Induction of G250-targeted and T-Cell-mediated Antitumor Activity against Renal Cell Carcinoma Using a Chimeric Fusion Protein Consisting of G250 and Granulocyte/Monocyte-Colony Stimulating Factor

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ABSTRACT

Immunotherapy targeting for the induction of a T-cell-mediated antitumor response in patients with renal cell carcinoma (RCC) appears to hold significant promise. Here we describe a novel RCC vaccine strategy that allows for the concomitant delivery of dual immune activators: G250, a widely expressed RCC associated antigen; and granulocyte/macrophage-colony stimulating factor (GM-CSF), an immunomodulatory factor for antigen-presenting cells. The G250-GM-CSF fusion gene was constructed and expressed in S9 cells using a baculovirus expression vector system. The M, 66,000 fusion protein (FP) was subsequently purified through a 6xHis-Ni2+ affinity column and SP Sepharose/fast protein liquid chromatography. The purified FP retains GM-CSF bioactivity, which is comparable, on a molar basis, to that of recombinant GM-CSF when tested in a GM-CSF-dependent cell line. When combined with interleukin 2 (IL-2; 100 units/ml), FP (0.34 μg/ml) induces differentiation of monocytes (CD14+) into dendritic cells (DCs) expressing surface markers characteristic for antigen-presenting cells. Up-regulation of mature DCs (CD83+CD19−; 17% versus 6%) with enhanced expression of HLA class I and class II antigens was detected in FP-cultured DCs as compared with DCs cultured with recombinant GM-CSF. Treatment of peripheral blood mononuclear cells (PBMCs) with FP alone (2.7 μg/10^6 cells) augments both T-cell helper 1 (Th1) and Th2 cytokine mRNA expression (IL-2, IL-4, GM-CSF, IFN-γ, and tumour necrosis factor-α). Comparison of various immune manipulation strategies in parallel, bulk PBMCs treated with FP (0.34 μg/ml) plus IL-2 (1000 units/ml) for 1 week and restimulated weekly with FP plus IL-2 (20 IU/ml) induced maximal growth expansion of active T cells expressing the T-cell receptor and specific anti-RCC cytoxicity, which could be blocked by the addition of anti-HLA class I, anti-CD3, or anti-CD8 antibodies. In one tested patient, an augmented cytoxicity against lymph node-derived RCC target was determined as compared with that against primary tumor targets, which corresponded to an 8-fold higher G250 mRNA expression in lymph node tumor as compared with primary tumor. The replacement of FP with recombinant GM-CSF as an immunostimulant completely abrogated the selection of RCC-specific killer cells in peripheral blood mononuclear cell cultures. All FP-modulated peripheral blood mononuclear cells cultures with antitumor activity showed an up-regulated CD3+CD4+ cell population. These results suggest that GM-CSF-G250 FP is a potent immunostimulant with the capacity for activating immunomodulatory DCs and inducing a T-helper cell-supported, G250-targeted, and CD8+-mediated antitumor response. These findings may have important implications for the use of GM-CSF-G250 FP as a tumor vaccine for the treatment of patients with advanced kidney cancer.

INTRODUCTION

Metastatic RCC2 poses a therapeutic challenge because of its resistance to conventional modes of therapy, such as chemotherapy and radiation (1). Advances in the treatment of metastatic RCC have evolved significantly in the last decade since the Food and Drug Administration’s approval of IL-2 in 1992. It has become clear that immunotherapy is capable of producing durable remissions in selected RCC patients; yet, the overall response rates of immunotherapy remain 25% at best (2), at the cost of considerable toxicities to the patient. The recent identification of MHC-restricted TAs and the understanding of the critical role of immunomodulatory DCs have provided the rationale for the development of tumor vaccines for cancer therapy (3, 4). Many cancer vaccine strategies have been designed and tested in both animal models and clinical trials with encouraging results. These include peptide-based (5, 6), DC-based (7–11), recombinant viruses/DNA/RNA-based (12–14), and gene modified tumor cell-based (15–17) vaccines. Despite the fact that RCC is thought to be a relatively immunogenic tumor, no RCC-associated antigens have been identified and characterized in association with a significant rationale for the development of a kidney cancer-targeted tumor vaccine (18–20).

The first widely expressed RCC TAA that contains HLA-A2-restricted CTL epitopes has been recently identified and cloned from a RCC cell line (21, 22). This RCC-associated transmembrane protein, designated as G250, has been proven to be identical to MN/CAIX, a cell adhesion molecule that was first identified in cervical cancer and that contains carbonic anhydrase activity (23, 24). Immunohistochemical staining with monoclonal antibody G250 revealed that >75% of primary and metastatic RCCs expressed G250, whereas little to no expression was detected in normal kidney (21). G250 expression is found in nearly all clear cell carcinomas of the kidney, the most common RCC variant, which provides further basis for the use of G250 as a significant immune target for anticancer immunotherapy. Antigen presentation is a crucial and initial step for vaccine-based immunotherapy to be achieved. We therefore hypothesized that a chimeric protein consisting of G250 and GM-CSF, an immunomodulatory factor for the generation of functional DCs, would augment vaccine potency as compared with the use of either agent alone. Several chimeric FPs containing GM-CSF have been reported and shown to exert a variety of complex biological effects dependent on their components (25–28). GM-CSF has been well characterized as a growth factor that induces the proliferation and maturation of myeloid progenitor cells (29). It enhances macrophage and granulocyte natural cytotoxicity against tumor cells (30). The function of GM-CSF as a
key factor for the differentiation of DCs further substantiates its use in immune-based vaccine therapy (31). Evidence for the immunoadjuvant impact of GM-CSF in vaccine-based immunotherapy has been demonstrated in animal models. Cutaneous immunization with tumor peptide at sites containing epidermal DCs newly recruited by pretreatment with DNA encoding GM-CSF elicited an antigen-specific T-cell response, whereas peptide immunization of control skin sites showed no immune response (32). Likewise, treatment of established tumor with a cellular vaccine consisting of GM-CSF gene-modified DCs hybridized with malignant melanoma cells showed superior therapeutic efficacy to the treatment with the same vaccine generated with nonmodified DCs (33). A Phase I trial further demonstrated that systemic injection of GM-CSF and IL-4 was capable of inducing tumor regression or stable disease response in patients with advanced RCC and prostate cancer (34). Similarly, vaccination of patients with irradiated autologous RCCs or melanoma cells engineered to secrete human GM-CSF also induced potent antitumor immunity (16, 17).

In this study, we describe our strategy to generate a FP consisting of G250 and GM-CSF. We tested its feasibility, bioactivity, and specificity as a nonviral, noncellular RCC tumor vaccine and define its role as an immunostimulant of a G250-targeted, antitumor response in PBMC cultures derived from patients with advanced kidney cancer.

MATERIALS AND METHODS

Cloning of GM-CSF-G250 Fusion Gene in pVL1393 Vector. Plasmid p91023/B-GM-CSF (kindly provided by Dr. Judith Gasson, UCLA School of Medicine) was digested with EcoRI and the 0.8-kb fragment containing the full-length GM-CSF cDNA was used to generate the 0.4-kb GM-CSF fragment, containing the functional epitope flanked by the EcoRI site on the 5’ side and NorI site on the 3’ side replacing the GM-CSF stop codon, by DNA PCR. The GM-CSF fragment from the PCR product was subcloned into the EcoRI and BglII sites of the polyhedrin gene locus-based baculovirus transfer vector pVL1393 (PharMingen, San Diego, CA). Similarly, pBMB2CMVG250 (kindly provided by Dr. Egbert Oosterwijk, University Hospital Nijmegen, Nijmegen, the Netherlands) was used to amplify the full-length G250 cDNA (1.6-kb) containing the NorI site, followed by a six-nucleotide linker coding for two arginines by PCR in the 5’-flanking region of the G250 after the removal of its start codon. The 3’-flanking region of G250 was designed to encode six histidines followed by the stop codon and BglII site. The G250 fragment was gel purified. Both the vector pVL1393 already containing the GM-CSF and the G250 PCR amplified fragments were cut out with NorI and BglII. The G250 fragment and the vector were ligated for 3 h at 16°C and later transformed and plated on LB plates. The colonies containing the correct plasmid were purified by cesium chloride buoyant ultracentrifugation. The plasmid was cut with a set of different restriction enzymes to verify the plasmids. The plasmid clones were further verified for histidine tag using an Amplicycle Sequencing kit (Perkin Elmer).

Generation and Purification of FP. The recombinant baculovirus containing the His-tagged GM-CSF-G250 fusion gene was generated by cotransfection of 0.5 μg of BaculoGold DNA (modified AcNPV baculovirus DNA; PharMingen) and 5 μg of pVL1393/GM-CSF-G250 in S9 cells (Spodoptera frugiperda). Viruses were further amplified at a low multiplicity of infection (<1) in adherent S9 insect cells, and the titers of the virus were determined by plaque assay. Expression of GM-CSF-G250 FP in S9 cells was determined by immunocytochemical analysis using anti-G250 monoclonal antibody (kindly provided by Dr. Egbert Oosterwijk), anti-GM-CSF antibody (Genzyme, Cambridge, MA), and irrelevant control antibody. S9 cells infected with pVL1392-XyIE recombinant virus (PharMingen) and uninfected S9 cells were used as negative controls for FP expression and analysis. The viruses used for protein production were isolated and amplified from a single plaque. Cell lysate was prepared from the S9 cells infected with viruses at a multiplicity of infection of 5 for 3 days with insect cell lysis buffer containing a protease inhibitor mixture (PharMingen). Filtered lysate (0.22 μm filter) was applied to a Ni2⁺-NTA agarose column with high affinity for 6xHis (Qiagen, Santa Clarita, CA). After extensive washing of the column (50 mM sodium phosphate, 300 mM NaCl, and 10% glycerol, pH 8.0), the FP was stepwise eluted in a column (50 mM sodium phosphate, 300 mM NaCl, and 10% glycerol, pH 6.0) at 4°C using increasing concentrations of imidazole from 0.1 M up to 0.5 M. Fractions were analyzed with Western blot using anti-GM-CSF antibody. The peak fractions were combined, dialyzed, and resubmitted to Ni2⁺-NTA agarose column. Fractions containing FP were pooled, dialyzed, and further applied to a FPLC column containing SP Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). FP was eluted with an increasing salt gradient from 50 mM to 1 M NaCl in buffer X (20 mM Tris, 1 mM EDTA, and 10% glycerol), and the fractions containing FP were pooled, dialyzed, and sterilized through 0.2 μm filter. The Coomassie blue and silver stains were used to analyze the purity of GM-CSF-G250 FP. The protein concentration was determined by Bradford DC Protein assay (Bio-Rad, Hercules, CA).

GM-CSF Bioassy. The biological activity of the GM-CSF component of the FP was determined by measuring the proliferation of GM-CSF-dependent TF-1 cells (35) in the presence of FP. TF-1 cells (2 × 10⁶ cells/well) seeded in triplicate in 96-well plates in culture medium (RPMI 1640 + 10% FBS) contained titrated concentrations of FP or the corresponding molar concentrations of recombinant human GM-CSF (rh-GM-CSF; Genetics Institute, Boston, MA). Cultures were incubated for 5 days, and [3H]thyminidine (0.1 μCi/well) was added 12 h before harvest. The incorporated [3H]thyminidine was measured by scintillation counting with a beta counter.

Phenotypic Analysis of DCs by FACS. The phenotype of DCs generated from both adherent and bulk PBMCs was determined by two-color immunofluorescence staining as described in our previous report (35). Both adherent PBMCs and nonfractionated bulk PBMCs were cultured with IL-4 (1000 units/ml; R&D Systems, Minneapolis, MN) plus either GM-CSF (800 units/ml; Genetics Institute, Boston, MA) or FP (0.34 μg/ml; equivalent to 800 units/ml GM-CSF) for 7 days, and the identity of the DCs was determined. Cell cultures (1 × 10⁶ cells) were resuspended in 50 μl of FACS buffer (PBS, 2% new born calf serum, and 0.1% sodium azide) and incubated with 10 μl of the appropriate FITC- or PE-labeled monoclonal antibodies for 30 min at 4°C. After staining, cells were washed twice with PBS and resuspended in 200 μl of FACS buffer plus 200 μl of 2% paraformaldehyde. Five to 10,000 events/sample were acquired on a Becton-Dickinson FACScan II flow cytometer that simultaneously acquires forward and side scatter, as well as FL1 (FITC) and FL2 (PE) data, and analyzed utilizing the CellQuest Software (Becton-Dickinson, San Jose, CA). Settings for all parameters were optimized at the initiation of the study and were maintained constant throughout all subsequent analyses.

The DC population in bulk PBMC culture was gated based on their size and granularity (36). The voltage on the photomultiplier tubes was decreased when the DC population was analyzed, because large cells tend to have greater autofluorescence. In all samples, the position of quadrant cursors was determined by setting them on samples stained with the appropriate isotype control antibody. The following antibodies were used for characterization of the DC phenotype: anti-CD86 (B7-2; PharMingen); anti-CD40 (Caltag, Burlingame, CA); anti-CD11c (Catkal Laboratories, San Francisco, CA); and isotype control IgG1/IgG2a (Beckton Dickinson). The CD83⁺ surface marker was used to delineate the maturation of DCs. To discriminate DCs (CD83⁺CD19⁻) from activated B cells (CD83⁺CD19⁺), dual color staining using CD19FITC and CD83PE (Immunotech, Marseille, France) was performed.

Semiquantitative RT-PCR Analysis of Cytokine Profile in PBMCs. Total RNA was extracted from PBMCs treated with FP (2.7 μg/10⁷ cells) for various time intervals up to 24 h at 37°C, using acid guanidiniothiocyanate-phenol-chloroform extraction. Reverse transcription of messenger RNA into cDNA was carried out by incubating titrated RNA with avian myeloblastosis virus reverse transcriptase, primer oligo(dT), deoxynucleotide triphosphate, and RNase inhibitor at 42°C for 1 h. One μl of each cDNA sample was amplified using PCR in a total volume of 25 μl (30 ng of 1²PS)-oligonucleotide, 100 ng of 3'-oligonucleotide primer, 2.5 μl of modified 10²X PCR buffer, 1.25 units of Taq polymerase, and autoclaved double distilled water to a volume of 25 μl). The PCR mixture was amplified for 25 cycles in a DNA Thermocycler (Perkin-Elmer, Norwalk, CT). Each cycle consisted of denaturation at 94°C for 1 min and annealing/extension at 65°C for 2 min. The 3²P-labeled PCR products were then visualized directly via acrylamide gel electrophoresis and autoradiography and then quantitated by excision of bands and subsequent scintillation counting. The signal intensity of each amplified
product was calibrated to its corresponding β-actin mRNA expression as an internal control for quantitation of expression levels. In addition, quantitative analysis was further elucidated by a serial dilution of mRNA (1:3, 1:10, 1:30, and 1:300) and compropilization of β-actin and GM-CSF mRNA. The sequences of the oligonucleotide primer pairs were as follows: β-actin: 5'-CTCCCTATCATGAACTGTTGAC, 3'-CCACACGGAATCTTGCTTC; GM-CSF: 5'-CCATGATGCGCCAGCACTAC, 3'-CTTGGTTCTAGGAGGAGCAGC; TFN-α: 5'-TCTCGAAACCCCGAGTGACAA, 3'-TACAGGCGCCAGATTACCTAC; IFN-γ: 5'-AGTAAATATACCTATTGATGCGTTTC; IL-4: 5'-GGAAATTAATAATTACAAGAATAATTCT, 3'-CGACCTCGAAACAGCAT; IL-2: 5'-GGAATTAATAATTACAAGAATAATTCT, 3'-GTTCTTCCT. 

Immunomodulation of PBMCs with FP. Fresh isolated PBMCs from patients with RCC expressing G250 were cultured in RPMI 1640 supplemented with 10% autologous serum. Various schedules of immunomodulatory protocols of PBMC cultures with FP were carried out as described in Table 1 and Fig. 5. The growth of PBMCs was determined by cell count, and the cytolytic activity of PBMCs was assayed for different targets in a prolonged 18-h 51Cr release assay to enhance the detection of T-cell activity (37). Five thousand 51Cr-labeled target cells/well were seeded in a 96-well microtitre plate (Costar, Cambridge, MA) and mixed with PBMCs (E:T ratios 40:1, 20:1, 10:1, 5:1, and 1:300) and coamplification of 18S actin and GM-CSF mRNA. The sequences of 18S actin and GM-CSF mRNA. The sequences of

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<th>Phenotype (%)</th>
<th>Precultured</th>
<th>IL-2</th>
<th>IL-2 + GM-CSF</th>
<th>IL-2 + IL-2 + FP*</th>
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<tr>
<td>CD56 CD3⁺</td>
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<td>13</td>
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<td>CD3 + TcR⁺</td>
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<td>60</td>
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*40 IU/ml IL-2; 1000 IU/ml IL-4; 0.34 µg/ml FP.

RESULTS

Generation of GM-CSF-G250 FP from Baculovirus-infected S9 Cells. Baculovirus expression technology and the 6xHis affinity purification system were used to generate GM-CSF-G250 FP as described in “Materials and Methods.” Evidence for successful gene cloning and generation of recombinant baculovirus was provided by immunohistochemical staining of infected S9 cells using anti-GM-CSF and anti-G250. Abundant G250 and GM-CSF protein expression were detected in S9 cells that were infected with GM-CSF-G250 recombinant baculovirus (Fig. 1A), whereas expression of neither GM-CSF nor G250 was detected in noninfected cells (Fig. 1A) or cells infected with pVL1392-XylE recombinant viruses (data not shown). Western blot analysis was used to evaluate the efficiency of the 6xHis affinity tag in FP for Ni²⁺-NTA agarose. The expected Mᵦ 66,000 band that was detected with anti-GM-CSF appeared in fractions numbered 5–25, with a peak concentration in fractions numbered 15–19 (Fig. 1B). The protein purity was further improved by a rerun of positive fractions through the Ni²⁺-NTA agarose column, and then the proteins were subjected to FPLC using SP Sepharose column. A major single Mᵦ 66,000 band was detected in SDS-PAGE analysis stained with Coomassie blue (Fig. 1C).

Purified GM-CSF-G250 FP Retained GM-CSF Bioactivity. To determine whether the bioactivity of the GM-CSF was retained in the purified FP, the FP was analyzed for its ability to support the proliferation of a GM-CSF-dependent cell line, TF-1. Serial dilutions of FP were performed to span the effective concentration range. The results from the [3H]thymidine incorporation assay demonstrated that the FP could support TF-1 cell growth in a biphasic dose-dependent manner (Fig. 2). When compared with recombinant GM-CSF (Fig. 2), comparable bioactivity was obtained with the FP and equivalent molar concentrations of recombinant GM-CSF in the range between 0 and 6.71 ng/ml (0–30.2 ng/ml FP). However, there was a >1 log difference in the specific activities/mol of GM-CSF and FP (favoring FP) when half-maximal activities were used for comparison. In the presence of FP concentrations >30.2 ng/ml, the growth of TF-1 exceeded recombinant GM-CSF-mediated growth by 1.3-fold.

Immunomodulatory Effect of FP on APCs in PBMC Culture. To study how the FP could affect the development of DCs, PBMCs derived from patients with RCC were cultured in the presence of FP (0.34 µg/ml) plus IL-4 (1000 units/ml) for 7 days and compared with that cultured in GM-CSF (800 units/ml) plus IL-4. FACS analysis revealed a high percentage of large granulocytes expressing B7-2⁺, CD40⁺, and HLA-DR⁺ in both conditions, whereas CD14⁺ cells were negligible (Fig. 3A). However, when compared with DCs that were cultured with recombinant cytokines, an enhanced expression of both HLA class I (mean relative linear fluorescence intensity, 4830 versus 3215) and HLA class II (6890 versus 6290) was detected in the FP-modulated DC cultures (Fig. 3B). Moreover, a 3-fold increase in the percentage of mature DCs (CD83⁺CD19⁻) was detected in FP-modulated DC cultures (Fig. 3C). This observation was consistent in several bulk PBMC cultures derived from three RCC patients and two healthy donors. A similar FP-mediated immunomodulatory profile was also obtained from the conventional adherent DC cultures treated with IL-4 and FP (data not shown). A lesser efficiency DC differentiation was observed when DCs were cultured in the presence of FP alone without IL-4; a mix of the CD14⁺ and CD14⁻ B7-2⁺ cell population was determined on day 7 (data not shown).

FP Induces Activation of Cytokine Genes in PBMCs. To identify whether the FP has a direct effect on the regulation of cytokine genes in PBMCs, freshly isolated PBMCs that were derived from RCC patients were treated with FP alone (2.7 µg/10⁷ cells) and compared with those that were treated with GM-CSF alone. The kinetics of cytokine gene activation was followed by analysis of multiple cytokine mRNA expression through the time course indicated in Fig. 4. Treatment of uncultured PBMCs with FP gradually enhanced GM-CSF, tumor necrosis factor-α, IFN-γ, IL-4, and IL-2 mRNA expression with the peak level at 24 h after treatment, except IL-4. Peak of IL-4 mRNA expression was detected at 6 h after the treatment (Fig. 4). A similar cytokine gene activation profile was also detected in cells that were treated with recombinant GM-CSF. How-
ever, the peak expression level was detected between 1 and 6 h of treatment.

**FP Induces T-Cell-mediated, G250-targeted Immune Response in PBMC Cultures.** Six immunomodulatory protocols with and without FP were tested and compared in PBMC cultures. These culture conditions included: (a) IL-2 alone (40 IU/ml); (b) IL-2 + GM-CSF (800 units/ml); (c) IL-2 + FP (0.34 µg/ml; restimulated weekly); (d) IL-2 + IL-4 (1000 units/ml) + FP for 1 week and then restimulated with FP + IL-2; (e) FP alone for 1 week and then restimulated with FP + IL-2; and (f) FP + IL-4 for 1 week and then restimulated with IL-2 + FP. As indicated in Fig. 5 (patient 1), among various immunomodulatory conditions tested, pretreatment of PBMCs with FP plus IL-4 for 1 week and subsequent restimulation with IL-2 (40 IU/ml) and FP weekly showed the highest growth expansion (6.0×; Fig. 5A). A similar enhanced growth profile with this particular condition was determined in three PBMC cultures derived from additional patients with RCC. Notably, an additive growth effect of GM-CSF with IL-2 was detected when compared with IL-2 alone (Fig. 5). GM-CSF alone, however, failed to propagate PBMCs in long-term culture. Patient 1 had RCC metastasis in a LN. Enhanced cytotoxicity against LN-derived tumor target was determined in all four FP-modulated PBMC cultures (three cycles of restimulation) when compared with the cytotoxicity against the primary tumor target (Fig. 5B). Remarkably, this enhanced killing activity corresponded with an 8-fold increase in G250 mRNA expression in the LN metastasis over the primary tumor, as determined by a semiquantitative

**Fig. 1. Expression and purification of GM-CSF-G250 FP.** A, immunohistochemical staining for G250 and GM-CSF expression with anti-G250 and anti-GM-CSF antibodies in Sf9 cells infected with and without fusion gene recombinant baculovirus. ×100. B, Western blot analysis of 6xHis-tagged GM-CSF-G250 FP eluted from the Ni²⁺-NTA affinity column using anti-GM-CSF antibody. L, loading; BT, break through; W, wash. C, Coomassie blue-stained SDS-PAGE of fusion protein eluted from Ni²⁺-NTA affinity column (Lane 1) and further purified with SP Sepharose/FPLC (Lanes 2 and 3; two batches of final products). MW, molecular weight.

**Fig. 2. Comparison of functional activity of recombinant GM-CSF and purified GM-CSF-G250 FP.** GM-CSF activity was measured using the GM-CSF-dependent human cell line, TF-1. The TF-1 cells (2 × 10⁴/well/ml) were cultured in the presence of a serially diluted amount of recombinant GM-CSF or purified GM-CSF-G250 FP as indicated. After a 5-day incubation, the cultures were pulsed with 0.1 µCi of tritiated thymidine for an additional 12 h. The cultures were then harvested, and the incorporated thymidine was measured by scintillation counting.
RT-PCR (Fig. 5D). By pretreating the LN tumor target cells with anti-HLA class I (77%) or, alternatively, by pretreating the effector cells with anti-CD3 (66%) or anti-CD8 (55%) prior to the cytotoxicity assay, RCC-targeted cytotoxicity was markedly reduced. On the other hand, anti-HLA class II (33%) or anti-CD4 (33%) treatment led only to a lesser inhibition of cytotoxicity (Fig. 5C). Although poor growth expansion (1.8×) was detected in the condition in which PBMCs were pretreated with FP alone for 1 week and then restimulated with IL-2 plus FP, the highest cytotoxicity against both primary and LN-derived RCC targets was detected when compared with other tested conditions (Fig. 5B). High killing activity against K562 was detected in most early cultures of FP-modulated PBMCs, which was noted to gradually decrease, whereas the killing activity against autologous RCC was maintained. In contrast, both non-FP-stimulated cultures, IL-2, and IL-2 plus GM-CSF showed a nonspecific killing activity with relatively high cytotoxicity against K562 (Fig. 5B).

To identify the phenotypic identity of FP-modulated PBMCs that possess antitumor activity, phenotypic analysis was performed on the
day when cytotoxicity was determined. A markedly increased T-cell population (70–94%) expressing T-cell receptor (72–96%) was detected in all FP-stimulated PBMC cultures, when compared with precultured (40%) or cytokine-cultured PBMCs (IL-2, 45%; IL-2/GM-CSF, 60%; Table 1). Notably, the T-cell population expressing the most IL-2 receptor (CD3/CD25; 86%) was found to occur after pretreatment with FP plus IL-4 followed by restimulation with IL-2 and FP. This also corresponded with the greatest T-cell growth expansion with this specific condition compared with all other tested immunomodulatory protocols (Fig. 5A). Correspondingly, those PBMCs pretreated with FP for 1 week demonstrated a minimal T-cell population expressing IL-2 receptor (19%) with the lowest growth expansion (Table 1 and Fig. 5A). In contrast, PBMCs cultured in the absence of FP (IL-2 ± GM-CSF) expressed a higher percentage of CD56+ (13%, 20%), which corresponded with a higher natural killer activity against K562 when compared with FP-modulated cultures (Fig. 5B).

Replacement of FP with GM-CSF Abrogates the Selection of RCC-targeted Cytotoxic T Cells. To confirm that the component of G250 in the FP is the determinant for the growth selection of CTLs against RCC, a cytotoxicity assay was performed with PBMCs that were cultured in the presence of GM-CSF and IL-4 for 1 week and then continuously restimulated with IL-2 and GM-CSF (800 units/ml). Minimal cytotoxicity against autologous RCC was determined in all tested PBMC cultures without FP stimulation, whereas the corresponding PBMC cultures stimulated with FP showed an MHC-restricted, T-cell-mediated cytotoxicity against autologous RCC (day 35; Fig. 6A) that expressed a high level of G250 (data not shown). The

Fig. 5. Growth and cytotoxicity profiles of patient-derived PBMCs stimulated with GM-CSF-G250 FP. A, growth expansion of PBMCs (patient 1) induced by various immunomodulatory strategies as indicated. Cell cultures were stimulated with FP on days 0, 6, 12, and 18. Culture medium was changed weekly but maintained in a constant volume. Cell counts were performed on day 20. Expansion fold was calculated by the division of final cell counts/ml with cell counts/ml seeded on day 0 (3 × 10⁵ cells/ml). Data, means of triplicate; bars, SD. This analysis is representative of four different PBMC cultures derived from four RCC patients showing a similar growth profile. B, cytotoxicity of PBMCs (patient 1) against autologous normal kidney cells, primary tumor cells, and LN-derived tumor cells. Cytotoxicity was determined by 18-h ⁵¹Cr-release assay on day 21. Killing activity was expressed as LU per 10⁶ effector cells. LU is defined as the number of effector cells capable of inducing 30% lysis. Spontaneous release for tumor target was <20% of the corresponding maximal release. Data, means of triplicate; bars, SD. C, inhibition of cytotoxicity against autologous LN tumor cells by antibodies specific to T-cell markers and HLA antigens. Tumor target cells or PBMCs were pretreated with the respective antibody as indicated prior to the cytotoxicity assay. Data, means of triplicate; bars, SD. D, semiquantitative RT-PCR analysis of G250 mRNA expression by normal kidney, primary tumor, and LN metastasis obtained from patient 1. Tu, tumor.

Fig. 6. FP-induced, G250-targeted, and MHC-restricted T-cell immunity. A–C, cytotoxicity of PBMCs against autologous and allogenic tumor targets as indicated. PBMC cultures were pretreated with IL-4 (1000 units/ml) and FP (0.34 μg/ml) or IL-4 and GM-CSF (800 units/ml) for 1 week and then restimulated with IL-2 (40 IU/ml) and FP or IL-2 and GM-CSF weekly. Cytotoxicity was determined by 18-h ⁵¹Cr-release assay on day 35. Cytotoxicity against autologous tumor target was measured in the presence of isotype control antibody or antibodies specific to HLA class I, HLA class II, CD3, CD4, or CD8. Data, means of triplicate; bars, SD. D, flow cytometric analysis of FP-modulated PBMC phenotypes that expressed antitumor activity (day 35). Bars, SD.
cytotoxic killing capacity against natural killer-sensitive (K562) cells was negligible (patient 2, 3.5 LU; patient 3, 2.5 LU; patient 4, 2.0 LU). A predominant CD3 + CD4 + cell population was detected in all three FP-modulated PBMC cultures (68, 74, and 66%) that expressed antitumor activity when compared with the CD3 + CD8 + cell population (25, 22, and 30%; Fig. 6B). Moreover, both Th1 and Th2 cytokine mRNA were detected in these FP-modulated PBMC cultures, which included GM-CSF, tumor necrosis factor-α, IFN-γ, IL-2, and IL-4 (data not shown).

DISCUSSION

RCC is responsive to immunotherapy. However, no documented immune-based treatment protocol is available to date that would effectively eradicate tumor lesions in the majority of patients. The means of immune strategy, the type of immune activators used, the given dose, the method of administration, and the pretreatment immune status of patients all could influence the ultimate immune response in cancer patients that are treated with immune-based therapy. Therefore, a critical issue for an effective cancer vaccine is the development of a potent adjuvant that can facilitate both induction and augmentation of an immune response with antitumor activity. To work toward this goal, we proposed a chimeric construct consisting of G250 and GM-CSF. The demonstration of G250 expression in Sf9 cells and GM-CSF bioactivity in the purified G250 and GM-CSF. The demonstration of G250 expression in Sf9 cells and GM-CSF bioactivity in the purified M, 66,000 protein band confirmed the efficacy of the gene construct and the effectiveness of the protein purification process used. When compared with recombinant GM-CSF, the enhanced effectiveness and biphasic behavior of FP in the stimulation of TF-1 cell growth may be attributable in part to the stabilizing effect of larger molecules that lead to a longer half-life, as well as to a reduction in the binding coefficient of the larger molecule to the GM-CSF receptor. This may also explain in part the delayed cytokine gene activation in FP-modulated PBMCs.

Antigen presentation by DCs is crucial not only for the induction of primary immune responses but is also important for the regulation of the type of T-cell-mediated immune response (38). We recently developed a nonfractionated bulk PBMC culture system for the study of the maturation and immunomodulatory function of CD14 + -derived DCs and the interaction between the DCs and cocultured lymphocytes (36). Using this system, antigen loading can be performed during the early culture period of PBMCs in the presence of GM-CSF and IL-4, when immature DC/monocytes can take up and process tumor antigen. We have demonstrated previously that DC-modulated, cocultured lymphocytes in bulk PBMC culture can be further expanded to CTLs by repetitive stimulation with low-dose IL-2 and RCC tumor lysate (36). Likewise, direct treatment of bulk PBMCs with IL-4 and FP not only induced the differentiation of CD14 + cells into DCs but also increased the maturation of DCs when compared with DCs generated in IL-4 and GM-CSF. This suggests that the signaling pathway in DC maturation can be induced by FP stimulation (39). Moreover, when compared with recombinant GM-CSF, up-regulation of HLA antigen expression was determined on FP-modulated DCs, indirectly suggesting that DCs are capable of internalizing, processing, and presenting FP determinants. Whether the G250 was taken up by APCs through the GM-CSF component/GM-CSF receptor internalization or via the G250 component or both remains to be determined.

It appears that precubation of PBMCs with FP and IL-4 prior to exposing IL-2 is favorable to achieve a better effector expansion. This may be explained if exogenous IL-4 could synergize the FP for the mobilization of DC differentiation and maturation and subsequently present the antigen peptides to the surrounding immune cells. Although a successful CTL selection also could be achieved by other tested immunomodulatory protocols with FP, the growth expansion of CTLs was not favorable. This may be partly associated with a “delayed” DC differentiation under the suboptimal concentration of IL-4 (FP can induce IL-4 secretion by PBMCs). High expression of IL-2R and T-cell receptor in FP-modulated immune cells indicates that they were highly activated and proliferative. In contrast, preexposure of IL-2 to nonantigen-stimulated PBMCs usually results in the expansion of nonspecific, lymphokine-activated killer cells with short-term killing activity (40). To rule out the possibility of other common TAA's participating in the RCC-targeted cytotoxicity, several other TAA's were screened, including RAGE, gp100, PRAME, and NY-ESO-1. Among the tested TAAs, G250 was the only common TAA that was expressed by all lysed RCC specimens in the study. The availability of purified G250 protein and G250 gene-transduced autologous normal cells would further verify the mechanism of FP-mediated, G250-targeted, and MHC-restricted cytolytic activity. Three of the “nonresponding” PBMC cultures were determined. One of them showed low activity of killing capacity against autologous RCC (day 35), whereas the other two showed a nonspecific killing activity against all tested tumor targets that included K562 and non-RCC tumor targets. Therefore, the optimal use of FP, in terms of dose for the activation of G250-targeted antitumor response remains to be further tested and characterized. The dose of FP used in this study was based on the molar concentration of GM-CSF in the FP as the optimal dose for the development of DCs we have tested. However, this has limited the ability to adjust doses of the G250. Recently, Huang et al. (41) demonstrated that even immunogenic tumors, such as those modified to express costimulatory molecules, fail to stimulate the immune system unless functional APCs are available to process and present the antigens. It thus appears that the most effective anti-cancer vaccine strategy should target the activation of T cells through the manipulation of APCs in patients.

The replacement of FP with an equivalent dose of recombinant GM-CSF abrogated the selection and propagation of RCC-specific CTLs, suggesting that activation and propagation of CTLs is antigen (G250) dependent and that the GM-CSF moiety of the FP has served as an effective adjuvant for antigen presentation, amplification of T-cell activity, and induction of cytokine response (42–44). Although FP-induced, G250-targeted antitumor activity is mainly mediated by CD8 + T cells, a predominant up-regulation of CD4 + T cells was detected in most cultures. The FP-mediated Th1 and Th2 cytokine release and enhancement of HLA class II expression in DC cells further suggests that FP-mediated antitumor immunity may involve priming of both CD4 + and CD8 + T cells specific for G250. In this response, CD4 + T helper cells may have a role in providing regulatory signals required for the priming of MHC class I-restricted CD8 + CTLs (15). Conversely, RCC-specific cytotoxic CD4 + cells have been reported (45). Therefore, partial blocking of cytotoxicity by anti-CD4 and anti-HLA class II may indicate that the CD4 cell population also participates in lysis of tumor cells.

Studies comparing the efficacy of various formulations of tumor vaccines in parallel demonstrated that the use of DCs transfected with DNA coding for TAAs is superior to peptide-pulsed DCs and naked DNA-based vaccine for eliciting both antigen-specific CD8 + and CD4 + T-cell responses (46). This observation indicates that computer-predicted peptides might not be naturally processed by DCs, because of their typical expression of immunoproteasomes that possess cleavage specificity for production of immunodominant peptides (47, 48). Thus, some in vitro peptide-reactive T cells could only lyse peptide-pulsed cell targets but not tumor cells expressing the entire tumor antigen (20, 49). Likewise, a peptide-based vaccine could effectively elicit expansion of vaccine-specific T cells in PBMCs of cancer patients, but such response does not necessarily correspond
a clinical tumor regression (50). Therefore, immunization with an alternative G250-targeted anti-cancer strategy, generation of DCs transduced with FP gene has been initiated. Using this strategy, we will be able to further investigate the immune mechanism manipulated by G250.

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Induction of G250-targeted and T-Cell-mediated Antitumor Activity against Renal Cell Carcinoma Using a Chimeric Fusion Protein Consisting of G250 and Granulocyte/Monocyte-Colony Stimulating Factor

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