1-1-2008

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Recommended Citation

Craig, R., Cutrera, J., Zhu, S., Xia, X., Lee, Y., & Li, S. (2008). Administering plasmid DNA encoding tumor vessel-anchored IFN-α for localizing gene product within or into tumors. *Molecular Therapy, 16* (5), 901-906. https://doi.org/10.1038/mt.2008.40

Administering Plasmid DNA Encoding Tumor Vessel–anchored IFN-α for Localizing Gene Product Within or Into Tumors

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Tumor-targeted gene delivery has been intensively studied in the field of gene therapy, but no attention has been given to targeting the therapeutic gene products, which are transcribed and translated from the injected genes, into tumors. Targeting immune stimulatory gene products into tumors is the key to triggering tumor-specific CD8+ T-cell responses and reducing systemic toxicity. To target the gene products generated from the injected genes into tumors, genes encoding the tumor-targeted fusion gene product were generated and administered locally and systemically via electroporation. As anticipated, administration of a therapeutic gene encoding IFN-α and the tumor vessel–targeted peptide CDGRC fusion gene product minimizes the leakage of immunostimulatory cytokine from tumors into the blood circulation, increases the infiltration of CD8+ T cells into tumors, induces a high magnitude of cytotoxic T-cell lysis (CTL) activity, and reduces tumor vessel density. As a result, tumor growth was more significantly inhibited by administering the *IFN-α-CDGRC* gene than by administering the wild-type *IFN-α* gene. The same result was obtained with the systemic administration of the tumor-targeted *IFN-α* gene. This gene product–based tumor-targeted gene therapy approach could complement any other tumor-targeted gene delivery method for improving tumor-targeting efficiency.

Received 16 October 2007; accepted 14 February 2008; published online 18 March 2008. doi:10.1038/mt.2008.40

INTRODUCTION

One of the most daunting tasks facing the field of cancer therapy today is finding an effective means of transporting therapeutic agents to the site of tumors. The most attractive method would be targeting cell surface proteins that are altered on cancer cells or in the tumor vasculature. These proteins could be growth factor receptors,[1] cell adhesion molecules,[2] integrins,[3] or other surface markers on endothelial cells.

One of the current interests in the field of cancer research lies in targeting the tumor vasculature, including tumor blood and lymphatic vessels.[4] Endothelial cells line blood vessels and serve as "gateways" to tumor cells. They contain surface proteins that function as vascular receptors able to transduce growth or angiogenic signals.[2] The main advantage of targeting endothelial cells is that they are highly accessible from the blood stream, thus simplifying the experimental design.

Several peptides express high affinity for endothelial markers, such as RGD-4C (A**C**D**C**RGD**C**F**C**G) for integrins[5] or CNGRC for aminopeptidase N/CD13.[2] Of the 25 known integrins, eight bind to peptides containing the sequence RGD, which serves as the main integrin recognition site in extracellular matrix proteins.[6] The RGD-4C peptide shows high affinity for $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins.[7] Integrin $\alpha_v\beta_3$ is highly expressed in endothelial cells of angiogenic vessels and exhibits high affinity for matrix metalloproteinase-2.[8] The $\alpha_v\beta_3$ receptor binds to a wide range of ligands including, but not limited to, fibronectin, vitronectin, osteopontin, and fibrinogen.[9]

The specificity of the sequence RGD-4C for $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins makes this peptide a competitive candidate for targeting DNA vectors to the tumor vasculature. In fact, this peptide has been integrated as part of adenovirus surface proteins and has been heavily explored for targeting the virus into $\alpha_v\beta_3$ expressing endothelial and tumor cells via the RGD-4C–$\alpha_v\beta_3$ interaction, instead of using the natural coxsackievirus and adenovirus receptors that are often lost in highly malignant tumor cells.[10] Curnis *et al.* showed that coupling tumor necrosis factor-α with $\alpha_v\beta_3$ ligands improves its antiangiogenic activity.[11] They reported that subnanogram doses are enough to induce antitumor effects when tumor necrosis factor is fused with A**C**D**C**RGD**C**F**C**G (RGD-4C) and coadministered with chemotherapeutic drugs like melphalan. de Groot *et al.* developed a doxorubicin prodrug conjugated to RGD-4C for reducing the systemic toxicity of doxorubicin.[12]

Interferon (IFN)-α has been shown to inhibit tumor growth by enhancing the antitumor immune response,[13] preventing angiogenesis in the tumor vasculature,[14] and inducing apoptosis of tumor cells.[15] It is effective in treating several cancers including renal cell carcinoma,[16] hairy cell leukemia,[17] malignant melanoma,[18] basal cell carcinoma,[19] squamous cell carcinoma of the head and neck,[20] and multiple myelomas.[21] However,

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IFN-α protein therapy is toxic and expensive.[22,23] A high level of IFN-α protein in the tumor is required for maximizing the therapeutic efficacy.[24]

To reduce the cost and increase the accumulation of IFN-α in tumors, we have generated a fusion gene encoding IFN-α and CDGRC peptide (IFNα-CDGRC). Administration of this fusion gene via intratumoral electroporation enhances anchorage of the fusion gene product into tumors and significantly improves the therapeutic efficacy. As far as we know, this is the first report of using a gene therapy approach to simultaneously express and target the gene product into tumors for increasing the accumulation of the gene product in the tumors and enhancing antitumor efficacy via either local or distant tumor delivery.

RESULTS

CDGRC, CNGRC, and RGD-4C containing reporter gene products illustrate similar tumor anchorage activity

Most tumor-targeted strategies have been used for systemic gene or vector delivery, but the study described here aims to anchor the tumor vessel–targeted gene product from the intratumoral injected *IFNα-CDGRC* gene at the tumor sites. The purpose of this strategy is to prevent the leakage of the therapeutic gene product from the injected tumor sites into blood circulation, thus increasing the accumulation of IFN-α in local tumors. An increased level of IFN-α and interleukin-12 in the tumor is required for maximizing the therapeutic efficacy.[25,26]

To yield a tumor vessel–anchored gene product, three reporter fusion genes encoding secreted alkaline phosphatase (SEAP) and tumor vessel–anchored peptides, CDGRC, CNGRC, and CDCRGDCFC (RGD-4C), were generated (**Figure 1a**). The selection of mini peptides, instead of a large antibody, for anchoring the therapeutic gene product may reduce the concern about immunogenicity. Also, the selected CDGRC shares its homology with both the core tumor vessel–targeted peptide, RGD-4C, and the other well-characterized tumor-targeted peptide, CNGRC.[27–29] To test whether the tumor-anchorage activity levels of SEAP gene products using RGD-4C, CDGRC, and CNGRC peptides were comparable, the expression of SEAP was determined in the liver, spleen, lung, heart, and tumors, and the expression indexes between tumors and livers and between tumors and overall tissues were determined. As expected, the SEAP activities were primarily detected in tumors as illustrated by the large ratios between tumors and tissues with no significant difference among the three tested peptides (**Figure 1b** and **c**).

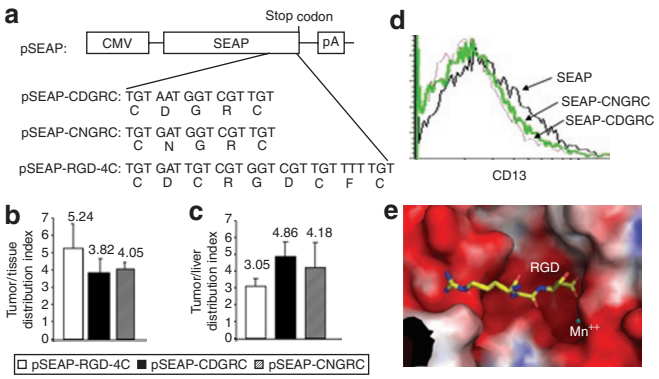

Figure 1 Tumor vessel–anchored binding activity from fusion gene products and targeted peptides. (**a**) Structures of wild-type and tumor vessel–anchored secreted alkaline phosphatase (SEAP) reporter genes encoding SEAP and CDGRC, CNGRC, or RGD-4C. (**b**) The ratio of SEAP expression between tumors and a combination of livers, spleens, and hearts. (**c**) The ratio of SEAP expression between tumors and livers. (**d**) Inhibition of anti-CD13 antibody binding to endothelial cells (EC40) with tumor vessel-anchored gene product. (**e**) Structural analysis of DGR peptide–binding activity using the known crystal structure of the integrin $\alpha_v\beta_3$. The ligand peptide and Mn^{++} ion are shown as a stick model and a ball, respectively, with color-coded atoms: red, oxygen; blue, nitrogen; yellow, carbon; and cyan, Mn^{++}. The ligand binding site in the integrin complex is represented as a surface diagram with color-coded surface electropotentials: red, negative; blue, positive; and white, neutral. CMV, cytomegalovirus.

It is known that DGR-containing peptides bind to the tumor vessel integrin $\alpha_v\beta_3$ (ref. 30). This binding is made by a strong salt bridge formed between Arg (R) of the peptide and the negatively charged residues in the integrin. Although the continued high negative potential in the ligand Asp (D) binding site may cause a negative effect on this peptide binding, this pocket is occupied by Mn^{++}. As a result, the ligand Asp is in direct contact with this metal ion, providing an additional salt bridge and, ultimately, enhancing binding of the peptide (**Figure 1e**). If Asn (N) is in the ligand, as in NGR, instead of Asp (D), the salt bridge between Asn and Mn^{++} may not be formed, resulting in a significant loss in binding force. Thus, NGR cannot effectively bind to the integrin due to the missing interaction between Mn^{++} and the Asp in the peptide ligand.

Next, we examined whether CDGRC binds to CD13, the receptor for CNGRC.[28] To determine the binding activity of CDGRC to CD13, a cell-binding competition assay was performed between reporter gene products (SEAP-CDGRC, SEAP-CNGRC, and SEAP) and the anti-CD13 antibody in a murine endothelial cell line (EC40). This strategy was chosen because antibodies in general have a very high–binding affinity compared to other competitor molecules, and illustration of any competition from the high-affinity antibody binding by these mini peptide–containing SEAP gene products would be a strong indicator that this peptide binds to the target CD13 receptor. Indeed, SEAP-CDGRC illustrates a similar level of inhibition on the anti-CD13 antibody-mediated binding to the endothelial cells as CNGRC-SEAP (**Figure 1d**). This result is a clear indication that the CDGRC not only binds $\alpha_v\beta_3$ as found by the other group[30] but also binds as effectively as CNGRC to the CD13 (**Figure 1d**).

The dual binding activity by the CDGRC illustrated above provided the basis for selecting this peptide encoding sequence to generate a CDGRC and IFN-α-encoding fusion gene (**Figure 2a**). To determine whether the fusion therapeutic gene product is able to anchor to tumors, the expression levels of IFN-α and tumor-anchored IFNα-CDGRC in tumors and blood were determined after intratumoral administration of the genes via electroporation. The expression index between tumors and blood illustrates a fourfold higher level of accumulation of IFNα-CDGRC than IFN-α (**Figure 2b**).

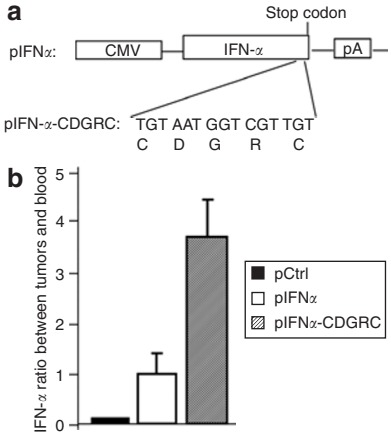

Figure 2 Expression of tumor-anchored IFNα-CDGRC via administration of plasmid DNA encoding IFN-α and the tumor vessel–anchored mini peptide, CDGRC. (**a**) Structure of wild-type *IFN-α* gene (*pIFNα*) and the tumor vessel–anchored *IFN-α* gene (*pIFNα-CDGRC*). (**b**) The expression ratio of IFN-α and tumor vessel–anchored IFN-α between tumors and blood. Plasmid DNA encoding IFN-α, IFN-α-CDGRC, or no transgene was administered into SCCVII tumors via electroporation (20 μg/tumor, n = 4) at the indicated electroporation parameters (see Materials and Methods). Tumors and blood were collected for determining the level of IFN-α 24 hours after the administration. CMV, cytomegalovirus; IFN-α, interferon-α.

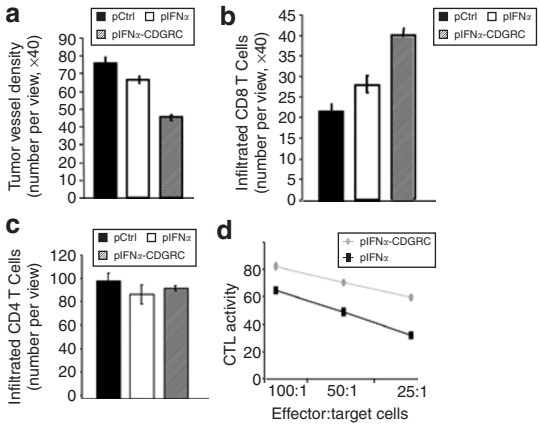

Figure 3 Inhibition of tumor vessel density and enhancement of CD8⁺ T-cell infiltration by administering *IFNα-CDGRC* over wild-type *IFN-α*. Plasmid DNA encoding wild-type IFN-α (pIFNα), tumor vessel–anchored IFNα-CDGRC (pIFNα-CDGRC), and no transgene (pCtrl), respectively, was administered into SCCVII tumors (20 μg/mouse) via electroporation. Ten days after one treatment, tumors were collected, sectioned, and immunostained with anti-CD31, -CD8, and -CD4 antibodies (n = 9). Five days after the second treatment (n = 5), spleens were collected for determining the cytotoxic T-cell lysis (CTL) activity against tumor cells in the presence of anti-NK antibody for avoiding detection of any NK cell–mediated tumor-cell death. (**a**) Tumor vessel density from different treatment groups (P = 0.00017 for pIFNα versus pIFNα-CDGRC). (**b**) Infiltration of CD8⁺ T cells from different treatment groups (P = 0.042 for *pIFNα* versus *pIFNα-CDGRC*). (**c**) Infiltration of CD4⁺ T cells from two treatment groups. (**d**) CTL activity in the two treatment groups (P = 0.0057). CMV, cytomegalovirus; IFN-α, interferon-α; NK cell, natural killer cell.

IFNα-CDGRC is more effective than wild-type IFN-α in reducing tumor vessel density and inducing CD8⁺ T–cell immune response

IFN-α plays multiple roles in the inhibition of tumor growth, including the induction of antitumor immune response,[13] inhibition of angiogenesis in the tumor vasculature,[14] and enhancement of tumor-cell apoptosis.[15] These effects by tumor vessel–anchored IFNα-CDGRC gene therapy and wild-type IFN-α gene therapy were compared in SCCVII tumors.

Intratumoral administration of plasmid DNA encoding tumor vessel–anchored IFNα-CDGRC reduces vessel density by 40%, and administration of the wild-type *IFN-α* gene reduces the vessel density by 13% (**Figure 3a**), which shows threefold more inhibition achieved by the tumor vessel–anchored *IFNα-CDGRC* gene therapy than wild-type IFN-α gene therapy (P = 0.00017). In fact, large necrosis areas were found in all tumors receiving tumor vessel–anchored *IFNα-CDGRC* gene therapy, but were found only in ~25% of tumors receiving wild-type *IFN-α* gene therapy. This necrosis by tumor vessel–anchored *IFNα-CDGRC* gene therapy may be partially caused by inhibition of tumor vessel development and partially caused by an enhanced cell death because the terminal uridine deoxynucleotidyl transferase dUTP nick end labeling assay results illustrated a larger area of apoptosis by *IFNα-CDGRC* than wild-type *IFN-α* gene therapy (data not shown).

To illustrate the antitumor immune response, the infiltration of both CD4⁺ and CD8⁺ T cells into tumors was compared between tumor vessel–anchored *IFNα-CDGRC* and wild-type *IFN-α* gene therapy. Tumor vessel–anchored *IFN-α* gene therapy was found to be three times more effective than wild-type *IFN-α* gene therapy in inducing infiltration of CD8⁺ T cells (P = 0.042), but no difference was detected in the infiltration of CD4⁺ T cells between these treatment groups (**Figure 3c**).

To determine whether tumor vessel–anchored *IFNα-CDGRC* gene therapy induces a higher level of cytotoxic T-cell lysis (CTL) activity than wild-type *IFN-α* gene therapy, CTL activity was compared in mice that received two types of therapeutic genes. As expected, tumor vessel–anchored IFNα-CDGRC gene therapy induces ~20–30% higher levels of CTL activity than wild-type *IFN-α* gene therapy (**Figure 3d**).

Tumor vessel–anchored *IFN-α* gene therapy is more effective in inhibition of tumor growth than wild-type *IFN-α* gene therapy

To examine the therapeutic efficacy, tumor volumes were measured following the administration of tumor vessel–anchored IFNα-CDGRC and wild-type IFN-α encoding genes. CDGRC alone was omitted in the treatment groups because the maximum level of IFNα-CDGRC protein was between 6 and 8 ng/mg protein in tumor tissues and this level of CDGRC was found to have no effect on tumor growth inhibition in our preliminary study (as determined by administering the reporter fusion gene encoding SEAP and SEAP-CDGRC) (data not shown).

An increased inhibition of tumor growth was detected by administration of the tumor vessel–anchored gene in two independent studies (**Figure 4a** and **b**; $P < 0.05$). The true tumor volume reduction by tumor vessel–anchored gene therapy may actually be more significant than the tumor volume shown in **Figure 4** because all tumors using this tumor-anchored gene therapy contain necrotic areas, but the tumor volume measurement is based on the diameters from the edges for easy measurement.

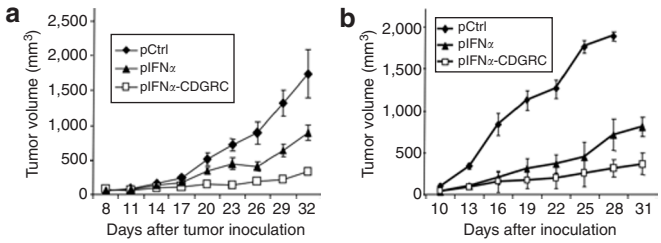

Figure 4 Comparison of tumor growth inhibition between pIFNα-CDGRC versus pIFNα gene therapy via local tumor administration. SCCVII tumor-bearing mice (n = 4) were treated with 20 µg of pCtrl (control), pIFNα, and pIFNα-CDGRC plasmid DNA in a volume of 30 µl saline via intratumoral administration and electroporation. (**a**,**b**) Two administrations were performed at 10 days apart. Two independent experiments were performed. IFN-α, interferon-α.

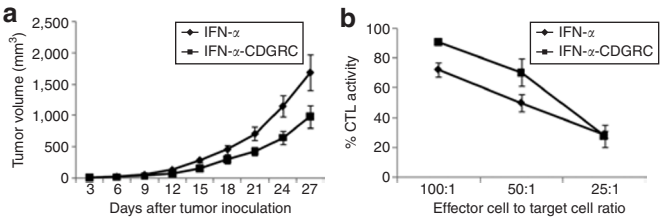

Figure 5 Comparison of tumor growth inhibition and tumor-specific CTL activity between systemic expression of tumor-anchored IFNα-CDGRC and wild-type IFN-α. DNA was injected into skins (the first administration) and muscles (the second administration) located distant from tumors (n = 6). Two administrations were performed at 10 days apart at a dose of 10 µg/mouse in a volume of 30 µl saline. (**a**) Inhibition of tumor growth after systemic expression of IFN-α and tumor-anchored IFNα-CDGRC. (**b**) Tumor-specific CTL activities following expression of two types of IFN-α. Spleen cells were isolated for CTL activity assay 7 days after the second administration. CTL, cytotoxic T-cell lysis; IFN-α, interferon-α.

Tumor vessel–targeted *IFN-α* gene therapy is also effective when delivered systemically

To determine whether the *IFNα-CDGRC* gene therapy demonstrates a stronger antitumor efficacy when it is administered systemically, both wild-type *IFN-α* and *IFNα-CDGRC* were administered into hindlimb muscles or skin via electroporation in a colon tumor–bearing model. Administration to muscles or skin was counted as systemic delivery because the injected tissues are distal from tumors and the gene products made from the injected tissues were secreted into blood circulation. After administration, inhibition levels of tumor growth and antitumor CTL activity were compared. Increased inhibition of tumor growth and increased CTL activity are the ultimate surrogate end points for the enhanced IFN-α accumulation because tumor-specific accumulation of immune stimulatory cytokines is the key to inducing effective antitumor response and CTL activity.[24–26] As is also found from intratumoral administration, IFNα-CDGRC yields a much better tumor growth inhibition from systemic delivery than the wild-type IFN-α (**Figure 5a**). Likewise, *IFNα-CDGRC* induces much stronger CTL activity than wild-type IFN-α with this systemic delivery (**Figure 5b**). These results illustrate that tumor-targeted *IFNα-CDGRC* gene therapy is beneficial for treating tumors with both systemic and local administrations.

DISCUSSION

The major goal of this study was to determine whether administering *IFN-α* gene encoding the tumor-targeted IFN-α gene product (*IFNα-CDGRC*) into tumors via intratumoral electroporation would increase the therapeutic efficacy and improve the antitumor immune response. Our results support the tumor-anchoring assumption because a fourfold higher level of IFNα-CDGRC was accumulated in tumors compared to wild-type IFN-α, in two independent experiments (**Figure 1**). Because this tumor-anchoring approach occurs after protein translation and protein secretion outside of cells, it can complement other tumor-targeted gene delivery approaches occurring at the delivery and transcriptional levels to achieve double or triple tumor-targeting effects. For example, this anchoring strategy complements the tumor-targeted electroporation injection for accumulating the therapeutic cytokine product IFNα-CDGRC at the injected tumors (**Figure 1**). Associated with this accumulation, a significant infiltration of $CD8^+$ T cells and inhibition of tumor angiogenesis were achieved by administering this *IFNα-CDGRC* gene, compared to administering the wild-type *IFN-α* gene (**Figure 3**).

As was found from local tumor delivery, systemic administration of tumor-targeted *IFNα-CDGRC* also enhances tumor growth inhibition and tumor-specific CTL activity (**Figure 5**). These results suggest that this tumor-targeted gene therapy not only can complement other tumor targeted delivery approaches for enhancing antitumor efficacy but also works independently for enhancing antitumor efficacy. In summary, the local tumor and distal administration results support the assumption that accumulation of immune stimulatory cytokines into tumor is the key to triggering a significant antitumor response. This notion was also found to be true for interleukin-12-mediated antitumor efficacy.[25,26] The results from this study and the interleukin-12 administration route study conducted previously suggest that different immune stimulatory cytokines may share the same principal,[25,26] which is that tumor-specific accumulation of these cytokines boost tumor-specific antitumor immune response.

Unlike antibody- or peptide-mediated recombinant cytokine protein tumor targeting,[31,32] this study uses a gene therapy approach to express the tumor-anchored gene product containing a mini-CDGRC peptide and IFN-α. This peptide shares the identical RGD-4C core sequence (**Figure 1**) and also has a high homology with the mini-tumor vessel–targeted peptide CNGRC that binds aminopeptidase N.[2] The structure of amino acids "D" and "N" that appear in CDGRC and CNGRC, respectively, is almost identical, which suggests that the CDGRC peptide not only binds the integrin $\alpha_v\beta_3$ (ref. 30) but also aminopeptidase N.[28] Indeed, the competition study demonstrates that CDGRC-SEAP is as effective as CNGRC-SEAP in inhibiting the binding of the high-affinity anti-CD13 antibody (5 µg total antibody) (**Figure 1d**). However, CNGRC does not bind $\alpha_v\beta_3$ favorably despite its similarity to CDGRC, according to the structural analysis (**Figure 1e**).

A cyclic peptide, CDGRC, was used because a cyclic structure is more effective than linear NGR motifs in receptor:ligand binding.[33] This short peptide, containing only five amino acids, has much less chance to induce immunogenicity than a large polypeptide as determined from the CNGRC study.[34] Therefore, this strategy has great potential for anchoring the gene product in tumors without triggering immunogenicity against the therapeutic gene product itself.

Others have demonstrated that the CNGRC-TNFα conjugated protein is more effective in treating tumors than the wild-type tumor necrosis factor-α at a low dose.[35] This observation may apply to gene therapy as illustrated in this study, in which tumor-anchored *IFNα-CDGRC* gene therapy is more effective than wild-type *IFN-α* gene therapy for treating tumors. The advantage of the electroporation gene therapy approach is that it is easier and less expensive than recombinant protein therapy. From an economic point of view, this gene therapy strategy has great potential.

MATERIALS AND METHODS

Gene constructs encoding tumor vessel–anchored reporter gene products and therapeutic gene product. SEAP gene constructs encoding fusion protein SEAP and tumor vessel–targeted peptides CDGRC, CNGRC, or RGD-4C were generated via direct PCR. The same forward primer 5′-GCATCATCCCAGTTGAGGAG-′ was used for generating fusion genes SEAP-CDGRC, SEAP-CNGRC, and SEAP-RGD-4C. The reverse primers were 5′-GTAGGATCCTTATCAGCATCTTCCATCGCATGTCTGC TCGAAGCGGCCGGC-3′ for generating SEAP-CDGRC fusion gene, 5′-TTATCAACAACGACCGTTACATGTCTGCTCGAAGCGGCCGG-3′ for generating SEAP-CNGRC fusion gene, and 5′-TTATCAGCAGAA ACAATCACCGCGGCAATCACA-3′ for generating SEAP-RGD-4C fusion gene. The PCR product was cloned into pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA), and then subcloned into a vector containing a cytomegalovirus promoter, a mini-intron, wild-type SEAP gene, and a human growth hormone polyadenylation signal using restriction enzymes *Bam*H1 and *Spe*I (**Figure 1**). The sequence was confirmed by sequence analysis and reporter gene activity assay.

Using the same strategy, IFNα-CDGRC encoding gene construct was generated (**Figure 2**). In brief, the forward and reverse primers used for generating DNA fragment encoding IFNα-CDGRC were 5′-GTAAGC TTATGGCTAGGCTCTGTGCTTTCC-3′ and 5′-TCAGCATCTTCCAT CGCACTCCTTCTCCTCACTCAGTCT-3′. The PCR product containing IFNα-CDGRC encoding sequence was cloned into pCR2.1 TA cloning vector. The IFNα-CNGRC fusion gene was released from this vector with *Hin*dIII and *Spe*I restriction digestion enzymes and subcloned into an expression vector cut with *Hin*dIII and *Spe*I. The expression vector for this fusion gene is the same as the one expressing the wild-type IFN-α (**Figure 2**). The fusion gene sequence was confirmed by sequence analysis.

Tumor models and DNA delivery via electroporation. Six- to eight-week-old female C3H/HeN or Balb/c mice, weighing 18–20 g, from the in-house animal breeding facility were used for this study and were maintained under National Institutes of Health guidelines, approved by the Institutional Animal Care and Use Committee of Louisiana State University.

SCCVII and CT26 cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Rockville, MD). SCCVII and CT26 tumors were generated by subcutaneously inoculating the mice with 2×10^5 tumor cells in a 30-μl volume into C3H and Balb/c mice, respectively. Tumor dimensions were measured with calipers, and volumes were calculated from the formula: $V = (\pi/8)(a \times b^2)$, where V = tumor volume, a = maximum tumor diameter, and b = diameter at 90° to "a".[36] Using the protocols described previously, plasmid DNA was injected into tumors, skins and muscles. Each injection was followed by immediate electroporation.[37,38] The parameters for intratumoral electroporation were previously set at 450 V/cm and 20 ms pulse duration for 2 pulses.[37] The parameters for intramuscular and intradermal electroporation were 350 V/cm, 20 ms pulse duration for 2 pulses.[39] The endotoxic free plasmid DNA was prepared using Qiagen Endotoxic free plasmid DNA preparation kit (Germantown, MD).

Gene expression analysis. In brief, tumors were injected with plasmids that expressed SEAP-CDGRC, SEAP-CNGRC, or SEAP-RGD-4C with a syringe followed by electroporation. After 1 day, the tumors and organs were harvested, sectioned at 10 μm, and the expression of reporter genes was measured using a SEAP activity assay kit (Tropix, Bedford, MA). Using the same administration approach, plasmid DNA encoding IFN-α and IFNα-CDGRC was administered and the level of IFN-α was detected using a kit from PBL Biomedical Laboratories (Piscataway, NJ).

Fluorescent microscope-based CTL assay. Cytotoxic T-lymphocyte (CTL) activity was evaluated using a CyToxiLux kit (OncoImmunin, Gaithersburg, MD), a single–cell–based fluorogenic cytotoxicity assay.[40] Splenocytes were obtained from SCCVII tumor-bearing C3H mice 2 weeks after treatment. These splenocytes are referred to as effector cells. The effector cells were primed by coculture with mitomycin C–treated SCCVII tumor cells in a ratio of 25:1 for 5 days in Rosewell Park Memorial Institute-1640 medium with 20% fetal calf serum. Effector cells were incubated with red fluorescence–labeled target SCCVII cells in the indicated ratios in a volume of 200 μl for 16 hours. The apoptotic target cells (red/yellow colored cells) were examined using an Olympus BX41 fluorescence microscope. CTL activity was calculated using the equation: percentage specific killing = 100 × (apoptotic target cells − spontaneous apoptotic target cells)/(total target cells).

Immunostaining of T-cell infiltration. The procedures for frozen-block preparation, tissue sectioning, and immunostaining were the same as described previously.[37–39,41] The primary antibody applied to the sections was anti-CD8 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA).

Flow cytometric analysis of CD13 expression. EC40 (American Type Culture collection, Manassas, VA) cells were stained with phycoerythrin-labeled anti-CD13 receptor antibody (BD Biosciences, San Jose, CA) in the presence of tumor lysates containing 50 ng protein of SEAP, SEAP-CDGRC, or SEAP-CNGRC. The stained cells were analyzed using FACscan cytometer (BD Biosciences, San Jose, CA). A total of 10,000 cells/sample were acquired and data samples were analyzed using Cellquest software.

Structural binding analysis. The binding activity of DGR and NGR to the known RGD-binding integrin $\alpha_v\beta_3$ was analyzed. The coordinates for the RGD peptide and $\alpha_v\beta_3$ integrin were obtained from the pdb "1L5G".[42] Protein surface electropotential was calculated using apbs-0.5.1 (ref. 43) and the protein surface diagram and the ligand stick model were drawn using PyMol Molecular Graphics System (DeLano Scientific, San Carlos, CA).

Statistical analysis. Tumor volume, $CD8^+$ T-cell infiltration, and vessel density were the primary outcomes measured. We used the two-sided Student's t-test to compare individual treatments. P values <0.05 were considered statistically significant.

ACKNOWLEDGMENTS

This work was supported by grants from National Cancer Institute/National Institutes of Health (RO1CA120895).

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