradiated on day 0 with 35 (A), 50 (B), or 100 Gy (C), respectively, and plated in 25-mL tissue-culture flasks. GM-CSF levels in 24-hour culture supernatants were determined by bioassay on days -1 (before irradiation) and days 1, 3, 6, 9, and 12 after irradiation and after supernatant collection cells were trypsinized and counted. Values are means ± SE of six (GM-CSF) or two (cell number) determinations.

Fig 2. GM-CSF serum levels in BALB/c mice after injection of GM-CSF-secreting CMS-5 cells, parental CMS-5 cells or recombinant murine GM-CSF. (A) GM-CSF serum levels 3 and 10 hours after subcutaneous injection of 100 ng rmGM-CSF. (B) GM-CSF serum levels 1, 2, 3, and 4 days after injection of 10^7 irradiated (35 Gy) N2/CMVGM-CSF/CMS5 #6 cells or untransduced CMS-5 cells. Values are means ± SE of four mice from two different experiments (days 1 and 2, and 10 hours) or 2 mice (days 3 and 4, and 3 hours). nd, not done; >, below limit of detection.

neally on days 0 and 2 with 150 mg/kg cyclophosphamide, the control group did not receive further treatment. One group of mice was injected subcutaneously with 10^7 irradiated N2/CMVGM-CSF/CMS5 #6 cells on day 3, another group was injected twice daily (d3-d10) subcutaneously with 100 ng of rmGM-CSF. Figure 3 shows hematologic changes in BALB/c mice after therapy. Baseline leukocyte counts in BALB/c mice were between 9,000 and 10,000/μL. Three days after initiation of cyclophosphamide therapy leukocyte counts dropped to nadir levels of 1,000 to 1,500/μL in all mice, persisting until day 6. Starting from day 7, a difference in absolute leukocyte counts between GM-CSF-treated mice and control mice injected with cyclophosphamide only was noted. On day 8, absolute white blood cell (WBC) counts
in mice injected subcutaneously twice daily with rmGM-CSF as well as in mice treated with a single injection of irradiated GM-CSF–secreting syngeneic cells were significantly higher as compared with control mice (cyclophosphamide v GM-CSF subcutaneously: \( P = 0.010 \), cyclophosphamide v N2/CMVGM-CSF/CMSS #6: \( P = 0.036 \)). Average WBC counts on day 7 were 4,380/μL in the cytokine treatment group compared with 3,005/μL in control mice. On day 8, WBC count of the cytokine treatment group had increased to 9,260/μL, and in controls to 6,340/μL. In mice treated with rmGM-CSF subcutaneously or with GM-CSF–secreting cells, leukocyte counts continued to increase until day 9 and returned toward baseline levels on day 10. After recovery of hematopoiesis (eg, after day 9) no significant difference in absolute leukocyte counts is expected.

Table 2 shows differential WBC counts of treatment groups as absolute values of leukocyte subpopulations on days 7 and 8 after initiation of treatment. Untreated BALB/c mice had a differential count of about 400 monocytes, 1,800 granulocytes and 7,100 lymphocytes. On day 3 through 5, ie, 1 to 3 days after cytotoxic therapy, blood smear examinations showed absolute neutropenia (<100/μL) in all treatment groups (data not shown). On day 7, mice treated with subcutaneous rmGM-CSF or GM-CSF–secreting cells showed almost twice the absolute leukocyte levels of control mice, accounted for by a higher proportion of myeloid cells. Baseline myeloid counts were reached in both GM-CSF–treated populations 7 days after the first cyclophosphamide injection or 4 days after initiation of cytokine treatment, respectively, whereas control mice had only reached approximately half-normal values at that time. No significant differences in absolute lymphocyte counts were noted between different treatment groups. On day 8, the difference in absolute myeloid cells between cyclophosphamide-only–treated mice and those with addition of hematopoietic growth factor treatment was even more pronounced (cyclophosphamide v GM-CSF subcutaneously: \( P = 0.019 \), cyclophosphamide v N2/CMVGM-CSF/CMSS #6: \( P = 0.044 \)).

**DISCUSSION**

The use of recombinant hematopoietic growth factors has reduced morbidity of cytotoxic chemotherapy in tumor pa-
tients by amelioration of neutropenia and its associated infectious complications.8-12 Currently, growth factors are applied by daily subcutaneous injections or continuous intravenous infusion. In this study, we show that a single injection of irradiated GM-CSF-transduced murine fibrosarcoma cells is as effective in accelerating hematopoietic recovery after cyclophosphamide chemotherapy as twice daily subcutaneous injections of recombinant GM-CSF.

Because long-term expression of GM-CSF is not desirable for the treatment of chemotherapy-induced neutropenia, non-proliferating cells with a finite life span should be used. Irradiation of transfected cells is one option and has the additional advantage of preventing outgrowth of possibly transformed cells in vivo.24 Here we show that after irradiation of GM-CSF gene-transduced cells with up to 100 Gy, cytokine production persisted for more than 12 days in vitro (Fig 1). After irradiation, GM-CSF secretion initially increased. This effect could possibly be caused by cytokine leakage from radiation-damaged cells or by radiation-induced activation of transcription factors.32,33 Irradiated cells were incapable of further proliferation in vitro, as shown by decreasing cell numbers over time. There was no evidence of tumor growth or other side effects in mice observed for up to 2 months after treatment (data not shown).

After injection of $10^7$ irradiated GM-CSF-secreting syngeneic cells, circulating GM-CSF was measurable for 4 days, reaching peak levels of $405 \pm 58$ pg/mL on the first day after injection and decreasing slowly thereafter (Fig 2). GM-CSF serum level 3 hours after subcutaneous injection of 100 ng rmGM-CSF was $93 \pm 11$ pg/mL, comparable with that found 3 and 4 days after injection of irradiated GM-CSF-secreting cells. Ten hours after subcutaneous injection, GM-CSF was no longer detectable in serum. These data show that after injection of cells that continuously secrete GM-CSF, sustained serum levels are achieved as opposed to only transient elevation found after subcutaneous injection.

Accelerated recovery of WBCs and total myeloid cells after twice daily subcutaneous GM-CSF treatment after cyclophosphamide therapy has been previously shown by Gamba-Vitalo et al.3 Here we confirm the effects of twice daily subcutaneous rmGM-CSF injections on WBC and myeloid recovery and show that injection of irradiated GM-CSF-secreting syngeneic cells is at least as efficacious (Fig 3 and Table 2). Although implanted devices as described by Tani et al29 or miniosmotic pumps are likely to be equally effective, both approaches require surgical interventions.14,15

With current technology, it is unlikely that cytokine-gene-transfected cells would replace administration of recombinant growth factors in transient, chemotherapy-induced neutropenia except in special circumstances when poor patient compliance or other factors make daily administration difficult or impossible. However, successful treatment of chronic neutropenic disorders like Kostmann's syndrome (congenital neutropenia) and cyclic or idiopathic neutropenias with genetically engineered cells or slow-release formulations of G-CSF might be possible in the near future.34,35 In this situation, the application of G-CSF gene-transfected unirradiated, non-proliferating autologous cells, possibly in conjunction with a suicide gene allowing interruption of cytokine secretion in case of detrimental effects or other clinical indications, appears to be a promising strategy, and is currently under investigation in our laboratory.22,23,36

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REFERENCES

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Table 2. Leukocyte Subpopulations

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>Cyclophosphamide</th>
<th>+ GM-CSF</th>
<th>+ GM/CMS5 #6</th>
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</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>Lymphocytes</td>
<td>7.100 ± 350</td>
<td>1.674 ± 102</td>
<td>1.869 ± 261</td>
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<tr>
<td>Day 8</td>
<td>Myeloid cells</td>
<td>2.200 ± 480</td>
<td>1.331 ± 415</td>
<td>2.771 ± 438</td>
</tr>
<tr>
<td>Day 8</td>
<td>Lymphocytes</td>
<td>7.100 ± 350</td>
<td>3.410 ± 316</td>
<td>4.078 ± 651</td>
</tr>
<tr>
<td>Day 8</td>
<td>Myeloid cells</td>
<td>2.200 ± 480</td>
<td>3.657 ± 163</td>
<td>5.304 ± 310</td>
</tr>
</tbody>
</table>

Results are shown for untreated control animals, and for days 7 and 8 after cyclophosphamide administration and subcutaneous injection of rmGM-CSF or irradiated GM-CSF-secreting syngeneic N2/CMVGM-CSF/CMS5 #6 cells (GM/CMS #6). Values are means ± SE of five mice from two different experiments. (P values for day 8, myeloid cells: cyclophosphamide v subcutaneous GM-CSF: P = .019, cyclophosphamide v N2/CMVGM-CSF/CMS5 #6: P = .044).


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