Transfer of the Tumor Necrosis Factor α Gene Into Hematopoietic Progenitor Cells as a Model for Site-Specific Cytokine Delivery After Marrow Transplantation

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Relapse of leukemia is the major cause of failure after autologous stem cell transplantation due to reinfusion of residual clonogenic cells and the absence of an immune-mediated graft-versus-leukemia effect. To provide an antileukemia effect, immune-activating cytokines have been given to patients after transplantation. Systemic administration of such cytokines early after transplantation is often accompanied by substantial side effects, and it is unknown whether sufficient concentrations reach the sites of residual disease in the marrow. As a method of site-directed immunotherapy provided early after stem cell transplantation, we have tested in a murine model whether (1) marrow can be retrovirally transduced with the tumor necrosis factor α (TNFα) gene, (2) local production of TNFα by marrow cells after transplantation can be achieved, and (3) adverse effects of TNFα occurred. Balb/c mice were treated with 5-fluorouracil and bone marrow (BM) was obtained 4 days later. Whole BM was transduced in the presence of interleukin-3 (IL-3), IL-6, and stem cell factor by coculture with the packaging cell line GP+E-86, producing the cDNA for TNFα. Irradiated (1,300 cGy) syngeneic recipient mice were given 10^6 transduced BM cells on day 0. Integration of the TNFα gene into the host genome was documented by Southern blotting in spleen and BM cells on days 7 and 12 and in BM on day 40 after marrow infusion, but was no longer found on day 90. Messenger RNA for TNFα was present on day 12, but could no longer be shown on day 40 or 90. Although no measurable (L929 bioassay) levels of TNFα were found in serum of mice who had received TNFα transduced marrow, the supernatant of 10^6 unstimulated BM cells obtained 12 days after marrow infusion was found to have 7 pg of TNFα compared with 1.8 pg in nontransduced marrow. Mice that had received TNFα transduced marrow showed no side effects suggestive of systemic TNFα release, and cellularity of the TNFα-transduced marrow was not different from control mice that had received unmanipulated marrow or cells transduced with the neomycin resistance gene only. The studies suggest that gene transfer of immune-activating cytokines into hematopoietic cells could be used as a means to achieve their temporary local production early after transplantation by cells located in the BM.

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by reverse-transcription polymerase chain reaction (RT-PCR) using RNA isolated from the murine fibrosarcoma Fsa-N (Dougherty ST, et al; manuscript in preparation). To generate the retroviral vector JmTNFa, the cDNA for TNFa was first inserted into the Xba I site of plasmid pTZ19Rtk-neo. A cassette containing both the TNFa gene and the Tk-promoted neomycin resistance gene was then isolated by Sma I/HindIII digestion and ligated into Hpa I/HindIII cut Jzen. The plasmid obtained was then introduced into the ecotropic packaging line GP+E-86 by calcium-phosphate precipitation and transfected cells selected using the neomycin analog G418 (0.5 mg/mL, active weight) (GIBCO-BRL, Grand Island, NY). Supernatants conditioned by the retroviral packaging line had active viral titers ranging between 2 x 10^4 and 3 x 10^5 colony-forming units (CFUs)/mL when assayed for their ability to generate G418-resistant NIH3T3 cells. A control virus designated Jzen-neo was also constructed by inserting a 954-bp Mlu II/HincII fragment containing the coding region of the neo^ gene from pMCNeo into the Hpa I site of Jzen. Supernatants of this construct had active viral titers of greater than 1 x 10^5 CFU/mL.

Transduction of murine BM with the TNFa gene. BM cells were isolated from normal male Balb/c mice (6 to 8 weeks old, Charles River, Montreal, Quebec, Canada) that had received 5-Fluorouracil (50 mg/kg body weight, intraperitoneally) (Sigma Chemical Co, St. Louis, MO) 4 days previously. Mice were kept in a standard animal facility and fed ad libitum in a nonprotective environment. BM was harvested by flushing the marrow cavities of the femurs and tibiae with α-minimal essential medium (αMEM) supplemented with 2 mmol/L glutamine, penicillin/streptomycin, and 2% fetal calf serum (FCS; catalog no. SLM-4100; StemCell Technologies Inc, Vancouver, BC, Canada). Marrow cells (2 x 10^6) without further separation were cocultured with the viral producer cell line (GP+E-86 cells) at 70% confluence in 100-mm tissue culture dishes (catalog no. 3025; Falcon, Cokesville, MD) in Dulbecco's modified medium supplemented with 10% newborn calf serum, 2 mmol/L glutamine and penicillin/streptomycin (catalog no. SLM-2000; StemCell Technologies) and then irradiated with 1,500 cGy. To increase transfection efficiency, recombinant murine steel factor (100 ng/mL) (provided by Dr Widmer [Immunex Corp, Seattle, WA]), IL-3 (20 ng/mL), IL-6 (15 ng/mL) (both cytokines provided by the Terry Fox Laboratory, Vancouver, BC), and 8 g/mL of poly-1-lysine were added to the cultures. After 36 hours of coculture, nonadherent cells were removed, washed twice with phosphate-buffered saline (PBS), and resuspended at 10^6 cells/0.5 mL for transplantation into irradiated recipients.

Transduction efficiency in the nonadherent cells was determined after plating 10^5 Jzen-neo or JmTNFa-infected viable cells in culture dishes (Greiner GmbH, Frickenhauen, Germany) containing Iscove's methylcellulose (catalog no. HCG-4100; StemCell Technologies) supplemented with 30% FCS, 10% M2-mercaptoethanol and 1 mmol/L L-glutamine supplemented with either or without 4G18 (neomycin sulfate) (Geneticin, GIBCO-BRL) at 1.2 mg/mL (active weight). This concentration of 4G18 completely suppressed growth of nontransfected hematopoietic cells. Cultures were incubated at 37°C in 5% CO₂ for 12 to 14 days and colonies (>50 cells) were counted under an inverted microscope (Nikon Canada Instruments Inc, Mississauga, Ontario, Canada). Transduction efficiency was calculated according to the formula:

% Efficiency = No. of Colonies Growing in the Presence of G418
No. of Colonies Growing in the Absence of G418

Transplantation protocol and documentation of engraftment. Recipient Balb/c mice were irradiated with 1,300 cGy of total body radiation given by a Cesium source overnight (1.45 cGy/min). This dose of irradiation has proven to be fatal to 100% of mice by day 15 if no marrow support is given. Syngeneic marrow cells (10^6 cells/mouse) were injected into the tail vein of recipient mice. On days 12 and 40 after transplant, recipient mice were killed by cervical dislocation and BM cells were harvested from all four limbs by flushing with PBS. Spleen cells on day 12 were obtained by stripping cells free from individual mouse spleens in αMEM supplemented with 10% FCS. Before counting nucleated cells, red blood cells were lysed with 3% acetic acid and remaining cells stained with trypan blue.

Southern blot analysis. Genomic DNA was isolated from spleen colonies on days 7 and 12 and BM on days 7, 12, 40, and 90 after marrow injection by enzymatic digestion followed by phenol/chloroform extraction and ethanol precipitation. Resuspended DNA was digested to completion with Xba I to excise the entire 3·75-kb TNFa cDNA. The digested DNA was separated in a 1% agarose gel, and the fragments were transferred to a nylon membrane and hybridized to a [32P]-labeled TNFa cDNA probe. Filters were imaged by autoradiography at −70°C using Kodak XR-5 film (Eastman-Kodak, Rochester, NY).

Northern blot analysis. For documentation of gene expression, total RNA was isolated from spleen and marrow cells using the acid guanidinium-isothiocyanate phenol-chloroform single-step extraction method as described previously. Samples prepared from spleen cells on day 12 and marrow cells on days 12, 40, and 90 were probed with the murine TNFa cDNA. A full-length murine β-actin cDNA probe was used as a control. Probes were oligolabeled with [32P] deoxycytidine triphosphate (dCTP), hybridized, and imaged by autoradiography as described above.

Determination of TNFa activity by L929 assay. Bioactivity of TNFa in serum and supernatants of cultured marrow cells was determined based on the lysis of the transformed mouse fibroblast cell line L929 (American Type Culture Collection, Rockville, MD) as described previously. Serum (1 mL) from mice that had received Jzen-neo or JmTNFa-transduced marrow was concentrated threefold using a Centricon 10 Microconcentrator (Amicon, Beverly, MA). BM cells (10^6) from the same mice were cultured in αMEM medium for 24 hours and the supernatant was also concentrated through a Centricon filter. For the L929 assay, target cells were incubated with the test samples in flat-bottom microtiter wells (Costar, Cambridge, MA). After 18 hours of incubation, 1,929 cells remaining intact were stained with crystal violet and dye uptake was measured using an enzyme-linked immunosorbent assay microplate reader (Microplate
A dose-response curve using a recombinant murine TNF-α standard (Genentech Inc., South San Francisco, CA) was included in each assay. TNF-α bioactivity was inversely related to the amount of staining, which represented viable nonlysed cells.

RESULTS

Transduction efficiency of transplanted marrow. Equivalent numbers of nontransfected, Jzen-neo or JmTNF-α-infected marrow cells were assayed for hematopoietic progenitor cell content in semisolid media in the presence of G418. Transduction efficiency was 30% for Jzen-neo and 10% for JmTNF-α. This difference in transduction efficiency was likely caused by the consistently lower viral titer for the JmTNF-α construct.

Integration and expression of the transduced TNF-α gene in spleen and BM cells. Several groups of recipient mice were injected with BM transduced with either the Jzen-neo or JmTNF-α retroviral vectors. On day 7, 12, 40, and 90 after transplantation, spleen and/or BM cells were isolated and the DNA analysed by Southern blotting. Both spleen and marrow cells obtained on day 12 after BMT showed the 0.75-kb DNA fragment that hybridized with a TNF-α specific DNA probe (Fig. 2). Data from day 7 were identical, but are not presented. The gene was also documented in BM cells on day 40 after transplantation, but was not found on day 90 (Fig 2).

These findings were correlated with the expression of the TNF-α gene as documented by Northern blotting. TNF-α mRNA was present in spleen cells on day 7 (result not shown) and day 12 (Fig 3). Despite the presence of the gene on day 40 (documented by Southern blotting), no expression of the gene in spleen or marrow cells could be documented at that timepoint.

Bioactivity of TNF-α in serum and marrow supernatant. To determine whether the expression of TNF-α mRNA on day 12 resulted in production of bioactive TNF-α production, 10⁶ marrow cells obtained from the femur and tibia of the mice were cultured for 24 hours in medium and the bioactivity of TNF-α in the supernatant measured using the L929 assay. Results were compared with those obtained with marrow cells obtained from mice that had received marrow transduced with the neo⁶ gene (Table 1). In addition, serum from recipients of transduced (neo⁶ or TNF-α) or nontransduced marrow was concentrated and TNF-α measured. No bioactive TNF-α could be detected in the serum of mice that were given the TNF-α transduced marrow. In contrast, their marrow cells produced an amount of TNF-α that was near that measured in the supernatant of the packaging cells and significantly higher than that found in control mice that had received Jzen-neo transduced marrow (Table 1).

Engraftment of Jzen-neo- and JmTNF-α-transduced marrow. Because TNF-α is believed to be myelosuppressive, the cellularity of spleen (obtained on day 12 after BMT) and BM (obtained on day 12 and 40 after BMT) was determined by counting the number of nucleated cells from the spleen and/or from all four limbs. Results suggest that the number of nucleated cells from the TNF-α-transduced BM early after BMT (day 12) was slightly lower than that from the Jzen-neo-transduced or control mice (Table 2). However, the difference was not statistically significant. By day 40 after BMT, marrow cellularity had increased in all mice compared with day 12, and no differences were found between the different treatment groups.

DISCUSSION

Immunotherapy is increasingly being considered as a non-cross-reactive treatment modality to prevent relapse, especially after autologous marrow transplantation, where no allogeneic GVL effect occurs. Because the function of cytotoxic cells recovers early after BMT, cytokines such as IL-2 and interferon α have been given to marrow recipients.
in an attempt to stimulate those cells toward antileukemic effects.\textsuperscript{25,26} The rationale for the experiments presented here is based on the observation that the systemic administration of such cytokines can cause a number of side effects; it is also uncertain whether high enough concentrations are obtained locally in the marrow where residual leukemia is located.

TNFα has not been given after BMT because of its known short half-life and significant toxicities when given systemically.\textsuperscript{27} On the other hand, even low concentrations of this cytokine have a potent antitumor effect. To overcome the drawbacks of systemic application, we exploited the possibility of using TNFα gene transfer into hematopoietic progenitor cells as a means to deliver this antileukemic cytokine locally to the marrow.\textsuperscript{16} Hematopoietic progenitor cells were chosen as target cells as they home to the marrow cavity after myeloablative treatment. The entire marrow harvest was cocultured with the retroviral vector-producing packaging cell line and then injected into irradiated syngeneic recipients. Stable integration and expression of the TNFα gene in BM and spleen cells for at least 2 weeks after marrow infusion was documented. At 6 weeks posttransplant, marrow cells still had the TNFα gene present, but no expression of the gene could be detected by Northern blotting at that time. The gene was below detectable level on day 90 after BMT. Using a bioassay, we could not detect any TNFα in the serum of mice that had received TNFα-transduced marrow cells. Conversely, significant TNFα activity was found in the supernatant of TNFα-transduced BM cells compared with cells transduced with the neo\textsuperscript{8} gene.

Although unproven at this point, it is hoped that these locally produced TNFα concentrations could be sufficient to have an antileukemic effect, either directly or through activation of secondary effector cells. The temporary expression of the TNFα gene confined to the time early after BMT may be desirable for the clinical situation, because immunotherapy should be delivered early after stem cell transplantation when only minimal disease is believed to be present. Hence, no attempt was made to achieve gene expression in early stem cells or devise strategies to maintain its expression. It is conceivable that gene integration and expression occurs only temporarily because of the lack of any selective pressure for maintaining this gene.

Before proceeding to any clinical studies, it was important to document in a murine model that marrow recovery after BMT is not delayed, and TNFα, produced locally, does not induce unwanted effects (such as wasting with weight loss and fever) in recipients.\textsuperscript{27} These side effects seem to occur only at higher serum concentrations of TNFα that are not achieved with this approach. A marrow-suppressive effect has been reported in some\textsuperscript{28,29} but not all studies.\textsuperscript{30} We have previously observed that TNFα has no negative effect on human long-term–culture initiating cells, which represent a cell population at the early stage of hematopoiesis (Gong and Klingemann, unpublished observation, February 1994). Hence, it was not unexpected to see maintained hematopoietic function in murine recipients of TNFα-transduced marrow.

Autologous hematopoietic progenitor cells could be useful vehicles for cytokine genes whose products can mediate anti-
leukemic effects, but which would cause side effects if given systemically.\textsuperscript{31} In addition to being an adjunct treatment modality to arrest or eliminate residual disease after stem cell grafting for leukemia, this approach might also be useful for other malignant diseases in which minimal residual disease may persist after stem cell transplantation.

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**REFERENCES**

Transfer of the tumor necrosis factor alpha gene into hematopoietic progenitor cells as a model for site-specific cytokine delivery after marrow transplantation

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