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ORIGINAL ARTICLE

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Improved survival in tumor-bearing SCID mice treated with interferon-γ-inducible protein 10 (IP-10/CXCL10)

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Abstract Tumor growth requires angiogenesis, which in turn requires an imbalance in the presence of angiogenic and angiostatic factors. We have shown that the CXC chemokine family, consisting of members that are either angiogenic or angiostatic, is a major determinant of tumor-derived angiogenesis in non-small-cell lung cancer (NSCLC). Intratumor injection of interferon-inducible protein 10 (IP-10, or CXCL10), an angiostatic CXC chemokine, led to reduced tumor growth in a SCID mouse model of NSCLC. In this study, we hypothesized that treatment with CXCL10 would, by restoring the angiostatic balance, improve long-term survival in NSCLC-bearing SCID mice. To test this hypothesis, A549 NSCLC cells were injected in the subcutis of the flank, followed by intratumor injections with CXCL10 continuously (group I), or for ten weeks (group II), or a control group (human serum albumin). Median survival was 169, 130, and 86 days respectively ($P < 0.0001$). We extended these studies to examine the mechanism of prolonged survival in CXCL10-treated mice. CXCL10 treatment inhibited lung metastases, but was dependent upon continued treatment, and was associated with an increased rate of apoptosis in the primary tumor, with no direct effect on the proliferation of the NSCLC cells. Furthermore, the inhibition of lung metastases was due to the angiostatic effect of CXCL10 on the primary tumor, since the rate of apoptosis within lung metastases was unaffected. These data suggest that anti-angiogenic therapy of human lung cancer should be continued indefinitely to realize persistent benefit, and confirms the anti-metastatic capacity of localized angiostatic therapy.

Keywords Cytokines · Angiogenesis · Chemokines · Tumor immunity

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Introduction

Lung cancer is responsible for over 150,000 deaths per year in the US, more than the next three most common cancers combined [2, 5, 8]. Currently available treatments for non-small-cell lung cancer (NSCLC) yield an expected 5-year survival rate of less than 15% [2, 5, 7]. In order to develop more effective treatment, newer therapies and further insights into the biology of NSCLC are required. Lung cancer, like other solid tumors, depends upon neovascularization to continually supply the tumor with nutrients and oxygen [10, 12]. Investigations of tumor angiogenesis have primarily focused on the role of angiogenic factors, such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), transforming growth factor-β (TGF-β), and basic fibroblast growth factor (bFGF) [16, 17, 25, 26]. However, recent studies have demonstrated an increasing role for endogenous angiostatic factors in the regulation of net tumor-associated neovascularization. For example, the loss of endogenous angiostatic molecules may contribute to the tumor-associated angiogenic activity that enhances tumorigenesis and spontaneous metastasis [13, 18, 20, 27]. This can be re-stated by saying that overall tumor-derived neovascularization is regulated by an imbalance characterized by over-expression of angiogenic factors relative to an under-expression of angiostatic factors within the tumor.

We have found that one such endogenous angiostatic factor in human NSCLC is the CXC chemokine interferon-γ inducible protein 10 (IP-10, or CXCL10 in the revised nomenclature [28] for the chemokine family) [3].

Levels of CXCL10 are reduced in surgical specimens of adenocarcinoma, a more aggressive form of NSCLC, compared to squamous cell carcinoma [3]. This was confirmed in an animal model where tumor growth of a squamous cell carcinoma cell line (Calu-1) was associated with higher levels of CXCL10 than an adenocarcinoma (A549) [3]. Regardless of the cell line, we found an inverse correlation between tumor growth and tumor-associated levels of CXCL10 [3]. Intratumor injection of CXCL10 reduced tumor growth via an angiostatic mechanism [3]. In this current study, we hypothesized that localized treatment with CXCL10 would lead to prolonged survival in a SCID mouse model of human NSCLC, by restoring the imbalance toward an angiostatic environment within the primary tumor. We confirmed that intratumor treatment with CXCL10 (100 ng every other day) resulted in significant inhibition of tumor growth, and found that prolonged treatment resulted in improved survival and reduction of spontaneous lung metastases. Typical of therapies directed at inhibition of angiogenesis, we found that treatment with intratumor CXCL10 was associated with no change in the rate of proliferation of tumor cells (as assessed by PCNA staining), but an increased rate of apoptosis (as assessed by TUNEL staining). The occurrence of metastases was inhibited by CXCL10, and this required continued treatment, as animals that were treated for only 10 weeks prior to stopping intratumor CXCL10 demonstrated reduced survival compared to those that received continued treatment.

Materials and methods

Reagents

Human IP-10 (CXCL10) was purchased from Peprotech (Rocky Hill, N.J.). Recombinant CXCL10 was diluted in PBS with 0.1% human serum albumin (HSA). The A549 (adenocarcinoma) cell line (American Type Culture Collection, Rockville, Md.) was maintained in sterile 150-cm^2 tissue culture flasks in RPMI-1640 (BioWhittaker, Whittaker, Calif.) supplemented with 1 mM glutamine, 25 mM HEPES buffer, 100 U/ml penicillin, 100 ng/ml streptomycin and 10% fetal calf serum. PCNA antibodies, coupled with peroxidase enzyme were purchased from DAKO (Carpenteria, Calif.). Reagents for TUNEL staining (in situ cell death detection kit) were purchased from Boehringer-Mannheim, Mannheim, Germany.

Human NSCLC-SCID mouse chimeras

Four- to six-week-old female CB17-SCID mice (Taconic Farms Germantown, N.Y.) with serum Ig <1 µg/ml were injected subcutaneously with A549 human NSCLC cells (1×10^6 cells in 100 µl) into each flank. The animals were maintained under sterile conditions in laminar flow rooms. Tumor-bearing mice received intratumor injections of either human recombinant CXCL10 (0.1, 1, 5 or 10 µg in 20 µl of sterile saline every other day) or control [0.1% HSA in PBS (Sigma)], beginning at the time of tumor inoculation. We have shown a high degree of correlation between tumor mass (g) or volume, and the area of a tumor (as measured by length × width; $r > 0.9$, data not shown); therefore, we report all tumor sizes as mm^2, since these measurements are more readily made on living animals. Tumor measurements were made weekly in all mice. In survival studies, animal care technicians and investigators, blinded to treatment group, monitored each cage for mice with signs of excessive morbidity. Investigators were notified of animals appearing moribund or experiencing difficulty reaching food and water and these animals were euthanased. The day of euthanasia was recorded as the day of death. The survival study was terminated and all remaining group I animals were euthanased when the control and group II mice had all succumbed to metastatic tumor burden. In subsequent studies, cohorts of mice were euthanased at selected time-points (weeks 6, 10, and 14; $n = 6$ per group per time-point) to enumerate the rate of occurrence of spontaneous metastases in different treatment groups ($n = 6$ per group). At time of death, tumors were dissected from the mice. The lungs were inflated and fixed with 4% paraformaldehyde for 24 h and transferred to 70% ethanol. Before paraffin embedding, each lung was examined under a 4× magnifier to count grossly visible metastases. All study animals were handled in accordance with the guidelines of the University Committee on the Use and Care of Laboratory Animals. Principles of laboratory animal care, as stipulated in NIH publication No. 85–23, 1985 revision, were adhered to in all studies.

Measurement of apoptosis and proliferation

Five-micrometer sections of paraffin-embedded A549 tumors from SCID mice were stained, using either the TUNEL method to detect apoptosis or proliferating cell nuclear antigen (PCNA) antibody conjugated to horseradish peroxidase (DAKO). Cells were counted in 3 high-power fields per tumor (400×; 5 high-power fields counted for PCNA), after scanning at low power (40×) to avoid areas of frank necrosis. Results were expressed as the number of apoptotic nuclei per 400× field, or the number of proliferating (PCNA-positive) cells/400× field.

Statistical analysis

Survival studies were performed with 10 animals in each treatment group. The subsequent studies involved a minimum of 12 human NSCLC tumors or 6 SCID mice at each time-point or for each treatment group. Groups of data were evaluated by analysis of variance to indicate groups with significant differences. The Mann-Whitney *U*-test was used to compare 2 groups in observations that were not normally distributed (numbers of metastases prior to 10 weeks), and the Kruskall-Wallis modified ANOVA was used for comparison of the three treatment groups (after 10 weeks). Results are presented as means ± SEM for normally distributed data. Non-normally distributed data are presented as median (with maximum and minimum range in parenthesis). All data were analyzed on a Dell computer using the Prism 3.0 software package (GraphPad Software Inc., San Diego, Calif.). Bonferoni's correction was applied to all multiple comparisons.

Results

Survival of A549 tumor-bearing mice treated with CXCL10

Since we have previously demonstrated the efficacy of CXCL10 (1 µg, intratumoral, every other day) in reducing tumor angiogenesis and growth, we performed preliminary experiments to determine the effectiveness of various doses of intratumoral CXCL10. This was to permit the prolonged administration of CXCL10 in a survival study for a minimum of three months. Over 8 weeks of tumor growth, we found that 0.1 µg of CXCL10 was of equal efficacy to 1, 5, or 10 µg in terms of the degree of inhibition of tumor growth (approxi-

mately 50% inhibition in tumor size at 8 weeks (data not shown). We used this dose in subsequent survival studies.

Tumor sizes from mice treated with CXCL10 continuously (group I), for 10 weeks (group II), or with control protein (0.1% HSA in PBS) are shown in Fig. 1A. Consistent with our previous findings, treatment with intratumor injections of the angiostatic factor, CXCL10, significantly inhibited tumor growth. Survival of mice in these treatment groups is depicted in Fig. 1B, demonstrating a doubling in median survival from 86 days in control mice, to over 169 days in mice treated continuously with CXCL10 (0.1 μg three days per week, $P<0.0001$ by log rank test; Table 1). Group II mice had an intermediate median survival of 130 days ($P=0.001$ by log rank test for trend). This suggests that in the absence of continued treatment, tumors of CXCL10-treated mice resume the growth rate of tumors from mice that had never been treated.

Metastases are inhibited in mice treated with CXCL10

In this animal model, mice eventually succumb to significant metastatic tumor burden in the lungs. We have previously shown that CXCL10 treatment is associated with a reduction in metastases. However, it is unclear if intratumor injection of CXCL10 at this low dose inhibits metastasis via a local mechanism, or if metastases were inhibited by a systemic effect of the injected CXCL10. In order to determine the mechanism by which CXCL10 treatment improved survival in A549 tumor-bearing mice, we treated cohorts of mice with either CXCL10, or control protein, and killed mice ($n=6$ per time-point per treatment group) at 6, 10, and 14 weeks to determine the effect of CXCL10 on metastases. Consistent with our previous findings, CXCL10 inhibited metastases through 14 weeks of treatment. At 10 weeks, the rate of development of metastases significantly increased in control-treated mice. Metastases also occurred in the group treated continuously with CXCL10, but at a markedly reduced rate. In group II (treated for only 10 weeks), after treatment was stopped, metastases continued to occur at a rate intermediate between the control group and the continuously treated group (Table 2).

These findings suggest that local inhibition of angiogenesis by CXCL10 prevented a step in the metastatic process, perhaps by reducing access of A549 cells within the primary tumor to the circulation. Balanced proliferation and apoptosis has been referred to as the hallmark of angiogenic therapy in solid tumors [14, 19]. In order to determine if the inhibition of metastases by intratumoral CXCL10 was due to a systemic angiostatic effect, or due to local inhibition of metastases in the primary tumor, we performed TUNEL staining on primary and metastatic tumors in the CXCL10- and control-treated tumors to compare the relative incidence of apoptosis in these conditions. Consistent with our previous data [3], apoptosis in the primary tumors was increased in 10-week tumors of mice treated with CXCL10 (Table 3), compared to tumors from control-treated mice. However, in the lung metastases taken from mice killed at week 10, there was no difference in the rate of apoptosis, as assessed by TUNEL staining between CXCL10- and control-treated mice (Table 3), suggesting that the lung metastases were not subjected to

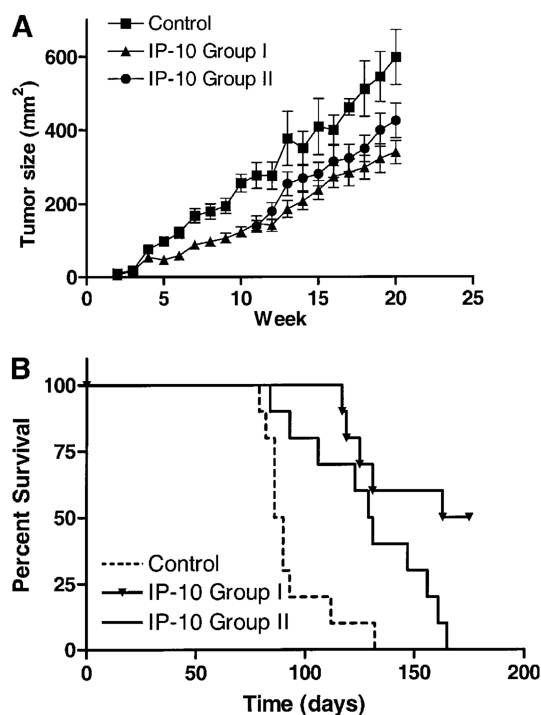

Fig. 1 **A** Sizes of tumors in mice treated with *control* (human serum albumin 0.1% in PBS), IP-10 (0.1 μg every other day) continuously (*Group I*), or for 10 weeks (*Group II*). **B** Survival of mice treated with HSA (human serum albumin 0.1% in PBS, vehicle *control*), IP-10 (0.1 μg every other day) continuously (*Group I*), or for 10 weeks (*Group II*). Median survival in these groups was 86, 169, and 130 days, respectively ($P<0.0001$ for trend by log rank test)

Table 1 Time of death for each treatment group. The time of death after tumor inoculation was recorded as the day of euthanasia of animals having significant tumor-related morbidity by animal technicians or investigators blinded to treatment groups. *C* censored data (the experiment was terminated when group II had reached 100% mortality)

Animal	Time of death (days)		
	Control (HSA)	CXCL10 group I (continuous)	CXCL10 group II (10 days)
1	79	117	84
2	82	119	93
3	86	125	106
4	86	131	123
5	86	169	129
6	90	C	131
7	90	C	147
8	93	C	156
9	112	C	161
10	132	C	165

Table 2 Occurrence of metastases in IP-10 treated and control mice. Lung metastases in A549 tumor-bearing mice treated with CXCL10 [continuously (*group I*) or for 10 weeks (*group II*)], or with control protein [0.1% human serum albumin in PBS (*HSA*)]. Data are presented as mean ± SEM, with median, and maximum presented in parenthesis, because of the non-parametric nature of the occurrence of metastases in this experiment groups

Group	Metastases [mean ± SE (median, maximum)]		
	Week 6	Week 10	Week 14
HSA	2.5 ± 1.0 (1.0, 10)	4.3 ± 1.6 (4.5, 10)	10.67 ± 2.6 (7.0, 22)
CXCL10 group I (continuous)	0.2 ± 0.2 (0.0, 1)	1.8 ± 1.3 (0.5, 8)	4.0 ± 0.6 (4.0, 6)
CXCL10 group II (10 weeks)	NA	NA	7.33 ± 4.0 (2.0, 21)

the angiostatic effect of intratumoral CXCL10. Also consistent with our previously published data, proliferative activity, as assessed by PCNA staining, was similar in primary tumors from each group [PCNA^{+} cells/hpf at 400×: HSA, 94 ± 5; group I, 97 ± 4; group II, 107 ± 7 ($P = 0.26$ by ANOVA)].

Discussion

In this study, we found improved survival in tumor-bearing SCID mice treated with low-dose intratumor injections of recombinant human CXCL10. In addition to extending our previous findings of CXCL10 as a potent angiostatic factor that inhibits the growth of NSCLC, these data demonstrate a clinically relevant endpoint, providing the rationale for further development of CXCL10 as an anti-tumor agent in humans. We found that the improved survival of CXCL10-treated mice was associated with a decrease in spontaneous metastases. It is unlikely that the quantity of CXCL10 administered (100 ng every other day), reaches sufficient systemic levels to have a direct effect on distant metastases. Rather, we propose that this is an indirect effect on metastases by inhibiting angiogenesis within the primary tumor. One hallmark of angiogenesis is increased permeability of the vessel wall [11], a feature that may facilitate metastases [9]. Inhibition of angiogenesis might also result in reduced access to the microvasculature by tumor cells migrating through the extracellular matrix. Our data suggest that localized inhibition of angiogenesis is sufficient to interfere with the metastatic process. This could be achieved through local delivery of gene therapy vectors via a bronchoscope, or the use of sustained-release polymers containing an angiostatic agent implanted in the vicinity of the tumor.

Table 3 Apoptosis, as determined by TUNEL staining, in primary and metastatic tumors. TUNEL detection of apoptotic cells in the 10-week primary tumors or lung metastases of A549 tumor-bearing mice treated with CXCL10 or control protein (*HSA* 0.1% human serum albumin in PBS)

Group	TUNEL^{+} cells/400× field (mean ± SE)	
	Primary tumors	Lung metastases
HSA	7.8 ± 0.4	5.0 ± 0.9
CXCL10 group I (continuous)	10.3 ± 1.0($P = 0.01$)	5.3 ± 1.0

In contrast to our data showing that continued treatment with CXCL10 is required for continued inhibition of tumor growth and metastases, Boehm et al, using endostatin to treat mice with a variety of human cancer cell lines, found that after several cycles of anti-angiogenic therapy, their mice could effectively be cured of their tumors [6]. The discrepancy could be related to many factors, including the mode of delivery (local injection versus systemic), the tumor model employed, (syngeneic cell line in immunocompetent vs. human tumor cell line immunocompromised mice), and the quantity of angiostatic agent used. In the study of Boehm and co-workers, mice received 20 mg/kg of endostatin daily, or 400 µg per day in a 20-g mouse [6]. In contrast, our study employed SCID mice receiving 0.1 µg or 100 ng of intratumor CXCL10 every other day. Therefore, these two studies do not allow direct comparisons of the potency of these two angiostatic agents. It is also notable that, in spite of continued treatment, most CXCL10-treated mice eventually succumbed to disease. It is possible that, even at the slower rate of tumor growth, the increased size of the tumor resulted in a "dilution" of the angiostatic effect of CXCL10, allowing some areas of the tumor to escape the angiostatic effect and to metastasize. Nevertheless, our experiment was conceived to test the hypothesis that the angiostatic activity of CXCL10 was capable of producing improved survival in tumor-bearing mice. We did not test whether increasing the dose of CXCL10 with time would have further improved survival.

In both our current and previous study, we found that CXCL10 treatment was associated with an increased rate of apoptosis within the primary tumors, with proliferation remaining unchanged [3]. We have previously found that IP-10 does not directly induce apoptosis of NSCLC cells in vitro [3]. The increase in apoptosis noted in vivo is more likely a result of the angiostatic activity of IP-10. This increased rate of apoptosis relative to proliferation has been referred to as the hallmark of angiostatic therapy [14, 19]. While the magnitude of the difference we detected in our study was small, O'Reilly and colleagues have demonstrated that similarly small differences in the rate of apoptosis (determined by TUNEL positivity) within tumors can account for dramatic differences in the rate of growth [19].

The metastases found in the lungs of mice treated with CXCL10 did not demonstrate this increase in apoptosis, suggesting that the reduction of metastases in CXCL10-treated mice was due to local inhibition of angiogenesis within the primary tumors, and not due to systemic angiostatic activity. Scarcity and expense of the reagent prevented us from determining if higher doses of CXCL10 delivered systemically would have demonstrated evidence of systemic inhibition of angiogenesis. However, our findings suggest that local angiostatic therapy can delay the occurrence of metastases, and that this translates into improved survival. This finding has implications for the design of gene therapy in that local activity (as opposed to systemic therapy) may be sufficient to provide meaningful improvement in clinically relevant outcomes (survival and prevention of metastases).

The cytokine cascade of IL-12, inducing interferon-γ, with subsequent production of interferon-inducible angiostatic chemokines (CXCL10 and monokine induced by interferon-γ, [MIG/CXCL9]) may be a critical anti-tumor pathway. Others have demonstrated that CXCL10 and the related angiostatic CXC chemokine, CXCL9, are critical mediators of the "downstream" anti-tumor effects of IL-12, a potent anti-tumor cytokine [15, 23]. In addition to CXCL10, we have previously shown that CXCL9 has potent anti-tumor activity, using both direct injection and adenovirus-mediated gene delivery to the tumor [1]. Systemic administration of chemokines may have fewer side effects than administration of more proximal members of this cascade, given the more pleiotropic biologic effects of interferons, but this has not been proven or tested in clinical trials. Clinical trials of interferon in cancer patients have demonstrated severe toxicity in up to 50% of patients [21]. Use of more distal mediators of this cascade could be expected to have fewer side effects than interferon or IL-12. Indeed, in our study we detected no signs of overt toxicity (weight loss, diminished activity level) after treatment of SCID mice for up to 5 months (data not shown). On the other hand, while IP-10 is chemotactic for Th1 lymphocytes, use of distal angiostatic mediators has the potential disadvantage of lacking other (presumably) beneficial immunomodulatory effects of IL-12 or IFNγ. Our study using SCID mice as the host for the growth of human tumor cell lines does not allow us to determine if CXCL10 also has anti-tumor activities that involve the recruitment of cytotoxic lymphocytes.

Other chemokines with angiostatic activity may also prove to be beneficial as anti-tumor agents, for example Sharma and colleagues have found that the CC chemokine secondary lymphoid chemokine (SLC; also known as exodus 2, 6C-kine, or CCL21) has potent anti-tumor activity that is related to increased dendritic cell-dependent activation of cytotoxic T-cells [24]. Using this same chemokine in a SCID mouse model, we have found that it also possesses angiostatic activity that inhibits tumor growth [4]. It is interesting to note that 6C-kine shares the property of binding to the CXC chemokine receptor-3 (CXCR3) in mice, with CXCL10, and CXCL9, also angiostatic factors. Another CXCR3 ligand has been identified: interferon-inducible T-cell attracting chemokine (ITAC, or CXCL11). As of yet, there is no data on whether this chemokine possesses anti-tumor activity. Recently, Romagnani and colleagues have identified CXCR3 as the receptor that mediates the angiostatic activity of CXCL10 [22]. Cell-cycle dependent expression of CXCR3 was demonstrated, and this expression of CXCR3 was greater in tissues taken from inflammatory or neoplastic conditions compared with normal tissues [22]. This finding is important in that it suggests that other ligands for CXCR3, either naturally occurring or synthetic, might be potent angiostatic agents for the treatment of cancer or angiogenesis-dependent inflammatory diseases.

In summary, we have confirmed our previous finding of potent anti-tumor activity of the angiostatic CXC chemokine IP-10/CXCL10. We have further found that even in low doses, the inhibition of angiogenesis in the primary tumor leads to meaningful improvement in survival and reduced metastases in a SCID mouse model of human lung cancer.

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