Granulocyte-Macrophage Colony-Stimulating Factor mRNA Stabilization Enhances Transgenic Expression in Normal Cells and Tissues

By Lakshman E. Rajagopalan, Joseph K. Burkholder, Joel Turner, Jerilyn Culp, Ning-Sun Yang, and James S. Malter

To increase transgenic production of granulocyte-macrophage colony-stimulating factor (GM-CSF), we mutated the mRNA's 3'-untranslated region, AUUUA instability elements. Expression vectors containing human or murine GM-CSF cDNAs coding for wild-type (GM-AUUUA) or mutant versions with reiterated AUGUA repeats (GM-AUGUA) were transfected into cells in culture or animals using particle-mediated gene-transfer technology. Normal peripheral blood mononuclear cells accumulated 20-fold greater levels of GM-CSF mRNA and secreted comparably greater amounts of cytokine after transfection with hGM-AUGUA expression vectors versus hGM-AUUUA. hGM-AUGUA mRNA was fivefold more stable (t1/2 = 95 minutes) than hGM-AUUUA mRNA (t1/2 = 20 minutes), accounting for elevated steady-state levels. Transfection site extracts and serum samples obtained 24 hours after gene transfer of hGM-AUGUA cDNA into mouse skin contained greater than 32 ng/mL and 650 pg/mL of GM-CSF protein, respectively, compared with 0.33 ng/mL and less than 8 pg/mL for hGM-AUUUA cDNA. GM-CSF produced from mGM-AUGUA cDNA transfected into rat abdominal epidermis induced a profound neutrophil infiltrate. These data suggest a novel strategy for enhanced production of biologically active cytokines by normal cells after in vivo gene transfer.

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In recent years cytokines have shown considerable therapeutic potential. Single clinical grade recombinant cytokines or combinations have been systemically administered to patients with a variety of neoplasms. For example, interferon-α (IFN-α) therapy has improved the long-term survival of patients with hairy cell leukemia and may be synergistic with conventional chemotherapy for the treatment of multiple myeloma, non-Hodgkin's lymphoma, and colorectal cancers. Mechanistically, exogenous cytokines, particularly interleukin-2 (IL-2), augment the proliferation and antitumor activity of cytotoxic T lymphocytes, natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and tumor-infiltrating lymphocytes. Adoptive immunotherapy with IL-2 and LAK cells has been effective against malignant melanoma and renal cell carcinoma. However, this approach is time consuming and expensive and, thus, not yet in routine clinical use.

Typically, cytokines have been administered systemically by either bolus injection or continuous infusion. However, the inability to specifically target cytokines to tumor cells requires the delivery of massive systemic doses. As such, many patients experience significant side effects that often prevent adequate dosage. This has impeded the widespread use of tumor necrosis factor-α (TNF-α). Daily, peritumor injection of low to moderate doses of IL-2 or TNF-α was associated with significant tumor shrinkage without debilitating systemic side effects. However, the short serum half-life of cytokines (t1/2, 15 to 30 minutes) has encouraged alternative delivery systems including ex vivo transfer of cytokine cDNAs into tumor cells. In such models, cytokine genes have induced impressive, local and systemic antitumor immune activity without incapacitating side effects. The success of peritumor administration has critically depended on the local cytokine concentrations obtained after direct injection or gene transfer.

Irrespective of gene delivery system, cytokine cDNAs tend to be very poorly expressed after gene transfer. The use of powerful viral or endogenous gene promoters has not circumvented this problem, suggesting posttranscriptional regulatory mechanisms may be limiting cytokine mRNA accumulation and translation. The 3' untranslated region (UTR) of most cytokine mRNAs including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IFN-α, TNF-α and IFN-γ contain adenosine-uridine (AU)-rich elements (AREs) that target the mRNAs for rapid degradation and inhibit translation. The highly structured 5' UTR and suboptimal start codon context of cytokine mRNAs are poor initiators of translation, further impeding cytokine production. We hypothesized that targeted mutations within the 3' UTR regulatory elements could stabilize cytokine mRNAs derived from transgenes thereby increasing the availability of templates and enhancing protein production and secretion. We have tested this by mutating the AREs of GM-CSF mRNA. The datashow that after gene transfer of mutant cDNAs, the steady-state level of GM-CSF mRNA was substantially higher and the production of cytokine proportionately greater than seen with wild-type cDNAs. We further show that transgenic GM-CSF derived from mutant constructs was biologically active and able to elicit a profound inflammatory response. These observations suggest alternative strategies to increase cytokine production after gene transfer for the treatment of human disease.

MATERIALS AND METHODS

cDNA constructs. cDNA coding for a human GM-CSF was obtained from the American Type Culture Collection, Rockville, MD.

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Mutagenesis of the construct (Fig 1) was performed by overlap extension polymerase chain reaction (PCR).25 Briefly, oligonucleotides complimentary to opposite strands of the most 5' and 3' regions of GM-CSF were constructed. GM-1 (nt 1 to 19) and GM-4 (nt 748 to 734) contained ~50% GC residues (melting temperature \( T_m \) = 45°C to 55°C). Mutagenic primers were constructed containing complimentary sequences to GM-CSF immediately 5' or immediately 3' to the flanking 3' UTR AUUUA pentamers (designated GM-2 (nt 620 to 638) and GM-3 (nt 721 to 704)). At the 5' end of the mutagenic oligomers were 17 bases containing four ATGTA or ATGTG sequences (GM-AUGUA). Dashed lines denote deleted sequences in the mutant cDNAs.

Fig 1. Targeted mutagenesis of wild-type human and murine GM-CSF AREs. Using overlap extension PCR (see Materials and Methods), the AREs of wild-type human (nt 630 to 703) and murine (nt 789 to 889) GM-CSF (GM-AUUUA) were replaced with four tandem AUGUA sequences (GM-AUGUA).

**Human GM-CSF**

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<thead>
<tr>
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<th>Wild Type</th>
<th>Mutant</th>
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<tr>
<td>Primers 782</td>
<td>GTAATTTTCTACTGATAGGGACCATTATATTATTTATATTTTT</td>
<td>GATA</td>
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<tr>
<td>Primers 832</td>
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<td>TAAATTTTTATTATTATTATTATTATTATTATTATTATATTATTATTATTATTATTTTT</td>
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**Murine GM-CSF**

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<th>Wild Type</th>
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Cells and cell culture. Normal peripheral blood mononuclear cells (PBMCs) were obtained after Institutional Review Board (IRB) approval from healthy volunteer blood donors. Whole blood (200 to 500 mL) was diluted 1:1 with phosphate-buffered saline (PBS) and layered over Ficoll-Hypaque before centrifugation at 200g for 30 minutes at room temperature. The PBMCs were carefully removed, transferred to 30-mL conical tubes and washed twice with PBS. Cells were greater than 95% viable by trypan blue exclusion with yields in the range of 1 x 10^9 cells per 500 mL of whole blood starting material. Before transfection, cells were cultured overnight at 37°C and 5% CO₂ at a density of 5 x 10^6 cells/mL in RPMI 1640 containing 10% fetal calf serum (FCS).

**RESULTS**

Steady-state accumulation of wild-type and mutant GM-CSF mRNAs in resting PBMCs. Particle-mediated gene de-
livery has been shown to successfully transfect normal cells under various in vivo, ex vivo, and in vitro experimental conditions providing an alternative gene transfer method to retroviruses or liposomes. Therefore, we observed whether ficol purified, normal human PBMCs could be transfected in vitro. Typically, 4% to 6% of normal PBMCs were successfully transfected by a single discharge of gold particles loaded with 0.8 μg of a cDNA construct. PBMCs were transfected with either wild-type hGM-AUUUA or the modified hGM-AUGUA cDNAs subcloned in identical CMV-driven expression vectors. RNA was isolated at 2, 4, 8, and 12 hours posttransfection, Northern blotted, and probed using random-primed, human GM-CSF cDNA. This experiment allowed direct comparison of the accumulation of wild-type hGM-AUUUA or mutant hGM-AUGUA mRNAs in normal PBMCs. Examination of the ethidium bromide–stained gels showed that the 28S and 18S rRNAs remained intact and stable over the duration of the experiment (Fig 2). hGM-AUGUA mRNA increased steadily over 12 hours posttransfection (Fig 2), whereas the steady-state level of hGM-AUUUA mRNA peaked at 2 hours and decreased steadily thereafter (Fig 2). At 12 hours posttransfection, the steady-state level of hGM-AUGUA mRNA was greater than 20-fold more abundant than that of hGM-AUUUA mRNA (based on phosphor-imager analysis). GM-CSF–specific signals were detected only in PBMC transfected with the wild-type or mutant construct, but not with the vector control or naked gold beads (data not shown). Therefore, GM-CSF signals must have originated from the transgene. ELISAs for GM-CSF were performed on conditioned culture medium and cell lysates from identical numbers of cells at 24 hours after transfection. The hGM-AUGUA transfectants secreted 520 ± 12 pg of GM-CSF protein/mL/1 × 10⁶ cells compared with 26 ± 2 pg/mL/1 × 10⁶ cells for hGM-AUUUA transfectants and 18 ± 6 pg/mL/1 × 10⁶ cells for vector control transfectants. In addition, cell extracts from the hGM-AUGUA transfected cells contained an additional 220 ± 16 pg of GM-CSF protein/1 × 10⁶ cells. GM-CSF was not detectable in cell pellets from wild-type or vector transfected cells. The Biosource GM-CSF ELISA used for these determinations has a lower limit of detection of ≥10 pg/mL.

Half-lives of hGM-CSF mRNAs in resting PBMCs. Because transcription of wild-type and mutant hGM-CSF mRNAs were both under the control of identical CMV promoters, it was unlikely that the higher steady-state mRNA levels and increased protein production in hGM-AUGUA transfected PBMCs was caused by differences in the transcription rates. Because we had specifically mutated the AU-UUA instability elements, we expected hGM-AUGUA mRNA to show enhanced stability. Therefore, we determined the turnover rates of these two mRNAs after blocking transcription with actinomycin D. For this purpose, PBMCs were isolated from single donors and cultured overnight in RPMI 1640 with 10% FCS. Equal quantities (2.5 μg DNA/ mg gold) of each plasmid were precipitated onto gold beads and delivered into resting PBMCs (1 × 10⁶ cells/transfection) using particle-mediated gene transfer. Replicate cultures were pooled immediately after transfection to normalize differences between individual transfections. After 4 hours in culture, actinomycin D was added (5 μg/mL final) to block transcription. Equal numbers of transfected cells were removed at the indicated times for RNA isolation and Northern blotting. Based on absorbance at 260 nm, 2 μg of total RNA/time point was loaded in each lane to determine the half-life (t₁/₂) of hGM-AUGUA mRNA (Fig 3A). Because of its lower abundance, five times as much RNA (10 μg/time point) was Northern blotted to measure the t₁/₂ of wild-type hGM-AUUUA mRNA (Fig 3A). Ethidium bromide–stained 28S and 18S ribosomal bands were intact and stable over the duration of the experiment. GM-CSF mRNA signals normalized to GAPDH signals at each time point (based upon phosphor-imager analysis) were plotted versus time to provide a calculated half-life of 20 minutes for wild-type hGM-CSF (Fig 3B). To our knowledge, this is the first report of the decay rate of GM-CSF mRNA in primary cell cultures of normal PBMCs. In contrast, hGM-AUGUA mRNA decayed with a calculated half life of 95 minutes (Fig 3B). Therefore, the enhanced stability of mutant GM-CSF mRNAs in normal cells accounts for their accumulation.

In vivo synthesis of GM-CSF protein. Although the in vitro studies are suggestive, they do not show if our constructs will be differentially active in vivo. To address this question, we introduced cDNAs coding for hGM-AUUUA or hGM-AUGUA mRNAs into mouse skin by particle-mediated gene transfer. Human cDNA constructs were used so that we could employ a human GM-CSF–specific ELISA to measure protein made only from the transgene. Based on control studies, murine and human GM-CSFs show no cross-reactivity (data not shown). Mouse (Balb/C) abdominal skin was shaved, treated with a depilatory, washed, and transfected with gold particles coated with identical amounts of CMV-driven hGM-AUUUA, hGM-AUGUA, or control vector containing a luciferase reporter cDNA. Three mice...
were used per treatment to evaluate reproducibility. No untoward effects were observed in treated animals other than a slight rash at the target site. After 24 hours serum samples were taken from animals, and transfected skin was obtained and homogenized to obtain a total volume of 1 mL tissue extract. Both serum and skin samples were assayed by human GM-CSF-specific ELISA to determine expression levels from the various gene constructs. Skin extracts and serum samples obtained at 24 hours after hGM-AUGUA gene transfer contained a 100-fold excess of human GM-CSF immunoreactive material compared with identically prepared samples of skin and serum from hGM-AUUUA cDNA transfected mice (Table 1). Animals that received luciferase cDNA did not produce any detectable human GM-CSF.

Table 1. Transgenic GM-CSF Production in Skin Extracts and Serum Samples of Mice

<table>
<thead>
<tr>
<th>Transfected cDNA</th>
<th>Skin GM-CSF* (ng/mL)</th>
<th>Serum GM-CSF* (pg/mL)</th>
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<tr>
<td>hGM-AUUUA</td>
<td>0.33 ± 0.074</td>
<td>&lt;8</td>
</tr>
<tr>
<td>hGM-AUGUA</td>
<td>&gt;32</td>
<td>650 ± 37</td>
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* A human GM-CSF-specific ELISA was used to measure protein made only from the transgene.

These data show that the GM-CSF protein detected in these experiments originated from the transgene. Second, they show that mutant hGM-AUGUA cDNAs are extremely active in vivo at expression levels at least 100-fold greater than wild-type hGM-AUUUA cDNAs.

**GM-CSF produced from mutant constructs is biologically active.** To verify that transgenic GM-CSF protein produced from the mutant constructs was equivalent in biologic activity to that produced from unmodified cDNA, we introduced both gene constructs into adjacent regions of rat epidermis. Because of interspecies specificity, human GM-CSF does not invoke a biologic response in rats. However, murine GM-CSF is biologically active in rats, permitting visualization by local redness and immune cell recruitment. In addition, there is adequate contiguous surface area on rat abdomens to perform multiple, discrete transfections. The abdomens of several animals were shaved and treated with a depilatory before the introduction by particle-mediated gene transfer of gold particles loaded with CMV-driven expression vectors containing mGM-AUUUA, mGM-AUGUA, or β-galactosidase (control) cDNAs. These experiments were performed in similar sized animals. Twenty-four hours after gene delivery, the abdominal skins were examined grossly and punch biopsy samples were obtained from each target site. Formalin-fixed paraffin sections were stained with hematoxylin and eosin and examined microscopically (Fig 4).

Skin regions that received β-galactosidase cDNA showed minimal redness and were not raised (data not shown). Identical levels of redness occurred in control, shaved animals. The sites that received wild-type mGM-AUUUA cDNA were grossly indistinguishable from the control that received β-galactosidase cDNA. However, the sites where mutant mGM-AUGUA cDNA was delivered were markedly red and raised. These differences were consistently observed at all transfection sites and in the different experimental animals. The suggestion that substantial inflammation was present where mutant mGM-AUGUA was introduced was confirmed by histologic examination. As shown in Fig 4, inflammation was absent in tissues where control cDNA was introduced. A single small focus of inflammation was observed in the central region where wild-type mGM-AUUUA cDNA was delivered. Polymorphonuclear cells (PMNs) were the dominant inflammatory cell type present, but the bulk of the epithelium or dermis was devoid of immune cells. However, the total tissue site underlying the mGM-AUGUA cDNA transfection was infiltrated with PMNs that formed a layer of 20 to 40 cells deep, effectively separating the epidermis...
GM-CSF mRNA STABILIZATION

Fig 4. Transgenic GM-CSF recruits neutrophils to transfection site. Contiguous 3.2-cm² regions of rat abdomen were transfected with pCMVβgal (top), pCMVmGM-AUUUA (middle) or pCMVmGM-AUGUA (bottom) by particle-mediated gene transfer. After 24 hours, punch biopsy samples from each site were collected and fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (original magnifications × 40 [left] and 200 [right]).

From the underlying dermis. Infiltration into the dermis could also be appreciated. A substantial percentage of the PMNs had released their granules suggesting that the PMNs were activated. These data clearly show that GM-CSF produced from mutant transgenic constructs is indeed biologically active. Furthermore, they showed that enhanced cytokine production can profoundly increase local, immune cell recruitment.

DISCUSSION

Cytokines such as IL-4, GM-CSF, IFN-γ, and TNF-α effectively retard tumor growth in animal models when expressed by tumor cells after retroviral-mediated gene transfer. Typically, cytokine gene transfer has been performed ex vivo followed by the subsequent reimplantation of the modified malignant cells into the host. Retroviral vector-mediated gene transfer has been successfully used to achieve
constitutive expression of cytokines in explanted human melanomas and real carcinomas. These experiments showed that peritumor cytokine production recruited a range of host immune effector cells with antitumor activity. As the growth rate of cytokine-producing tumor cells was normal in severe combined immunodeficient mice, antitumor effects likely require an intact immune system. Occasionally, the local eradication of cytokine-modified tumor cells was accompanied by a systemic antitumor response capable of destroying unmodified parental cells at distant sites. Thus, local gene therapy with cytokines may be able to induce systemic antitumor immunity.

Unfortunately, ex vivo retroviral-mediated gene therapies are often costly and time consuming, requiring weeks for the selection of stably transduced tumor cells. In addition, not all tumors can be surgically explanted, cultured, and transduced ex vivo. Retroviral vectors are unable to infect nondividing cells or targets lacking specific cell-surface viral receptors. Insertional mutagenesis with activation of proto-oncogenes has been described. Cytokine production cannot be modulated and continues until all stably transduced tumor cells are destroyed or expression stops for generally unknown reasons. The sequential delivery of cytokines, which may be therapeutically advantageous, is technically very difficult.

The inadequacies of retroviral delivery systems have fueled attempts to develop alternative means of delivering cytokine genes in vivo to the vicinity of tumors. In this report, we have used a promising new technology that utilizes a high voltage discharge to deliver microscopic gold particles loaded with nucleic acids into normal, nondividing cells in culture or into animals in vivo, eliminating the need for ex vivo manipulations. Significant advantages of the gene gun include the ability (1) to physically target gene expression to the site of particle delivery; (2) to transfect nondividing cells irrespective of cell lineage; (3) to allow sequential delivery of different cytokines; and (4) to transiently produce transgenic proteins for short duration (days) rather than permanently. Previously we have shown that reiterative cycles of gene delivery can readily and safely be performed on transformed Therefore particle-mediated gene transfer permits the study of mRNA decay in normal nontransformed cells.

However, mRNA instability can be circumvented by at least one set of mutations. We disrupted the AUUUA boxes in the 3' UTR of GM-CSF mRNA by inserting guanosines in the third position (Fig 1). This change had previously been shown to prevent mutant GM-CSF mRNAs from binding to the AUUUA sequence-specific AU binding factor. Recently, fine mutagenesis of the ARE of c-fos mRNA showed that guanosine insertions at a comparable site as reported here prevented rapid decay. Therefore, it appears likely that RNAses that normally degrade AUUUA-containing cytokine or proto-oncogene mRNAs are also unable to recognize mutant versions.

As shown in Fig 2, mutant GM-CSF mRNA accumulated to 20-fold greater levels than wild-type GM-CSF mRNA. Because both mRNAs were transcribed from the same CMV promoter it was unlikely that the difference in steady-state levels was caused by a difference in the rate of transcription. However, the calculated half-life (t) of hGM-AUUUA mRNA was 20 minutes (Fig 3B), whereas hGM-AUGUA mRNA was significantly more stable with a half-life of 95 minutes. As decay is exponential, the fivefold greater stability of hGM-AUGUA mRNA accounts for its accumulation in PBMC after gene transfer. ELISA for GM-CSF performed on supernatants from identical numbers of cells at 24 hours after transfection showed that hGM-AUGUA transfectants secreted 20- to 25-fold more immunologically detectable protein (550 pg/mL/10^6 cells) than the hGM-AUUUA transfectants. As approximately 5% of PBMCs are typically transfected by the gene gun, a fully transfected population (100%) would generate about 11 ng GM-CSF/mL/10^6 cells. The linear correlation between increased steady-state levels of hGM-AUGUA mRNA and synthesis of GM-CSF protein was somewhat unexpected. Previous work has shown that the 3' UTR AREs interfere with the translatability of IFN-β mRNA. Our data suggest that the U to G mutation altered GM-CSF mRNA stability without changing its translatability. Therefore, additional ARE mutations may be effective in increasing translation as well as enhancing mRNA stability.

We have adopted a cautious approach of targeted mutagenesis of the AREs. The substantial homology between the 3' UTR of human and murine GM-CSF suggests that regions outside of the AREs may also have functional importance. Recent work with actin, bicoid, and nanos mRNAs shows that elements within the 3' UTRs mediate intracellular trafficking or localization. Based on these data and the paucity of information regarding additional 3' UTR elements within cytokine mRNAs, we preferred targeted ARE mutations rather than 3' UTR deletions.
remains that other advantageous mutations can also be identified and tested. Kozak has shown that cytokine mRNAs are poorly translated because of the presence of stable stem-loop structures in their 5' UTR. Thus, substitution of the 5' UTRs with less structured sequences could further enhance translational efficiencies. As many cytokine mRNAs are under similar posttranscriptional regulation, selective mutagenesis may be beneficially applied to enhance the expression of other transgenic growth factors.

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