MIG (CXCL9) Chemokine Gene Therapy Combinesto Antibody-Cytokine Fusion Protein to Suppress Growth and Dissemination of Murine Colon Carcinoma

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ABSTRACT

The induction of a CTL response capable of eradicating disseminated tumor metastases and the establishment of a persistent tumor-protective immunity remain major goals of cancer immunotherapy. Here, we demonstrate for the first time that the combination of interleukin 2 (IL-2)-targeted to the tumor microenvironment by a recombinant antibody-IL-2 fusion protein (huKS1/4-IL-2) with gene therapy by the murine chemokine MIG (CXCL9) markedly reduced s.c. tumor burden and decisively suppressed dissemination of experimental lung metastases of CT26-KSA colon carcinoma in syngeneic BALB/c mice. This combined therapy significantly prolonged the life span of these mice 3-4-fold by concurrently delivering MIG and IL-2 to the tumor site and thereby achieving chemoattraction of T cells together with their activation. The antitumor effect obtained was mediated predominantly by MHC class I antigen-restricted CD8+ T cells with help from MHC class II antigen-restricted CD4+ T lymphocytes. In addition, the MIG chemokine also induced angiostatic effects in the tumor vasculature. Taken together, this combination of MIG chemokine gene therapy with tumor-targeted cytokine IL-2 provides an approach for the rational design of novel cancer immunotherapy modalities.

INTRODUCTION

The possibility to effectively modify and enhance immune responses against infectious and malignant diseases has become a major focus of interest for the development of novel treatment modalities. In this regard, the application of cytokines and chemokines was demonstrated to be beneficial in a large number of experimental animal models of infectious and malignant diseases (1, 2), and has become an alternative or an addendum to chemotherapy in the treatment of some human malignancies. Thus, cytokines such as IL-2 (3), IL-12 (4, 5), IFN-α, and IFN-γ (6) were found useful in the treatment of renal cell carcinoma (7) and melanoma (8). Other potential candidates for such immunotherapeutic interventions are chemokines, known to chemoattract specific leukocyte subpopulations to sites of infection or the tumor microenvironment (9). However, chemokines and chemokine receptors have been studied mainly in infectious diseases, particularly HIV and AIDS, whereas relatively little is known about their role in cancer therapy (10, 11). Chemokines, extensively described in several recent reviews (12-14), represent an extended family of cytokine-inducible secreted proteins of low molecular weight, which are characterized by four invariant cysteine residues at their NH2 termini (12). Among a variety of chemokines, MIG, i.e., monokine induced by IFN-γ (14-16), is a member of the CXC (α-) subfamily, characterized by its first two cysteines being separated by a single amino acid. Important for our purpose, the M12,000 chemokine MIG functions in vivo as a potent chemoattractant for tumor-infiltrating lymphocytes (17), activated peripheral blood lymphocytes (12), as well as NK cells and Tγδ lymphocytes (18). Among the CXC chemokines, MIG belongs to a subgroup lacking the characteristic, proangiogenic ELR-motif and, thus, functions as an angiostatic agent (19). MIG is also known to promote tumor necrosis when administered intratumorally (20). CXCR3 serves as the receptor for MIG, which is expressed exclusively on T and NK cells after activation by IFN-γ (13) but is absent from resting T or B lymphocytes as well as monocytes and granulocytes (21).

On the basis of our earlier application of the recombinant antibody-IL-2 fusion protein (huKS1/4-IL-2) in the treatment of murine colon carcinoma (22-24), we now combined both gene therapy by the CXC chemokine MIG with immunotherapy by the huKS1/4-IL-2 fusion protein, which targets IL-2 specifically to the tumor microenvironment.

MATERIALS AND METHODS

Construction of an Expression Plasmid Encoding MIG (CXCL9).

The murine MIG gene was cloned by RT-PCR using murine splenocytes as a template that were pre-stimulated for 48 h with ConA. The following primers were used: CCGGGG TAACCCAGGACTGTCGCCGTCCCTTTCG (forward); and ACCTCTATGTATGCCTCCCTTT (reverse). The design of these primers results in cloning of the MIG gene downstream of the Kozak sequence (25, 26), which is required for the initiation of transcription. Using KpnI and XbaI as restriction sites, this construct was inserted into the plasmid pcDNA3.1 (+/-; Invitrogen, Carlsbad, CA), which was subsequently used for transfection of the murine colon carcinoma cell line CT26-KSA.

Cell Lines. The murine colon carcinoma cell line CT26, kindly provided by Dr. Isaiah J. Fidler, M.D. Anderson Cancer Center, Houston, TX, was described previously (22, 23). This cell line was stably transfected with the gene encoding the murine chemokine MIG with the LipofectAMINE transfection system (Life Technologies, Inc., Grand Island, NY). Positive clones were selected by using 200 μg/ml zeocin (Invitrogen). All of the cell lines used were analyzed periodically and tested negatively for Mycoplasma.

RT-PCR. Total RNA was extracted from 3 x 106 double-transfected colon carcinoma cells CT26-KSA-MIG using the RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription was performed with 1 μg of total RNA followed by PCR with the same oligonucleotides as described above. The PCR was cycled 30 times at an annealing temperature of 60°C to result in a 380-bp fragment. RNA quantity and PCR were monitored by PCR for glyceraldehydes-3-phosphate dehydrogenase resulting in a 295-bp fragment.

Western Blot Analysis. To detect protein production, COS-7 kidney cells were transfected with the plasmid encoding the murine MIG gene using a calcium phosphate transfection kit (Invitrogen) following the manufacturer’s instructions. After 48 h, COS-7 and COS-7-MIG cells were harvested for the detection of protein expression. Western blot analysis was done with total protein of cell lysate homogenates using a polyclonal primary rabbit antibody directed against murine MIG (kindly provided by Dr. Lili Feng, The Scripps Research Institute). Specific protein was detected by a goat antirabbit, horseradish-peroxidase conjugated IgG antibody (Calbiochem, La Jolla, CA).
Migration Assay. To assess the chemoattractive properties of MIG we used cell culture supernatants harvested from CT26-KSA and CT26-KSA-MIG tumor cells after 72 h of fetal bovine serum-deprivation. Migration of splenocytes was evaluated in a 24-well chemotaxis chamber. The lower wells of these chambers were filled with 600 µl of supernatants obtained as described above. A 5-µm pore size, fibronectin coated Transwell Polycarbonate Membrane (Costar, Cambridge, MA), was placed above the wells and then 100-µl of cell suspension (1 x 10⁵ splenocytes/ml migration medium) was added to the upper compartment of the migration chamber and incubated for 5 h at 37°C in a humidified CO₂ incubator. Cells that had migrated through the polycarbonate membrane were harvested from the lower chamber and counted under a microscope. Results are expressed as the mean value of triplicates ± SD; the assay was repeated three times with a variability of <10%.

Animal Experiments. Tumors were generated by s.c. injection of 5 x 10⁴ tumor cells in 100 µl of PBS into the left flank of 6–8-week-old syngeneic, female BALB/c mice. Subsequent tumor size was measured by caliper and the tumor volume calculated using the formula ½(length x width x depth). Subcutaneous tumors were treated by i.v. injections of 5 µg of huKS1/4-IL-2 fusion protein each on days 1–5 after tumor cell inoculation. The humanized antibody was constructed as described previously (28) as was the fusion protein (27–29). A chimeric antibody-cytokine fusion protein was applied to reduce the formation of murine antihuman antibodies. This chimeric fusion protein is well tolerated for at least 7 consecutive i.v. applications of ≤20 µg fusion protein/injection (data not shown).

Survival studies were initiated by inducing experimental pulmonary metastases after i.v. injection of 5 x 10⁶ cells in 100 µl of PBS into 6–8-week-old syngeneic, female BALB/c mice. Established metastases were treated with subcutaneous doses of six i.v. injections of 5 µg/day of huKS1/4-IL-2 fusion protein on days 4–9 after tumor cell inoculation. Two control groups of mice received a mixture of 5 µg/day of huKS1/4-IL-2, a dose equivalent to that applied with 5 µg/day of the huKS1/4-IL-2 fusion protein. For immunohistochemical staining, tumor-bearing mice were sacrificed on day 12 and lungs removed and snap frozen. Each experimental group consisted of 8–10 BALB/c mice. All of the animal experiments were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and approved by The Scripps Research Institute.

In Vivo Depletion of CD4⁺ or CD8⁺ T Lymphocytes. As described earlier (22), female BALB/c mice were depleted of CD4⁺ or CD8⁺ T-lymphocyte subsets using rat IgG₂b, antimurine CD4 (clone GK1.5; National Cell Culture Center, Minneapolis, MN) or rat IgG₂b, antimurine CD8 (clone 2.43; National Cell Culture Center) antibodies, respectively. Depletion was achieved by i.p. injections of 500 µg each of anti-CD4⁻ or anti-CD8⁻-specific mAbs on days −4, −1, 2, 6, 10, and 14. FACS analyses of lymph node- and spleen-derived lymphocytes confirmed the depletion of >95% of the respective T-cell subsets before the treatment of mice was initiated.

Cytotoxicity Assay. Cytotoxicity was measured in a standard 3¹Cr-release assay with CT26-KSA cells serving as target cells. Effector cells were isolated from spleens of BALB/c mice bearing pulmonary metastases 3 days after cessation of therapy. Coincubation of effector and target cells (5 x 10⁴) at 37°C was done at different E:T ratios for 4 h. To investigate the MHC class I and class II antigen-dependent inhibition of cytotoxicity, 40 µg/ml of anti-mouse MHC antigen class I (H-2K¹, Pharmingen, San Diego, CA) and antimouse MHC antigen class II (I-A⁻¹/I-E⁻¹, Pharmingen) antibodies were used. Nonspecific antibodies, directed against H-2Db and H-2Kb failed to inhibit cytotoxicity (data not shown). The percentage of specific CT26-KSA target cell lysis was calculated using the formula [(E-S)/(T-S)] x 100 with E being the average experimental release, S the average spontaneous release, and T the average total release.

In Vivo Matrigel Plug Angiogenesis Assay. Liquefied Matrigel (400 µl/mouse; Becton Dickinson, Bedford, MA) mixed with 1 x 10⁵ CT26-KSA or CT26-KSA-MIG tumor cells were injected s.c. into the flank of BALB/c mice. On day 6, mice were sacrificed and plugs removed, weighed, and fixed in 3.7% formaldehyde/PBS. The formation of blood vessels during tumor growth and the abrogation thereof by MIG chemokine gene therapy was quantified by determining the hemoglobin content of Matrigel plaques according to the methods described by Passaniti et al. (30) and Drukin and Austin (31).

Immunohistochemical Analyses. Frozen sections of Matrigel were fixed in acetone, and endogenous peroxidase was removed by incubation with 0.03% H₂O₂. Nonspecific antibody binding was prevented by blocking with 10% normal swine serum in PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specific serum in 1% BSA/PBS. After incubation with the appropriate species-specifi
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Fig. 2. Suppression of s.c. tumor growth in syngeneic BALB/c mice treated with mMIG gene therapy combined with huKS1/4-IL-2 fusion protein therapy. Comparisons are shown between mMIG ( ● ); CT26-KSA-MIG ( ○ ); and CT26-KSA-MIG + huKS1/4-IL-2 fusion protein therapy ( ○ ). Data are shown as mean ( n = 6); bars, ± SD. The statistical differences between CT26-KSA and CT26-KSA-MIG as well as between CT26-KSA-MIG and CT26-KSA-MIG + huKS1/4-IL-2 fusion protein therapy are P < 0.0001.

CT26-KSA-MIG
+ IL-2-FP
CT26-KSA-MIG
+ IL-2-FP + anti-CD8+ mAb
CT26-KSA-MIG
+ anti-CD8+ mAb
CT26-KSA-MIG
+ IL-2-FP
CT26-KSA-MIG
+ IL-2-FP + anti-CD4+ mAb
CT26-KSA-MIG
CT26-KSA-MIG
+ anti-CD4+ mAb

Fig. 3. Role of CD8+ and CD4+ T-effector cells in the suppression of s.c. tumor growth. Comparisons are shown between mMIG gene therapy (CT26-KSA-MIG) + huKS1/4-IL-2 fusion protein therapy (IL-2-FP) and mMIG gene therapy alone (CT26-KSA-MIG). Data are shown as mean ( n = 6); bars, ± SD. Effect on treatment efficacy is indicated by in vivo depletion of (A) CD8+ or (B) CD4+ T lymphocytes.

To determine the role of effector T cells, we depleted mice of CD8+ and CD4+ T lymphocytes, respectively (Fig. 3). The depletion of CD8+ T lymphocytes in mice that thereafter were injected with the CT26-KSA-MIG cell line resulted in a decisive 4-fold increase in s.c. tumor volumes within 25 days reaching up to 1900 mm3. The subsequent treatment of CD8+ T-cell-depleted mice with subcurative doses of huKS1/4-IL-2 fusion protein resulted in s.c. tumor volumes of 1400 mm3 (P < 0.001) by the combination therapy.

The suppression of s.c. tumor growth by chemokine gene therapy per se, because the volume of s.c. growing tumors was additionally reduced from ~450 mm3 to 150 mm3 (P < 0.001) by the combination therapy.

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Prolongation of Survival by MIG Gene Therapy in Combination with huKS1/4-IL-2 Fusion Protein Therapy. To assess the efficacy of MIG gene therapy combined with huKS1/4-IL-2 fusion protein in a relevant minimal residual disease setting, we determined the life span of mice after treatment of their established experimental pulmonary metastases (Fig. 4). Control animals that received no treatment survived only an average of 26.9 days. In contrast, the combination of mMIG gene therapy with i.v. injections of 5 µg/day of huKS1/4-IL-2 fusion protein on days 4–9 after tumor cell inoculation resulted in a significant 3-fold increase in life span to >85 days in 100% of experimental animals (average survival: 108.8 days; P < 0.0001). Although 4 of 8 animals died between days 86 and 106 after tumor cell inoculation, the remaining 4 mice survived >108 days and, thus, attained a 4-fold increase in life span. In contrast, MIG gene therapy alone resulted only in an average life span of 33.5 days, whereas mice that received only subcurative doses of the huKS1/4-IL-2 fusion protein for 6 days survived 36.5 days. An additional control group of mice that received a mixture of 5 µg/day huKS1/4 mAb + 15,000 IU/day rhIL-2, a dose equivalent to that provided by 5 µg/day of huKS1/4-IL-2 fusion protein, reached only an average life span of 27.4 days. The combination of mMIG gene therapy with a mixture of huKS1/4 antibody and rhIL-2 cytokine had little effect and resulted in an average life span of 44.6 days. Taken together, the data from these experiments indicate that the combination of mMIG gene therapy plus the huKS1/4-IL-2 fusion protein was optimal, resulting in a 3-fold increase in life span to >85 days in 100% of mice and in a 4-fold increase in life span in 50% of experimental animals.

Effects of MIG Gene Therapy on Infiltration of Matrigel-embedded Tumor Cells by Immune Effector Cells. As documented in Fig. 5, chemokine gene therapy with mMIG leads to a marked increase in the number of CD8+ and CD4+ T lymphocytes (Fig. 5, B and D) infiltrating the Matrigel-embedded colon carcino-
noma cells in comparison with CT26-KSA wild-type tumor cells (Fig. 5, A and C). Furthermore, we also investigated the infiltration of Matrigel-embedded tumor cells by NK cells but did not find significant differences between MIG-transfected tumor cells and controls (data not shown).

**Effects of MIG Gene Therapy on Neovascularization of Matrigel-embedded Tumor Cells.** As documented by the data shown in Fig. 6, MIG chemokine gene therapy leads to a marked decrease in neovascularization of tumor cells embedded in Matrigel. This was shown by a decrease in the number of CD31+ endothelial cells (Fig. 6D) compared with untreated controls (Fig. 6C). Additionally, H&E staining confirmed this angiostatic effect of MIG by indicating newly formed blood vessels in controls (Fig. 6A) that disappeared after MIG gene therapy (Fig. 6B).

As documented by the data shown in Fig. 6E, Matrigel plaques that contain CT26-KSA-MIG tumor cells display a lower content of hemoglobin than control Matrigel plaques that contain CT26-KSA tumor cells. The difference between our MIG gene therapy group and a control group is statistically significant (P < 0.005). As the hemoglobin content of Matrigel plaques parallels the neovascularization of Matrigel-embedded tumor cells, this finding provides additional evidence for the angiostatic effect of MIG gene therapy.
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Effect of Combination Therapy on CTL Activity. Cytotoxicity studies provided a second line of evidence indicating the immunological mechanisms involved in the antitumor effects achieved by the combination of huKS1/4-IL-2 fusion protein and mMIG gene therapy (Fig. 7). Thus, splenocytes isolated from BALB/c mice 12 days after induction of pulmonary metastases with CT26-KSA-MIG tumor cells and subsequent treatment with huKS1/4-IL-2 fusion protein specifically lysed ≤44% of CT26-KSA tumor target cells. In contrast, only 29% lysis of target cells was achieved by splenocytes obtained from mice treated with mMIG gene therapy alone (P < 0.001). Furthermore, the involvement of MHC class I antigen restricted CD8+ T-effector cells was clearly indicated by a specific inhibition of cytotoxicity. Thus, tumor target cell lysis was essentially abrogated when splenocytes were coincubated with an anti-MHC class I antibody and decreased to only 22% when coincubated with an anti-MHC class II antibody (P < 0.001), suggesting a helper effect of CD4+ T cells.

DISCUSSION

We hypothesized that MIG (CXCL9) chemokine gene therapy aided by small but noncurative doses of huKS1/4-IL-2 might be effective in suppressing s.c. growth of murine colon carcinoma and inhibiting their pulmonary metastases. The rationale for this type of tumor immunotherapy is first to induce chemotraction of immune effector cells to the tumor microenvironment by MIG chemokine gene therapy and then to activate these cells at the tumor site with an antibody–IL-2 fusion protein.

Here, we demonstrate that the combination of a noncurative dose of huKS1/4-IL-2 fusion protein with mMIG chemokine-based gene therapy is far superior to the antitumor effect achieved by either therapy alone at these dose levels. In fact, this combination therapy is more effective in markedly decreasing s.c. CT26-KSA colon carcinoma tumor growth as well as in suppressing the dissemination of established pulmonary metastases, resulting in a ≤4-fold prolongation of life span. The antitumor effect achieved in this murine colon carcinoma model is based primarily on immunological mechanisms involving the chemotraction and subsequent activation of both CD8+ and CD4+ T lymphocytes, combined with an angiostatic effect induced by murine MIG.

These conclusions are supported by four lines of evidence. First, the treatment effect of the combination therapy was specifically abrogated in mice depleted of CD8+ cells and markedly suppressed in mice depleted of CD4+ T lymphocytes. Second, cytotoxicity studies indicated the involvement of MHC class I antigen-restricted CD8+ and MHC class II antigen-restricted CD4+ T cells. Third, immunohistochemical analyses demonstrated infiltration of tumor tissues by both CD8+ and CD4+ T cells. Fourth, an angiostatic effect of MIG was indicated by H&E staining and immunohistochemical analyses of Matrigel-embedded tumor cells, and by hemoglobin measurements.

Only a limited number of studies have thus far analyzed the role of chemokines in cancer immunotherapy. Specifically, the role of MIG gene therapy was investigated in two studies. First, in an immunodeficient (SCID) mouse model of human non-small cell lung carcinoma A549 (32), intratumoral injections of recombinant human MIG (rhMIG) or a recombinant adenoviral vector encoding the human MIG gene resulted in suppression of s.c. tumor growth in SCID mice. This effect was thought to be attributable to a decrease in tumor-derived blood vessel growth but was not accompanied by significantly increased numbers of tumor-infiltrating neutrophils or macrophages. The second study (20), in a model of human Burkitt’s lymphoma CA46 in immunodeficient BALB/c nu/nu mice, demonstrated that s.c. tumor growth could be suppressed, although not completely halted; however, this antitumor effect could be achieved only by intratumoral injections using a combination therapy of EBV-immortalized human lymphocytes and rhMIG. These results were mainly attributed to extensive tumor necrosis, including marked vascular damage and intravascular thrombosis but without any apparent difference in lymphocyte, neutrophil, or monocyte infiltration compared with controls.

In contradistinction, our own study used a syngeneic immunocompetent model of murine colon carcinoma, which allows the critical evaluation of immune effector functions induced by chemokine gene therapy, that are likely among the most important biological properties of chemokines. Furthermore, our survival studies are based on the growth and dissemination of pulmonary metastases and, thus, mimic a clinical setting much more closely than the correlation of life span with the growth of s.c. tumors.

To our knowledge, only one other study examined the effects of MIG chemokine gene therapy combined with cytokine gene therapy. In this case, cDNA for both mMIG and miIL12 were encoded in the same recombinant adenoviral vector and applied by intratumoral injections to a s.c. syngeneic immunocompetent model of murine colon carcinoma, which allows the critical evaluation of immune effector functions induced by MIG chemokine gene therapy. This study showed that the combination of both gene therapies only slightly improved the growth delay achieved with either gene therapy alone, and ≤50% of animals achieved a 2-fold increase in life span.

The results of our study clearly demonstrate that the combined application of mMIG gene therapy with an antibody–IL-2 fusion protein targeted to the tumor microenvironment is superior in its antitumor effects to the application of these respective agents alone. In addition, a critical feature of our approach is the tumor-targeting of IL-2 with a recombinant antibody cytokine fusion protein, which takes advantage of the paracrine nature of IL-2 to locally activate those T cells that were specifically chemottracted to the tumor microenvironment by MIG gene therapy. This maneuver resulted in an increase in life span and, importantly, in an abrogation of disseminated pulmonary metastases as well as a marked suppression of s.c. tumor growth. Furthermore, by targeting small doses of IL-2 to the tumor microenvironment, we reduce potentially dose-limiting toxicities of systemic IL-2 administration. In this regard, we demonstrated previously that huKS1/4-IL-2 fusion protein administered at high dose levels (7 × 15 μg) can eradicate experimental metastases of CT26-KSA colon carcinoma. In contrast, the application of a suboptimal dose (6 × 5 μg) did not inhibit tumor growth (22). However, by adding chemokine gene therapy to the already established IL-2 fusion protein treatment we
were able to significantly lower the dose of fusion protein required to achieve antitumor effects in mice. Taken together, these findings are of potential importance for future clinical applications where minimal amounts of optimally effective cytokines or chemokines are crucial in avoiding toxicities caused by these potent immune stimulators and in achieving successful cancer immunotherapy.

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