Effect of Interleukin 1 Receptor Antagonist Gene Transduction on Human Melanoma Xenografts in Nude Mice


INTRODUCTION

Tumors secrete multiple cytokines or factors that can alter the tumor microenvironment and promote tumor growth and metastases. Many of these factors promote an angiogenic phenotype in stromal or infiltrating host cells, resulting in the development of a neovasculature that provides nutrients for continued tumor growth and access to the vasculature for potential metastatic spread. Expression or overexpression of these tumor-derived angiogenic or inflammatory proteins in vivo has been shown to reflect an aggressive phenotype with respect to tumor growth, metastatic potential, and shortened survival (1–5). Antiangiogenic agents that inhibit proteins such as vascular endothelial growth factor or block matrix metalloproteinases are in clinical trials, alone or in combination with other antiangiogenic agents, in vitro proliferation of each parental cell line was comparable to the proliferative rate of each transduced cell line. IL-1ra-transduced SMEL (SMEL/IL-1ra) showed significantly slower tumor growth compared with null-transduced and parental cell lines (P < 0.001, ANOVA-Bonferroni/Dunn). There was no difference in growth rates between PMEL and IL-1ra-transduced PMEL (PMEL/IL-1ra). A mixing study of SMEL and SMEL/IL-1ra showed significant inhibition of tumor growth at various ratios (P < 0.001, ANOVA-Bonferroni/Dunn). There were significantly fewer lung metastases with SMEL/IL-1ra versus SMEL (P < 0.002). IL-1ra decreases in vivo growth and metastatic potential of a human melanoma xenograft that constitutively secretes IL-1. This effect may be exploitable using clinically available IL-1ra for the treatment of human cancers.

MATERIALS AND METHODS

Isolation of the IL-1ra Gene and Cloning Strategy. Frozen peripheral blood mononuclear cells were obtained from a leukopheresis of a volunteer donor and frozen. The cells were subsequently thawed and plated in a culture flask containing Iscove’s media supplemented with 10% AB serum and DNase. The cells were incubated for 3 h and washed twice with PBS. The RNA of the adherent monocytes was purified using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. cDNA was created from the purified RNA using SuperScript II (Invitrogen). The IL-1ra gene was amplified by PCR using gene-specific primers (5’-GATCAAGCTTATGCGATTA-GAGAC-3’ and 5’-GATCTCGATCTACCTGTCCTCTCTT-3’) and high-fidelity Pfx polymerase (Invitrogen). The amplified product was inserted into the blunt-4-TOPO cloning vector (Invitrogen) for sequencing using the manufacturer’s protocol. The vector containing the IL-1ra gene was transduced into Max efficiency DH5α cells (Invitrogen) and expanded overnight in LB broth with ampicillin (Strategene, La Jolla, CA) to provide selection pressure. The plasmid was purified using a mini plasmid prep kit (Qiagen, Valencia, CA). The portion of the vector containing the IL-1ra gene was sequenced using primers specific for the recombinant protein (ATG-CTGAGTTAGCCAAAAGGTTA-GAGAC-3’ and 5’-GATCTCGATCTACCTGTCCTCTCTT-3’). The sequencing reactions were analyzed on an ABI Prism sequencer (Applied Biosystems, Foster City, CA). DNA sequence determination was performed using the manufacturer’s protocol. The vector containing the IL-1ra gene was transduced into SMEL and PMEL (SMEL and PMEL) with differential (high versus low, respectively) constitutive production of IL-1 by ELISA. The IL-1ra gene was isolated from monocytic RNA by PCR and retrovirally transduced into SMEL and PMEL. In vitro cell proliferation was evaluated using a WST-1 assay. A thymic nude mice received s.c. or i.v. injection with parental, vector-transduced, or IL-1ra-transduced melanoma cells, and tumor growth, lung metastases, and histology were characterized. IL-1 was produced by SMEL in vitro and ex vivo (117 and 67 pg/ml/10⁶ cells/24 h, respectively), but not by PMEL (15 and 0 pg/ml/10⁶ cells/24 h, respectively). Neither made IL-1ra natively. Gene-transduced cell lines secreted >1000 pg/ml/10⁶ cells/24 h of IL-1ra by ELISA. In vitro proliferation of each parental cell line was comparable to the proliferative rate of each transduced cell line. IL-1ra-transduced SMEL (SMEL/IL-1ra) showed significantly slower tumor growth compared with null-transduced and parental cell lines (P < 0.001, ANOVA-Bonferroni/Dunn). There was no difference in growth rates between PMEL and IL-1ra-transduced PMEL (PMEL/IL-1ra). A mixing study of SMEL and SMEL/IL-1ra showed significant inhibition of tumor growth at various ratios (P < 0.001, ANOVA-Bonferroni/Dunn). There were significantly fewer lung metastases with SMEL/IL-1ra versus SMEL (P < 0.002). IL-1ra decreases in vivo growth and metastatic potential of a human melanoma xenograft that constitutively secretes IL-1. This effect may be exploitable using clinically available IL-1ra for the treatment of human cancers.

There is growing evidence that IL-1 promotes production of angiogenic proteins from host stromal or infiltrating cells in the tumor microenvironment that enhance tumor growth and metastases (12, 13). Investigators have shown an association between IL-1 production and altered tumor phenotype characterized by accelerated growth rates and increased metastases in experimental models (14). IL-1 production by tumors has been associated with a worse prognosis in patients with breast, lung, or head and neck cancers (15–17).

A naturally occurring antagonist to IL-1, the IL-1ra, has been extensively characterized and competitively blocks both isoforms of IL-1 at the receptor level (18–20). Previous work has shown that exogenously administered IL-1ra can block IL-1-induced experimental tumor metastases (14). Vidal-Vanaclocha et al. (21) have shown that development of hepatic metastases in a murine melanoma model could be inhibited by IL-1ra. Of note, others have shown that IL-1 may serve as an autocrine growth factor for some tumors (22), and La et al. (23) showed that IL-1ra directly inhibits proliferation of a murine skin carcinoma, suggesting that direct effects of IL-1ra on tumor cell proliferation may be responsible in part for effects on tumor growth or metastatic potential in vivo.

The current studies were performed to characterize the effects of IL-1ra gene transduction and protein overexpression on human melanoma cell lines and xenografts in nude mice. We selected two lines that differed in constitutive expression of IL-1 to demonstrate the specific effects of IL-1ra and show that IL-1ra inhibits tumor growth and metastases without direct effects on cellular proliferation in vitro or in vivo. The results suggest that clinically available recombinant IL-1ra may be useful in the treatment of human cancers that constitutively produce IL-1 and that further work with the recombinant protein is warranted.
Selection of the Tumor Lines and Assay for IL-1 Expression. Two human melanoma tumor lines, SMEL and PMEL, which have been derived from patients and characterized previously (26), were grown in culture and plated at 1 × 10^5 cells/well in a 6-well plate with 2 ml of RPMI-1640 for 12 h, and the supernatant was harvested. Simultaneously, 2 × 10^6 cells were injected s.c. into the flanks of athymic nude mice. The tumors were allowed to grow to 1 cm^2; harvested; digested at 37°C with a triple digest mix of hyaluronidase, collagenase, and DNase for 3 h; filtered; and plated at 1 × 10^5 cells/well in a 6-well plate with 2 ml of RPMI-1640 for 12 h, and the supernatant was harvested. Simultaneously, 2 × 10^6 cells were injected s.c. into the flanks of athymic nude mice. The tumors were allowed to grow to 1 cm^2; harvested; digested at 37°C with a triple digest mix of hyaluronidase, collagenase, and DNase for 3 h; filtered; and plated at 1 × 10^5 cells/well in a 6-well plate with 2 ml of RPMI-1640 for 12 h, and the supernatant was harvested.

Fig. 1. A, vector map of the retroviral expression plasmid pCLNCX with the IL-1ra gene inserted 3′ to the cytomegalovirus promoter. B, ELISA from the supernatant of each of the transduced and wild-type cell lines confirming production and secretion of IL-1ra by only the gene-transduced cell lines. C, PCR of the various clones demonstrating the presence of the neomycin resistance gene in all transduced cells and the presence of the gene encoding IL-1ra only in PMEL/IL-1ra and SMEL/IL-1ra (bottom). PMEL (wild type, Lane 1) and SMEL (wild type, Lane 4) do not contain either the IL-1ra or NeoR genes. PMEL/null (Lane 2) and SMEL/null (Lane 3) contain only the NeoR gene and lack the IL-1ra gene. PMEL/IL-1ra (Lane 3) and SMEL/IL-1ra (Lane 6) contain both the NeoR gene and the gene encoding IL-1ra. D, WST-1 reaction showing no difference in proliferation rates in vitro.
SMEL/IL-1ra cells to evaluate for a paracrine effect of IL-1ra production on experiments, parental SMEL cells were mixed (S. M. H.) who was blinded to the nature of the experimental groups. In other experiments, parental SMEL cells and SMEL/IL-1ra cells were injected s.c. into athymic nude mice that were irradiated with 500 cGy in a Gammarcell-40 irradiator (Nordian) just before tumor injection. Mice were euthanized on day 28, their trachea were cannulated with a 19-gauge needle, and the lungs were insufflated with India ink. The lungs were then harvested and washed in sterile PBS followed by Fecedes’ solution. The lobes were then dissected, and the number of lung metastases was counted. For all animal experiments, the observer conducting the measurements of tumor size or counting of metastases was blinded to design of the experiment.

RESULTS

A vector map for the pCLNCX expression plasmid following insertion of the IL-1ra gene by restriction digest is shown in Fig. 1A. After transduction of SMEL and PMEL with either a null pCLNCX retroviral vector or pCLNCX-IL-1ra, only the cell lines transduced with pCLNCX-IL-1ra produced IL-1ra as detected by ELISA of the cell supernatant (Fig. 1B) or by PCR (Fig. 1C). All four transduced cell lines expressed the neomycin resistance gene, indicating a successful transduction (Fig. 1C). There were no differences in proliferation rates in vitro between the transduced and nontransduced cell lines, indicating that there were no autocrine proliferative effects of IL-1ra on these tumors (Fig. 1D).

To determine the amount of secretion of IL-1α in vitro and in the tumor stroma ex vivo, quantitative ELISA for IL-1α was performed on supernatant from SMEL and PMEL melanoma cultured in vitro and ex vivo from cells harvested from tumor-bearing mice. The SMEL tumor line showed secretion of IL-1α both in vitro and ex vivo (117 and 67 pg/ml/10⁶ cells/24 h, respectively) whereas the PMEL tumor line produced very low or no detectable IL-1α in vitro or ex vivo (15 and 0 pg/ml/10⁶ cells/24 h, respectively). Transduction of the gene encoding IL-1α did not alter the amount of IL-1α produced compared with the respective wild-type cell lines, and none of the cell lines produced IL-1β by ELISA (data not shown).

Growth of s.c. SMEL/IL-1ra was statistically significantly decreased compared with the null-transduced or parental xenografts (P < 0.0001, ANOVA-Bonferroni/Dunn). However, PMEL/IL-1ra, PMEL/null, and parental PMEL xenografts, which do not produce IL-1α, all grew at identical rates (Fig. 2). When SMEL was mixed at various ratios with SMEL/IL-1ra ex vivo, there was a significant inhibition of tumor growth in vivo, indicating a marked paracrine effect of locally produced IL-1α on growth of tumor stroma.

Animal Models for Evaluation of Tumor Xenografts. Tumor cells (2 × 10⁶) were injected s.c. into the flanks of athymic nude mice, and the tumors were measured. Tumor areas were calculated by taking the product of the perpendicular diameters of each tumor over time. In a separate experiment, parental SMEL cells and SMEL/IL-1ra cells were injected s.c. into the flanks of athymic nude mice, and the mice were sacrificed, and tumors were harvested at approximately 16 mm² for quantitative vessel counts by a pathologist (S. M. H.) who was blinded to the nature of the experimental groups. In other experiments, parental SMEL cells were mixed ex vivo in different ratios with SMEL/IL-1ra cells to evaluate for a paracrine effect of IL-1ra production on tumor growth. In other mice, 1 × 10⁶ cells (SMEL, SMEL/null, or SMEL/IL-1ra) were injected via tail vein into athymic nude mice that were irradiated with 500 cGy in a Gammarcell-40 irradiator (Nordian) just before tumor injection. Mice were euthanized on day 28, their trachea were cannulated with a 19-gauge needle, and the lungs were insufflated with India ink. The lungs were then harvested and washed in sterile PBS followed by Fecedes’ solution. The lobes were then dissected, and the number of lung metastases was counted.

For all animal experiments, the observer conducting the measurements of tumor size or counting of metastases was blinded to design of the experiment.
(n = 6 mice/group) showed a large amount of central necrosis consistently present in SMEL/IL-1ra tumors that was not present in the parental SMEL tumors of equal size (Fig. 4). The mean number of mitotic tumor cells/high-powered field was 2.8 ± 0.37 in parental SMEL cells and 4.7 ± 0.53 in SMEL/IL-1ra cells (P2 = 0.054), supporting the in vitro findings that IL-1ra did not alter tumor size by affecting cellular proliferation rates in vivo.

The SMEL/IL-1ra clones had decreased metastatic potential compared with SMEL cells as evaluated in a lung metastasis model. The numbers of lung metastases 28 days after i.v. tumor injection were significantly lower in SMEL/IL-1ra compared with both SMEL/null and parental SMEL (3.0 ± 0.4 versus 45.6 ± 4.7 and 35.7 ± 2.1, respectively; P < 0.002; Fig. 5).

DISCUSSION

IL-1 is a pleiotropic angiogenic cytokine associated with an increase in the growth and metastatic potential of some human cancers such as squamous cell cancer of the head and neck or adenocarcinoma of the breast or lung (15, 16, 27). Previous work has shown that exogenously administered IL-1 can promote tumor growth and metastases in experimental tumor models (14, 17, 28), and IL-1ra administration has been shown to reduce the incidence of hepatic metastases in a murine melanoma model (21). The mechanisms for these observations are not known, but others have shown that IL-1 can cause autocrine proliferative effects on murine and human cancer cell lines, suggesting that IL-1ra may exert some antitumor activity in vivo by inhibiting IL-1-mediated tumor cell proliferation directly. The data from the current study support the prevailing theory that IL-1 produced by tumors in vitro and present in the tumor microenvironment in vivo may be important in tumor growth and metastatic potential. IL-1ra had no direct antiproliferative effects in vitro in two human melanoma lines that differed in their constitutive production of IL-1 and inhibited tumor growth in vivo selectively in the cell line that produced IL-1 natively.

It is also noteworthy that there was a significantly lower incidence of pulmonary metastases in SMEL/IL-1ra compared with wild-type or null-transduced SMEL; this supports a direct role for IL-1 in the metastatic phenotype of some human cancers. The mechanism of metastatic spread is a complex multistep process involving adhesion or trapping of tumor in the microcirculation, extravasation into the interstitium, and subsequent recruitment of host cells to establish a neovasculature (29–33). IL-1 has been shown to promote metastases in experimental tumors and may promote this process by up-regulation of endothelial cell surface expression of adhesion molecules or tissue factor, increased matrix metalloproteinase activity to promote tumor migration, and induction of localized vascular permeability with efflux of nutrient plasma and proteins into the interstitium (34–37).

The data reported in this study may have relevance to clinical cancer treatment. IL-1 is a known mediator of various acute and chronic inflammatory conditions, and a recombinant IL-1ra has been shown to have significant activity in vivo in abrogating the deleterious effects of endogenously produced IL-1 in experimental models of endotoxic shock and Gram-negative sepsis or models of chronic inflammation such as inflammatory bowel disease or arthritis (38, 39). The data suggest that IL-1 acts as an early mediator in the pathophysiology of these processes and promotes production of a cascade of downstream effector proteins from various cell types. It therefore represents an ideal target to abrogate or reverse these deleterious effects. In support of this concept, elevated levels of IL-1 in synovial...
fluid of patients with RA are thought to mediate the characteristic joint destruction and neovessel formation in this disease (40, 41). Recombinant IL-1ra has been shown to ameliorate this condition and is now approved by the Food and Drug Administration for the treatment of patients with moderate to severe RA. The mechanisms through which IL-1ra may exert its therapeutic effects are not entirely known but most likely are related to inhibition of inflammation and neovessel formation in the articular space, a pathophysiology that shares some features with the neovascularization of growing tumors.

Our data demonstrated a substantial bystander effect of IL-1ra in vivo, suggesting that local concentrations of the recombinant IL-1ra protein, when administered systemically, might also be sufficient to inhibit tumor growth or metastases. Further work characterizing the recombinant IL-1ra in experimental models of tumor growth and metastases is warranted, and we support further evaluation of the use of IL-1 blockade for the treatment of human cancers.

References


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