The Role of Interleukin 1 in Growth and Metastasis of Human Cancer Xenografts

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Abstract **Background:** Interleukin 1 (IL-1) is a pluripotent cytokine that promotes angiogenesis, tumor growth, and metastasis in experimental models; its presence in some human cancers is associated with aggressive tumor biology. The purpose of these studies was to characterize the role of IL-1 in human cancers and determine if inhibition of IL-1 via its receptor antagonist, IL-1Ra, alters tumor growth and metastatic potential.

Methods: IL-1 mRNA or protein levels were determined in clinical tumor samples, cancer cell lines, and xenografts using quantitative reverse transcription-PCR or ELISA. Biological activity of tumor-derived IL-1 protein was shown via induction of permeability across endothelial cell monolayers. The effects of recombinant IL-1Ra on tumor lines in culture (cell proliferation and IL-8 secretion) and in xenograft models (tumor growth, metastatic potential, and intratumoral levels of IL-8 and VEGF) were characterized. The effects of IL-1Ra-mediated regression of xenograft growth on angiogenic proteins (IL-8 and VEGF) were evaluated in an IL-1-producing melanoma (SMEL) xenograft model.

Results: IL-1 mRNA was highly expressed in more than half of all tested metastatic human tumor specimens including non – small-cell lung carcinoma, colorectal adenocarcinoma, and melanoma tumor samples. Constitutive IL-1 mRNA expression was identified in several cancer cell lines; tumor supernatant from these cell lines produced a significant increase in endothelial cell monolayer permeability, a hallmark event in early angiogenesis, in an IL-1-dependent manner. Moreover, systemic recombinant IL-1Ra resulted in significant inhibition of xenograft growth and neovessel density of IL-1-producing, but not non-IL-1-producing, tumor cell lines. Subsequent analysis of SMEL, a melanoma cell line with constitutive IL-1 production, showed that neither exogenous IL-1 nor IL-1Ra altered tumor cell proliferation rates *in vitro*. Gene expression analyses of IL-1Ra-treated SMEL xenografts showed a >3-fold down-regulation of 100 genes compared with control including a marked down-regulation of IL-8 and VEGF.

Conclusions: These data show that the *IL-1* gene is frequently expressed in metastases from patients with several types of human cancers. IL-1Ra inhibits xenograft growth in IL-1-producing tumors but has no direct antiproliferative effects *in vitro*; decreased tumor levels of IL-8 and VEGF may be an early surrogate of IL-1Ra-mediated antitumor activity. IL-1Ra may have a role alone or with other agents in the treatment of human cancers.

Many proteins or other factors present in the tumor microenvironment directly promote tumor cell proliferation and survival or induce processes, such as angiogenesis, that indirectly promote tumor growth and metastasis. Experimental models have shown that local production of interleukin 1 (IL-1) influences tumor growth and metastases either through direct proliferative effects or by promoting inflammatory and angiogenic pathways in host cells (1 – 4). IL-1 induces an angiogenic phenotype in endothelial tissue *in vitro* and angiogenesis *in vivo* (4 – 6); many of its angiogenic activities seem to be mediated indirectly via induction of proteins from other cell types including fibroblasts and immune cells typically present in the tumor microenvironment (7 – 11).

In a murine model, the IL-1 receptor antagonist (IL-1Ra) inhibits hepatic metastases and blocks accelerated tumor growth produced by simultaneously administered exogenous IL-1 (12); inhibition of IL-1 using microencapsulated genetically engineered cells that constitutively produce IL-1Ra implanted in mice will inhibit tumor angiogenesis and growth (13). We have shown that gene transduction and expression of IL-1Ra significantly inhibits s.c. growth and metastatic potential of a human melanoma xenograft that produces IL-1 constitutively while having no effect on a human melanoma

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Note: D.M. Elaraj and D.M. Weinreich contributed equally to this work.

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xenograft that does not produce IL-1 (14). In clinical breast and gastric cancer biopsies, *IL-1* gene expression has been shown to be an adverse prognostic factor (15, 16).

The current studies were done to further characterize the role of IL-1 in human cancer by quantifying the frequency of *IL-1* gene expression in metastatic tumor biopsies from patients with colon or lung cancers or melanoma. In addition, we evaluated the utility of systemically administered IL-1Ra, a protein in clinical use for patients with severe rheumatoid arthritis (17, 18), on human cancer xenografts in mice and explored the cellular and molecular mechanisms associated with its antitumor activity. The results indicate that IL-1 is expressed in a high percentage of several types of common human cancers and that systemically administered recombinant IL-1Ra can result in sufficient inhibition of local IL-1 to inhibit tumor growth via antiangiogenic actions. Moreover, the antitumor activities of IL-1Ra are strongly associated with marked inhibition of *IL-8* gene expression and protein production exclusively *in vivo* and suggest that local IL-8 inhibition may be a useful surrogate for confirming adequate IL-1 blockade in diseases mediated by this cytokine.

Materials and Methods

Materials. Anakinra (Kineret, rh-met-IL-1Ra, Amgen, Inc., Thousand Oaks, CA) is a recombinant, nonglycosylated form of human IL-1Ra. IL-1Ra is provided in prefilled syringes of 100 mg (150 mg/mL) diluted in water along with sodium citrate (1.29 mg), sodium chloride (5.48 mg), disodium EDTA (0.12 mg), and polysorbate 80 (0.70 mg). SMEL and PMEL are melanoma cell lines derived in our laboratory from biopsies of in transit metastases from two patients. WIDR and H20320, colon adenocarcinoma and lung non–small-cell carcinoma, respectively, were supplied by David S. Schrump (Surgery Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD) and SL-2 is a head and neck squamous cell carcinoma supplied by Carter VanWaes (National Institute on Deafness and Other Communication Disorders, NIH, Bethesda, MD).

Screening of biopsy samples and human tumor cell lines for IL-1 production. Quantitative reverse transcription-PCR (RT-PCR) was used to screen multiple human tumor samples and cell lines for gene expression of IL-1α and IL-1β. RNA was isolated from snap-frozen tumor biopsies of lung or liver metastases obtained from patients for clinical indications or research purposes after enrollment on Institutional Review Board–approved clinical research protocols at the Center for Cancer Research, National Cancer Institute using RNeasy kit (Qiagen, Valencia, CA). To determine IL-1 expression in cell lines, cells were grown in complete medium (DMEM or RPMI supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin) in a 5% CO_2 incubator at 37°C. Cells were washed twice with PBS and harvested with trypsin/EDTA. These cells were centrifuged at 1,500 × *g* for 15 minutes to collect cell pellet. RNA was isolated from the cell pellet using the RNeasy kit. The types of cancers evaluated included melanoma, colon adenocarcinoma, and non–small-cell lung cancer. The isolated RNA was reverse transcribed into cDNA using SuperScript II (Invitrogen, Carlsbad, CA) and the cDNAs obtained were used for *IL-1* gene expression. β-Actin was used to normalize IL-1 copy number (19). Custom primer and probe sequences were constructed (Biosource, Camarillo, CA). IL-1α: forward primer, ATTCATCCTGAATGACGCCT; reverse primer, ACCCATGTCAAATTTCACTGCTT; probe (FAM-TAMRA), TCAGTACCTCACGGCTGCTGCATTACATAA. IL-1β: forward primer, TGATGGCCCTAAACAGATGAAGT; reverse primer, GCCTGAAGCCCTTGCTFTAGT; probe (FAM-TAMRA), CATCCAGCTACGAATCTCCGACCACC. β-Actin: forward primer, GCGAGAAGATGACCCAGATC; reverse primer, CCAGTGGTACGGCCAGAGG; probe (FAM-TAMRA), CCAGCCATGTACGTTGCTATCCAGGC for use in a Prism Model 7700 Sequence Detector (Applied Biosystems, Foster City, CA). A cutoff of 1×10^3 copies of either IL-1α or IL-1β per 10^5 copies of β-actin was used to denote positive expression. Standards were run simultaneously with all samples and a tolerance of $R^2 > 0.99$ on the linear regression of the standards was required to accept the results of the reaction.

Table 1. Results of IL-1α or IL-1β mRNA expression in patient tumor biopsies evaluated by quantitative RT-PCR

Sample no.	IL-1α*	IL-1β*
Melanoma		
1	52	**3,750**
2	24	**1,520**
3	3	**1,000**
4	3	75
5	107	**1,530**
6	61	**14,100**
7	78	**6,900**
8	34	**10,900**
9	7	**1,670**
10	4	**3,050**
11	4	912
12	15	**3,580**
13	85	**2,020**
14	102	568
15	51	**22,500**
16	2	216
Colon adenocarcinoma		
1	31	87
2	17	53
3	13	87
4	46	54
5	88	98
6	149	**26,800**
7	14	144
8	**1,720**	**129,000**
9	253	**66,700**
10	5	501
11	**1,990**	**4,900**
12	14	**2,490**
13	7	**1,620**
14	75	**9,540**
NSCLC		
1	516	**15,100**
2	55	**3,333**
3	94	**3,540**
4	111	**1,590**
5	27	**5,560**
6	29	637
7	118	**1,390**
8	87	900

Abbreviation: NSCLC, non – small-cell lung cancer.
*Gene expression is expressed as copy number per 10^5 copies of β-actin; copy number >1000/10^5 copies of β-actin are in boldface.

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Table 2. Results of IL-1α or IL-1β mRNA expression in tumor cells lines evaluated by quantitative RT-PCR

Human tumor cell lines	IL-1α*	IL-1β*
SMEL (melanoma)	**3,267**	0
WIDR (colon cancer)	1	**122,932**
H2030 (NSCLC)	0	**29,084**
PMEL (melanoma)	35	67
SL-2 (squamous cell cancer)	140	0

*Gene expression is expressed as copy number per 10^5 copies of β-actin; copy number >1,000/10^5 copies β-actin are in boldface.

Endothelial cell permeability assay. Human umbilical vein endothelial cells (Clonetics, Walkersville, MD) were cultured in a 5% CO_2 incubator at 37°C in EGM-2 medium [basal endothelial cell medium with hydrocortisone, fibroblast growth factor, insulin-like growth factor 1, ascorbic acid, epidermal growth factor, GA-1000 (gentamicin/amphotericin), and heparin]. Human umbilical vein endothelial cells were passed for three generations and then plated on Transwell polycarbonate membranes for permeability assay and the Transwell filters were inserted in a six-well plate (Corning Costar Corp., Cambridge, MA) as previously described (20). Cells were cultured for 72 hours in EGM-2 medium and nonadherent cells were removed. Cells were treated with either 1 mL of conditioned supernatant from SMEL, H2030, or PMEL cell culture in the upper chamber or conditioned supernatant with IL-1Ra (10 mg/mL) in triplicate. Control cells were treated with 1 mL of fresh medium and fresh medium with human recombinant tumor necrosis factor (0.1 μg/mL, Knoll Pharmaceuticals, Whippany, New Jersey) served as positive control. Cells were incubated at 37°C for 90 minutes in a 5% CO_2 incubator. The inserts with membrane were washed with 2 mL of PBS with Ca^{2+} and Mg^{2+} once and fresh EBM-2 medium or medium with 1% factor VIII–deficient plasma and incubated for 1 hour. After the incubation period, inserts were washed with 2 mL of PBS with Ca^{2+} and Mg^{2+}, and 1.5 of mL Evans blue bound to 0.1% bovine serum albumin in PBS with Ca^{2+} and Mg^{2+} were added to the luminal chamber; 2 mL of PBS with Ca^{2+} and Mg^{2+} were added to the abluminal chamber and incubated for 1 hour. After 1 hour, PBS (100 μL) was removed from the abluminal chamber and the absorbance was measured with a spectrophotometer at 620 nm (DU530, Beckman, Fullerton, CA).

In vitro ***effects of IL-1Ra on cell proliferation.*** SMEL and WIDR cells were plated on flat-bottomed 96-well plates (Corning, Inc., Corning, NY) at a density of 1,500 per well and allowed to grow overnight in complete RPMI medium. Fresh medium alone or medium containing either IL-1Ra at concentrations of 0.1, 1, and 10 μg/mL or IL-1β (National Cancer Institute, Frederick, MD) at concentrations of 0.001, 1, and 1,000 ng/mL was added after incubation and cell proliferation was assayed after 1 hour and once daily for 4 days by WST-1 assay (Roche Diagnostics Corp., Indianapolis, IN). Proliferation was quantified by measuring the absorbance at 450 nm with a reference wavelength of 650 nm on a Multiskan MCC/340 microtiter plate reader (Titertek, Huntsville, AL).

Western blot for IL-1 receptor type I. Cells were homogenized in lysis buffer containing 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and 50 mmol/L Tris (pH 8.0), 1 mmol/L phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), incubated at 4°C for 20 minutes and then centrifuged at 10,000 × *g* for 15 minutes. Total protein concentration was determined in the supernatant by bicinchoninic acid protein assay (Pierce Biotechnology, Inc., Rockford, IL). The lysate (25 μg of total protein) was loaded in a 10% Bis-Tris NuPAGE gel (Invitrogen) and then transferred onto a 0.25-μm nitrocellulose membrane. Medium alone was used as a negative control and cell lysate prepared from the CCRF-CEM T-lymphoblastoid cell line (American Type Culture Collection, Manassas, VA) served as a positive control. IL-1 receptor type I (IL-1RI) rabbit anti-human polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as the primary antibody. The immunoreactive peptides

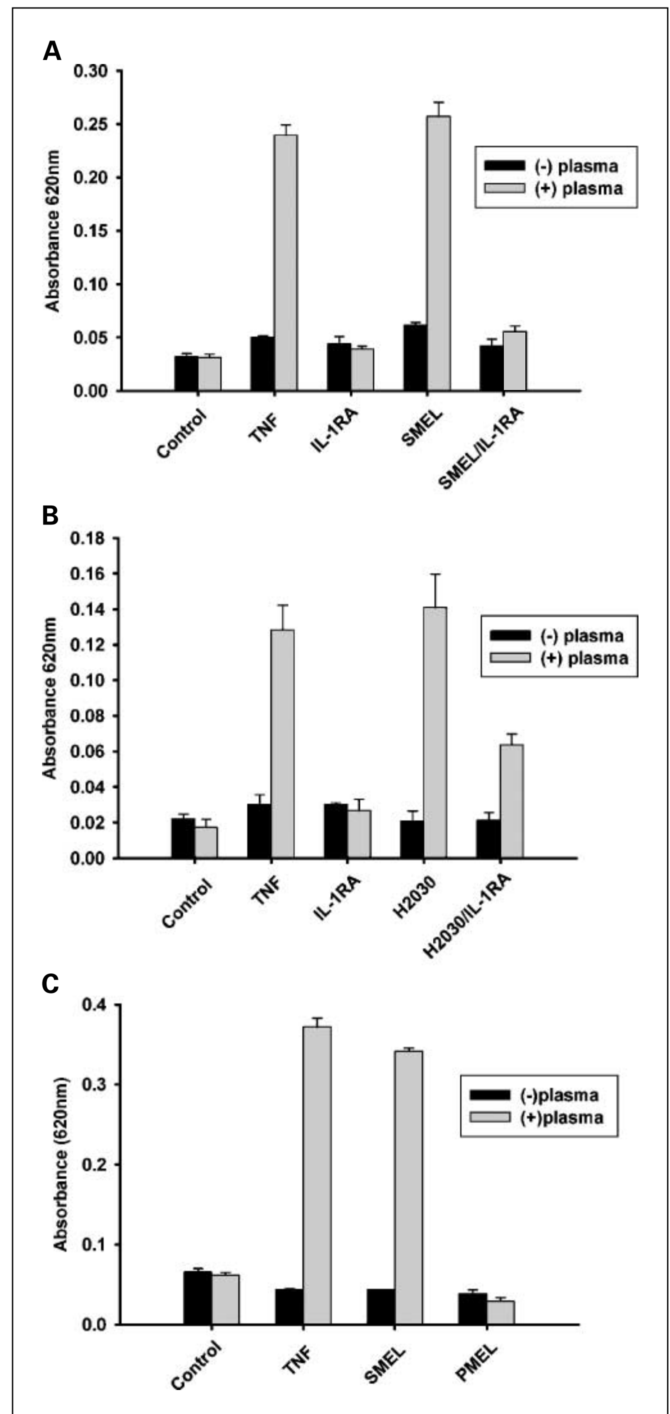

Fig. 1. Induction of endothelial cell monolayer permeability by tumor conditioned supernatant in an IL-1-dependent manner. Permeability across functional endothelial cell monolayers was determined by measuring absorbance at 620 nm 1 hour following supernatant exposure as described. Induction of permeability by SMEL (*A*) and H2030 (*B*) was completely or partially blocked by coincubation with 10 mg/mL IL-1Ra. PMEL did not induce permeability under identical experimental conditions (*C*). Tumor necrosis factor (*TNF*) at 10 ng/mL served as positive control. Columns, mean; bars, SE.

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were detected by using the WesternBreeze Chemiluminescent kit according to the protocol of the manufacturer (Invitrogen).

ELISA for IL-8. SMEL cells were plated at 1,500 per well in 96-well plates and allowed to incubate overnight. IL-1β at concentrations of 0.001, 1, and 1,000 ng/mL or IL-1Ra at concentrations of 0.1, 1, and 10 μg/mL was added on the next day and the control groups were fed with fresh medium. Supernatants from triplicate wells for each treatment condition were collected at 0, 1, 3, 6, 12, and 24 hours and frozen immediately at −80°C for IL-8 assay by ELISA (R&D Systems, Inc., Minneapolis, MN).

Effects of IL-1Ra on human tumor xenografts. Female NCr-*nu/nu* mice of ages 10 to 12 weeks, purchased from the National Cancer Institute (Frederick, MD), were used for this study. Mice were housed in a controlled environment of 12:12 hour dark and light cycle and provided food and water *ad libitum*. The NIH Animal Care and Use Committee approved all animal experimental procedures in advance. All mice from a single experiment were from the same litter. Two times 10^6 tumor cells were injected s.c. into the flanks of athymic nude mice. On the day of tumor injection, s.c. IL-1Ra therapy or placebo was started (n = 8-10 per group) and administered daily (2.4 mg/d for PMEL and SMEL and 5 mg/d for SL-2, WIDR, and H2030) into the opposite flank from tumor injection and tumor measurements were taken over time and compared with control group. Tumor measurements were made by an investigator who was blinded to the experimental groups; the same perpendicular diameters were recorded every other day and the tumor area was calculated as the product of the two.

To evaluate the effects of IL-1Ra on metastatic potential, SMEL cells were injected (1×10^6 cells) i.v. into tail vein of mice and IL-1Ra therapy was started on the day of tumor injection. On day 28, the mice were sacrificed, their trachea cannulated with a 19-gauge needle, and the lungs 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 counted. In a separate experiment, 1×10^6 SMEL cells were injected s.c. into the flanks of athymic nude mice and once-daily IL-1Ra therapy was initiated. Tumors were allowed to grow to ~ 10 mm^2 and then harvested. Vascular density and cellular proliferation in xenografts were quantitated as previously described (21, 22). H&E-stained and immunostained sections from harvested SMEL in each treatment group were analyzed by a pathologist (S.H.) who was blinded to the identity of the groups. Only good quality sections with well-demarcated staining and low background were analyzed. Microvascular density of SMEL tumors was calculated as the mean number of vWF-positive microvessels per high-power field (total magnification, ×600) based on a minimum of five high-power field evaluations. Mitoses were similarly counted as number of cells with positive nuclear staining for proliferating cell nuclear antigen per high-power field.

cDNA microarray. SMEL xenografts of IL-1Ra treatment and control groups were used for this study. Total RNA was isolated from pooled tumor samples derived from the SMEL xenografts using Trizol (Invitrogen) and the RNA samples were amplified as previously described (23). Amplified RNAs from four median-sized control tumors and from four median-sized IL-1Ra tumors were pooled by group and fluorescently labeled with Cy3- or Cy5-labeled dUTP (Perkin-Elmer Life Science Inc., Boston, MA). Six micrograms of antisense RNA were used for Cy3 labeling and 9 μg of aRNA for Cy5 labeling. We carried out

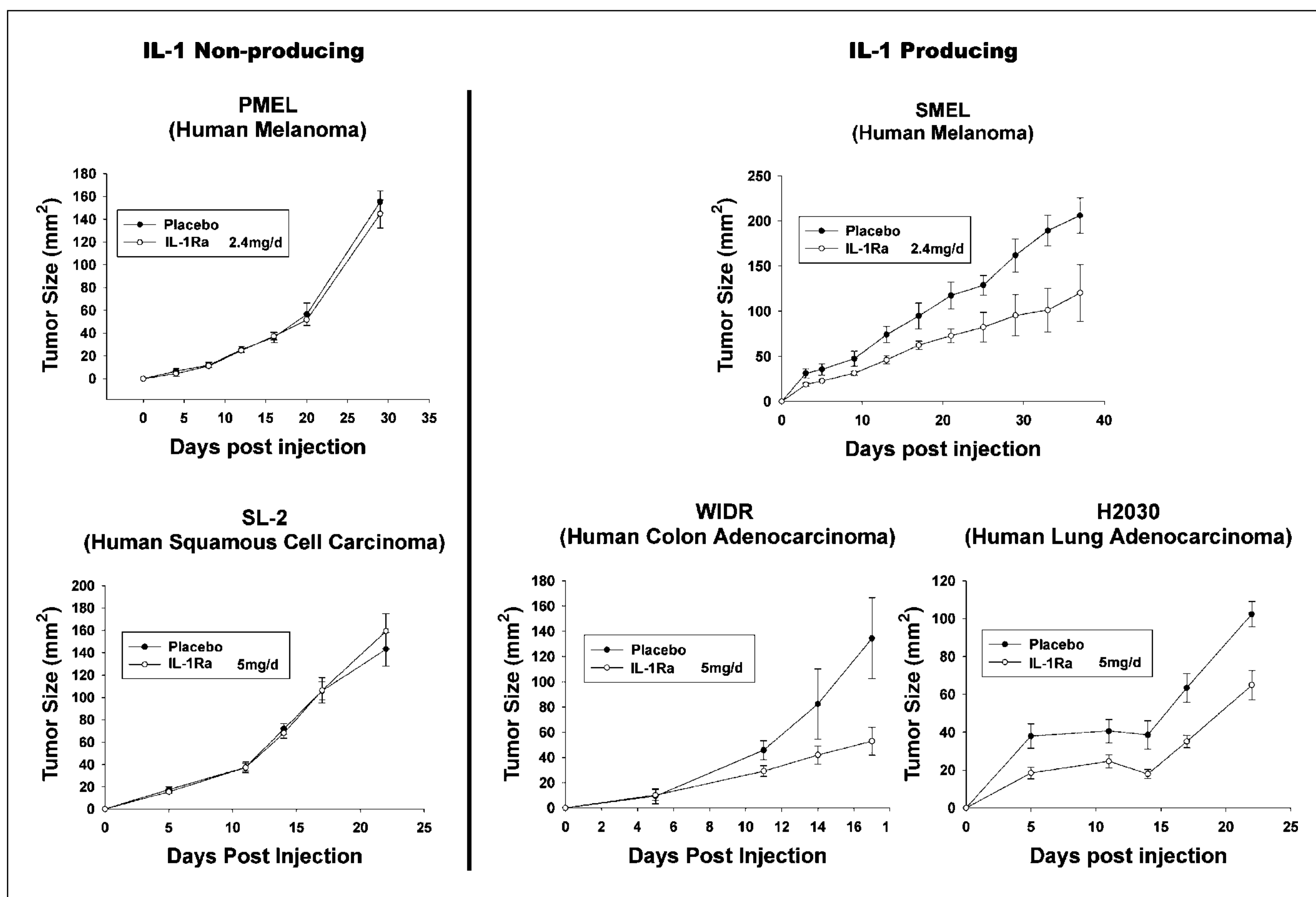

Fig. 2. Two times 10^6 tumor cells were injected s.c. into the flanks of athymic nude mice. On the day of tumor injection, s.c. IL-1Ra therapy at various doses or placebo began and was administered daily into the opposite flank from tumor injection. IL-1Ra consistently resulted in a statistically significant decrease in growth rate of all three IL-1-producing tumors tested but had no effect on two non-IL-1-producing tumors.

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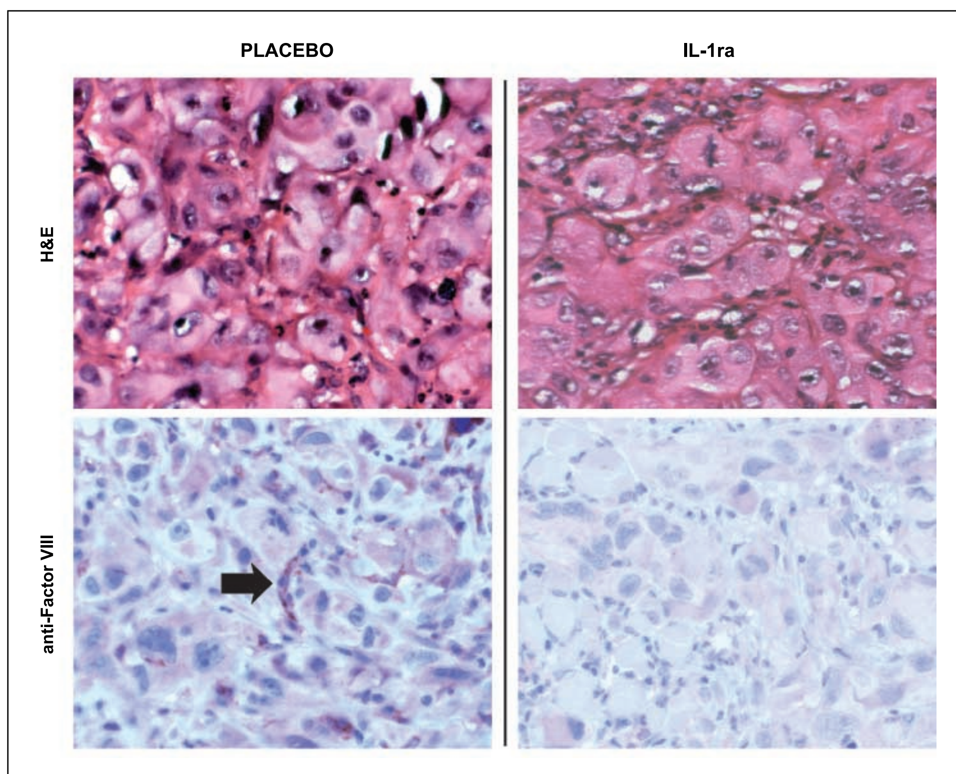

Fig. 3. SMEL was injected s.c. into the flanks of athymic nude mice and daily IL-1Ra therapy was initiated. Tumors were allowed to grow to ~16 mm^2 and then harvested for histologic and quantitative vessel counts by a pathologist (S.M.H.) who was blinded to the nature of the experimental groups. Histologic evaluation of SMEL tumors showed a marked decrease in vessel counts as assessed by factor VIII staining in the IL-1Ra-treated group compared with controls.

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at least two hybridizations for each sample using a dye-swap strategy to eliminate dye labeling bias. Hybridizations were carried out on a 10K human cDNA array slide (National Cancer Institute, Gaithersburg, MD). The hybridized arrays were scanned at 10-μm resolution using a GenePix 4000 scanner (Axon Instruments, Inc., Foster City, CA). Genes differentially expressed ≥3-fold were considered significant.

Validation of gene expression profile by quantitative RT-PCR. Quantitative RT-PCR was done to validate changes in gene expression identified by microarray analysis. cDNA synthesis was carried out using 1 μg of total RNA, oligo(dT)$_{12-18}$ primer, and SuperScript II reverse transcriptase (Invitrogen). Quantification of gene expression was done using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described (24). Probes were labeled with a reporter dye, 6-carboxyfluorescein (FAM), and a quencher, 6-carboxytetramethylrhodamine (TAMRA), at the 5′ and 3′ region, respectively. The primer and probe sequences for IL-8 were forward primer, 5′-GAACCATCTCACTGTGTGTAAACATG-3′; reverse primer, 5′-TTCACACAGAGCTGCAGAAATCA-3′; and probe, 5′-FAM-TCCAAGCTGGCCGTGGCTCTCTT-TAMRA-3′. Primers and probes for 18S rRNA were purchased from Applied Biosystems and used as internal control. Primer-specific amplifications of mRNA of relevant genes were carried out using Platinum Taq DNA polymerase (Invitrogen) and the following cycling conditions were used: 94°C for 3 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Amplicons were confirmed as single bands of appropriate molecular weight by agarose gel electrophoresis. The amount of amplified cDNA was then quantified using a spectrophotometer (Beckman DU530) and copy numbers were calculated using the molecular weight of each amplicon. RT-PCR reactions were carried out in triplicate in a total reaction volume of 25 μL containing TaqMan Universal Master Mix (Applied Biosystems) with primer concentrations of 20 μmol/L and probe concentrations of 10 μmol/L. Amplification conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Standard curves were generated for each gene evaluated and required to have $r^2 > 0.99$ by linear regression analysis. Copy number of each gene was calculated from standard curves and expressed as copy number per 10^5 copies of housekeeping gene, 18S rRNA. We also determined IL-8 levels in IL-1Ra "escape"–treated tumors using quantitative RT-PCR. Vascular endothelial growth factor (VEGF) mRNA levels were also determined in IL-1Ra- and control-treated xenografts and using specific primers and probes (Hs00173626, Applied Biosystems).

IL-8 protein quantification in IL-1Ra-treated mice. IL-8 levels were measured in two groups of IL-1Ra-treated tumors (median-sized and escape or larger tumors) by ELISA and compared with the control group. Tumors obtained from mice of the respective groups were pooled together ($n = 4$ for control and median-sized IL-1Ra-treated tumors and $n = 3$ for IL-1Ra escape), homogenized in lysis buffer, and centrifuged at 13,000 × g for 10 minutes in a Sorvall Biofuge Freco centrifuge (Kendro Laboratory Products, Asheville, NC). Protease inhibitor cocktail (Sigma-Aldrich, Inc., Milwaukee, WI) was added to the supernatant and stored at −80°C until ready for the assay. Total protein concentration in the supernatant was determined by bicinchoninic acid protein assay. Absorbance was determined on a Multiskan MCC/340 microtiter plate reader (Titertek) for both ELISA and bicinchoninic acid assays and the amount of IL-8 in tumor homogenates was expressed as picograms per milligram of total protein.

Statistical analysis. Statistical analysis was done using ANOVA with Bonferroni-Dunn correction using StatView platform (SAS Institute, Cary, NC) and $P < 0.05$ was considered significant. Results are expressed as mean ± SE.

Results

Quantitative RT-PCR for IL-1 was done on tumor specimens obtained from patients with metastatic colon adenocarcinoma, non–small-cell lung cancer, or melanoma. *IL-1* gene expression (≥1,000 copies/10^5 copies β-actin) was detected in ≥50% of all samples tested (Table 1). In addition, five human tumor cell lines were also screened for production of IL-1 (Table 2).

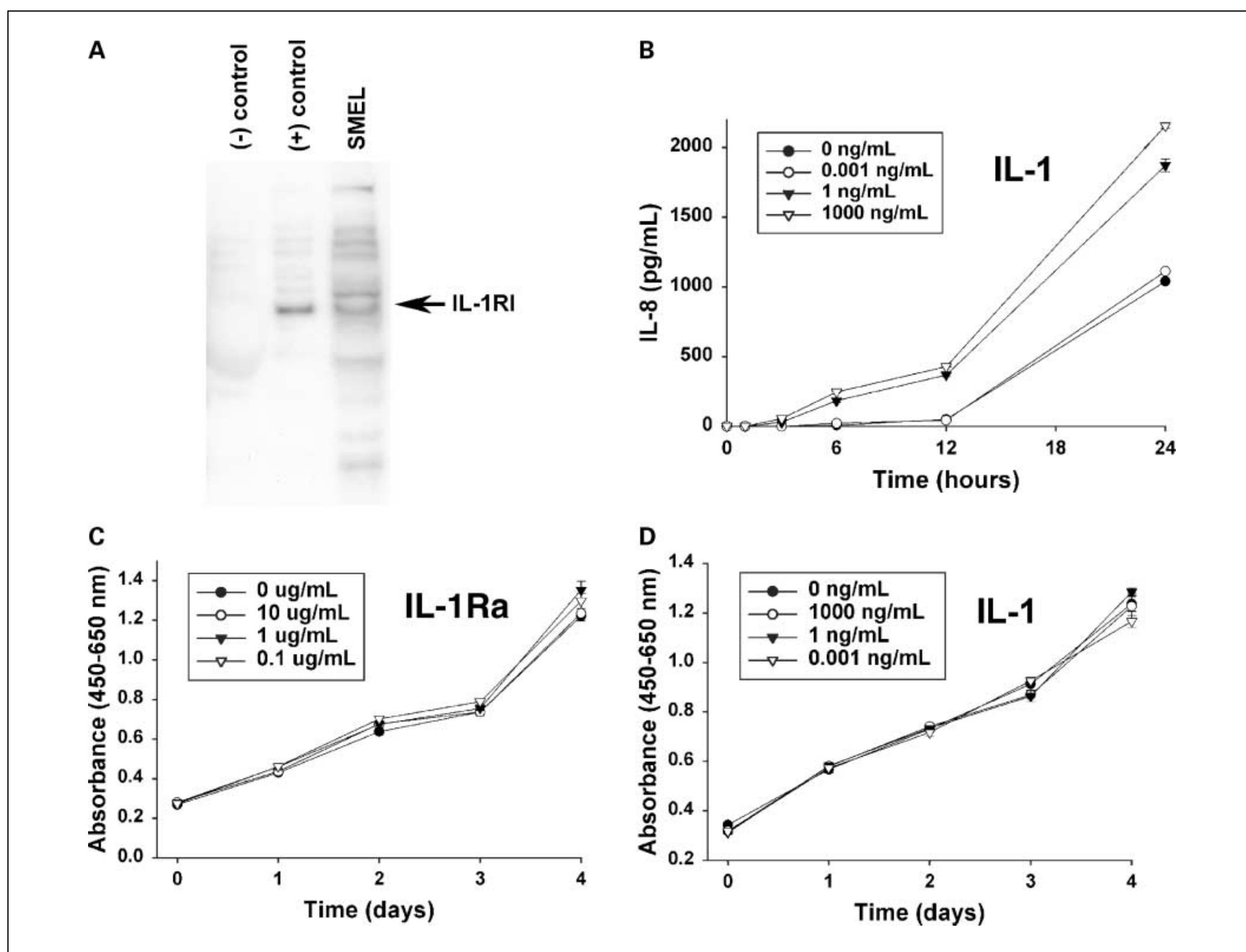

Fig. 4. Western blot of SMEL whole-cell lysate shows a band at 80 kDa representing IL-1RI. Whole-cell lysate prepared from the CCRF-CEM T-lymphoblastoid cell line was used as a positive control (*A*). IL-1β significantly induced ($P < 0.001$) IL-8 production in cultured SMEL cells in a dose- and time-dependent manner in two high doses selected (*B*). ELISA for IL-8 done on cell culture supernatants of SMEL incubated with 0.001 to 1,000 ng/mL of IL-1β. Supernatants from triplicate wells per treatment condition were assayed in duplicate. Points, mean from one representative experiment repeated at least twice; bars, SE. IL-1β augmented constitutive IL-8 production in both a dose- and time-dependent manner [$P < 0.001$, comparison of 1,000 and 1 ng/mL of IL-1β versus no treatment (ANOVA with Bonferroni-Dunn test)]. Neither IL-1 nor IL-1Ra altered proliferation of SMEL in culture (*C* and *D*). SMEL cells were seeded at a density of 1,500 per well in 96-well plates, incubated overnight, and treated with 0.1, 1, and 10 μg/mL of IL-1Ra (*C*) or 0.001, 1.0, and 1000 ng/mL of IL-1β (*D*). WST-1 assay, done in duplicate samples ($n = 8$), showed no significant difference in cell proliferation compared with untreated controls in any of the treatment groups as determined by ANOVA with Bonferroni-Dunn test. Points, mean; bars, SE.

WIDR, a human colon adenocarcinoma cell line, and H2030, a human lung adenocarcinoma cell line, constitutively expressed IL-1β m RNA under basal culture conditions. SMEL and PMEL are human melanoma cell lines; SMEL expressed only IL-1α and no IL-1β mRNA whereas PMEL and SL-2, a human squamous cell carcinoma line, expressed neither IL-1 isoform. To determine if *IL-1* gene expression was associated with production of biologically active protein, 1 mL of conditioned supernatant from two IL-1 expressing cell lines (SMEL and H2030) and one nonexpressing cell line (PMEL) was used to test for effects on endothelial cell permeability *in vitro*. IL-1-induced endothelial cell permeability in this model has been shown to be dependent on the presence of plasma and we conducted the experiments both with and without plasma to ensure the specificity of the findings (25). After a 90-minute exposure, both SMEL and H2030 conditioned supernatant resulted in a significant increase in endothelial cell permeability in a plasma-dependent fashion that was either completely (SMEL) or largely (H2030) abrogated by cotreatment with IL-1Ra (Fig. 1A and B). PMEL had no effect on endothelial cell permeability under identical experimental conditions (Fig. 1C). Together, these data indicate that constitutive gene expression of IL-1 in these tumors results in production of biologically active IL-1 protein that activates endothelial tissue as reflected in increased endothelial cell permeability, a hallmark of early angiogenesis (26).

The effects of s.c. administered IL-1Ra on human tumor xenografts of both IL-1-producing and non-IL-1-producing tumor lines were then determined. IL-1Ra resulted in a statistically significant decrease in growth rate of 3 IL-1-producing tumors tested but had no effect on two non-IL-1-producing tumors [SMEL, $P < 0.05$; WIDR and H2030, $P < 0.01$ (ANOVA with Bonferroni-Dunn test); Fig. 2]. When SMEL, an IL-1-producing melanoma cell line, was injected i.v. into the tail

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vein of mice, there was a statistically significant decrease in the number of lung metastases at 35 days in the IL-1Ra-treated group compared with controls (6.3 ± 2.6 versus 35.7 ± 5.5, respectively; $P = 0.008$, Student's t test). Histologic evaluation of s.c. SMEL tumors showed no difference in mitotic rate per high-powered field in tumor cells obtained from mice treated with IL-1Ra versus control (2.4 ± 0.6 versus 2.8 ± 0.9, respectively) but there was a markedly lower number of vessels per high-powered field observed in the IL-1Ra-treated tumors (4.4 ± 2.0 versus 19.1 ± 9.9, respectively; Fig. 3) as read by a pathologist blinded to the nature of the experimental groups.

The presence of functional IL-1 receptors on SMEL was confirmed by Western blot analysis; IL-1 induced production of IL-8 in a dose- and time-dependent fashion (Fig. 4). However, various concentrations of IL-1 or IL-1Ra were tested on SMEL *in vitro* and showed no direct effects on proliferation, indicating that neither cytokine is a growth-promoting or inhibitory factor for SMEL cells [Fig. 4; identical data (not shown) were obtained for WIDR]. To test whether IL-1Ra mediates down-regulation of tumor-derived IL-8 *in vitro*, SMEL cells were treated with three concentrations of IL-1Ra and tested at different time periods for IL-8 protein. There was no effect of IL-1Ra on SMEL proliferation (Fig. 4C) or IL-8 production at any dose at any time point [IL-8 (pg/mL), control: 2,477 ± 91; with 10 μg/mL of IL-1Ra: 2,409 ± 108 at 48 hours; other data not shown]. Together, these data indicate that inhibition of SMEL xenograft growth is not a consequence of direct effects of IL-1 or IL-1Ra on tumor cell proliferation.

cDNA microarray of SMEL xenografts. Because IL-1 enhances SMEL IL-8 *in vitro*, we determined whether the antitumor effects of IL-1Ra were associated with inhibition of tumor-derived IL-8 *in vivo*. We quantified changes in gene expression profiles from median-sized SMEL xenografts obtained from IL-1Ra and saline control animals ($n = 4$ per group). The results revealed a total of 134 genes differentially expressed by ≥3-fold. Among these, 100 genes, including *IL-8*, were down-regulated by IL-1Ra by a factor of almost 5-fold (Table 3). The magnitude of *IL-8* gene down-regulation in IL-1Ra-treated tumors was further validated by quantitative RT-PCR; IL-8 mRNA copy numbers were 4,489 ± 123/10^5 copies 18S rRNA in control versus 1,109 ± 47/10^5 copies 18S rRNA in IL-1Ra-treated tumors. Similarly, IL-8 protein levels in tumor homogenates also decreased significantly in mice treated with IL-1Ra (921 ± 23 versus 201 ± 15 pg IL-8/mg total protein, respectively).

IL-1Ra-mediated IL-8 and VEGF mRNA levels in xenografts. To further characterize the relationship between decreased *IL-8* or *VEGF* gene expression and IL-1Ra-mediated xenograft growth inhibition, we measured *IL-8* and *VEGF* gene expression from tumors of different sizes obtained from mice treated with IL-1Ra. When individual SMEL xenograft growth curves were plotted, ~25% of mice treated with IL-1Ra had tumor growth rates that were comparable to control treated mice (Fig. 5). We assessed gene expression profiles in the IL-1Ra escape tumors ($n = 3$) compared with median-sized tumors in the IL-1Ra and control groups. Interestingly, *IL-8* gene expression and protein levels in IL-1Ra escape tumors were similar to control xenografts; there was a 4-fold higher IL-8 mRNA level and 7-fold higher protein level in IL-1Ra escape tumors compared with xenografts that showed growth inhibition in response to IL-1Ra treatment (Fig. 6). Interestingly, in median-sized IL-1Ra-treated xenografts, relative VEGF mRNA levels were lower than median-sized control tumors and IL-1Ra-treated escape xenografts (control: 5,710 ± 500; IL-1Ra: 50 ± 0.3; and IL-1Ra escape: 1,000 ± 80; values normalized to β-actin expression).

Discussion

Production of IL-1 by tumor or other host cells in the tumor microenvironment has been associated with a virulent tumor phenotype in several types of murine or human cancers (2, 12, 15, 27, 28). These alterations in tumor biology by IL-1 are likely mediated directly or indirectly via induction of an angiogenic phenotype in endothelial tissue (1, 3, 9, 10, 29). The data from the current study provide further evidence that IL-1 plays an important role in promoting tumor growth and metastases in human cancers that produce IL-1. Because IL-1Ra is a competitive antagonist for IL-1 receptor (30–33), sustained saturation of IL-1 receptors in the tumor microenvironment is necessary to completely antagonize the actions of IL-1. One hypothesis for the partial inhibition of xenograft growth in mice treated with recombinant IL-1Ra is that there was only partial blockade of IL-1 receptors based on intermittent systemically administered recombinant IL-1Ra. In SMEL melanoma xenografts retrovirally transduced to overexpress IL-1Ra, growth inhibition is almost complete (14). To that end, more

Table 3. Gene categories and selected genes down-regulated by ≥3-fold by cDNA microarray analysis

Genes	Fold down-regulation
Genes involved in cell adhesion	
α-Catenin	3.85
Laminin receptor 1	3.30
Chemokines	
IL-8	4.97
Chemokine (C-C motif) ligand 20	4.42
Genes involved in signal transduction	
Protein kinase C-like 2	3.69
Annexin A2	3.38
Genes involved in cell cycling	
Cyclin C	3.01
Transcription factors	
TAF12 RNA polymerase II	3.22
Translation factors	
Eukaryotic translation initiation factor 3	3.46
Genes coding for ribosomal proteins	
Ribosomal protein L37	4.48
Ribosomal protein L10a	4.23
Genes related to endoplasmic reticulum	
Protein translocation complex β	3.08
Genes involved in oxidative phosphorylation	
Cytochrome *c* oxidase	5.40
NADH dehydrogenase	5.24
H^+ transporting ATP synthase	3.27

NOTE: Median-sized tumor samples from control and IL-1Ra treatment groups were used for gene expression profiling.

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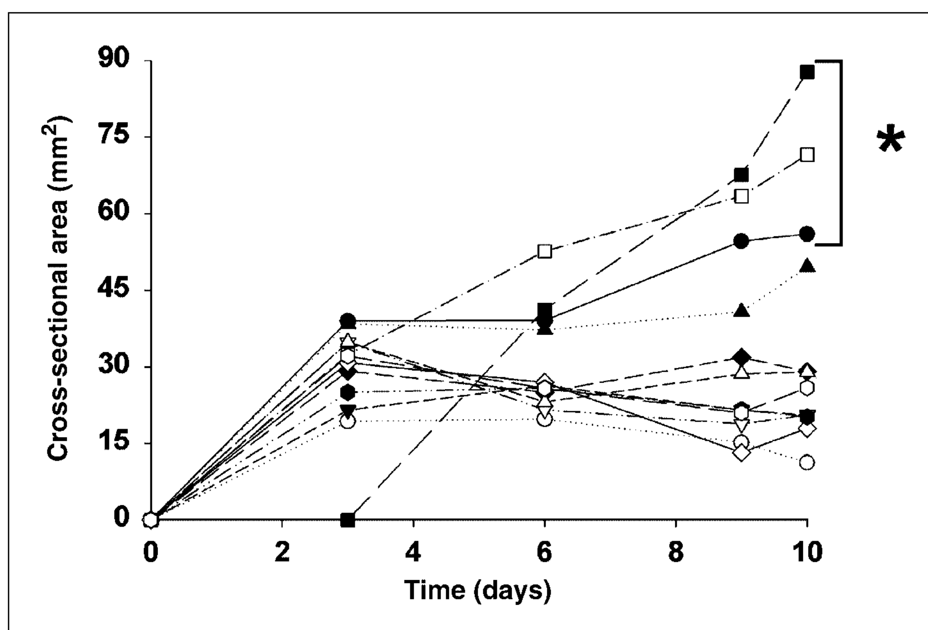

Fig. 5. Individual tumor growth curves for SMEL xenografts treated with IL-1Ra. A few tumors escaped the effects of IL-1Ra (*) and grew at rates comparable to control.

effective inhibition of IL-1 in the tumor microenvironment may be possible with a continuous delivery system for IL-1Ra (13) or by using a higher-affinity IL-1 receptor binding protein that has sustained bioavailability following systemic administration, such as the recently described IL-1 trap (34).

IL-1Ra inhibition of tumor growth is not principally related to tumor histology but rather to the presence of IL-1 in the tumor microenvironment. We evaluated both human cancer cell lines and tumor specimens obtained from patients for *IL-1* gene expression; it is not known whether IL-1 in the metastatic cancer samples was principally from tumor cells or host infiltrating cells. Because IL-1Ra did not alter tumor cell proliferation rates *in vitro* or mitotic rates *in vivo*, the effects of IL-1Ra on s.c. xenograft growth and metastatic potential must be a consequence of indirect effects mediated through a host-tumor interaction. This is evidenced in the fact that IL-8 levels within IL-1Ra growth-inhibited xenografts were very low but IL-8 production was not affected by IL-1Ra *in vitro*. One implication of these findings is that the actual source of the IL-1 in the tumor microenvironment, tumor cells versus host infiltrating cells, may not be consequential as the protein from either source will produce the identical downstream angiogenic host response. Relative VEGF mRNA levels were also reduced in median-sized but not in escape IL-1Ra–treated xenografts compared with control-treated, suggesting that IL-1Ra may mediate antiangiogenic effects by down-regulation of VEGF.

There is also increasing evidence that IL-8 plays a central role in cancer growth; IL-8 is a growth factor for human melanoma cells and endothelial cells specifically secrete IL-8 that can induce melanoma cell chemotaxis (35). It plays a role in vascular permeability via activation of G proteins in endothelial cells, leading to actin stress fiber formation, cell retraction, and gap formation (36). In melanoma cells, tumor-derived IL-8 induces endothelial cell chemotaxis. Transfection of IL-8 in poorly vascularized and weakly metastasized human melanoma xenografts induces the metastatic potential and vascularization, and thus the xenografts grow quickly (37). IL-1β induces IL-8 mRNA expression in human gastric TMK-1

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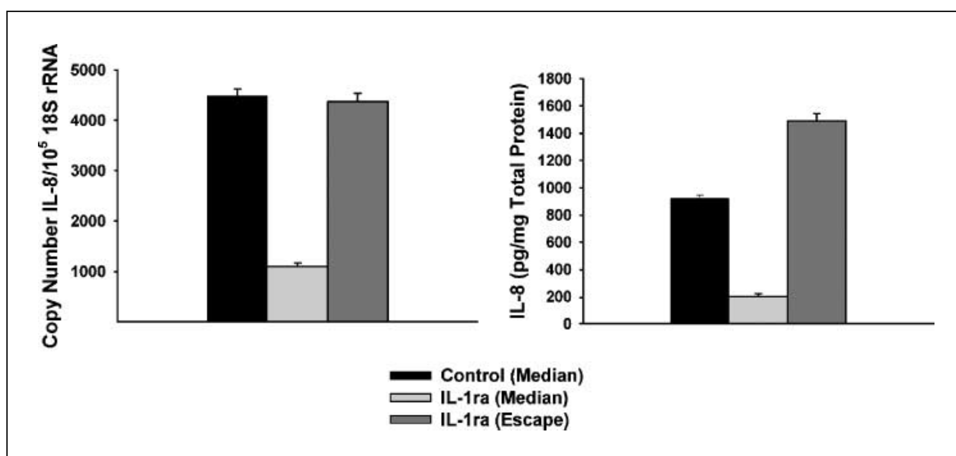

Fig. 6. IL-8 mRNA (*left*) and protein (*right*) were determined from total RNA and protein, respectively, isolated from tumors that responded and escaped IL-1Ra treatment (Fig. 5, *asterisk*), and compared with the control group. IL-8 mRNA copy number and protein were markedly lower in median-sized tumors that responded to IL-1Ra treatment whereas no significant change was found in tumors that escaped IL-1Ra treatment.

cells and this can be directly correlated with the vascularity of the gastric tumors (38). Overexpression of IL-8 mRNA in radical prostatectomy specimens is directly correlated with progression of prostate cancer (39). Our data show that down-regulation of IL-8 in the tumor microenvironment is strongly associated with IL-1Ra-mediated inhibition of tumor growth. Of note, IL-1Ra-mediated down-regulation of IL-8 in this model seems to be exclusively an *in vivo* phenomenon as IL-1Ra had no effect on constitutive IL-8 production by SMEL *in vitro*. This result is consistent with a previous study showing no effect on constitutive IL-8 production by a head and neck squamous cell carcinoma cell line incubated with IL-1Ra or with an anti-IL-1 neutralizing antibody *in vitro* (40). The mechanism responsible for IL-1Ra-associated IL-8 down-regulation *in vivo*, therefore, is not mediated through IL-1 receptor blockade of the tumor cell, but likely through other cells and/or factors in the tumor microenvironment.

Together, these data suggest that IL-1Ra, alone or in combination with chemotherapy regimens, may be a clinically useful therapy for patients with a variety of cancer histologies if IL-1 production in tumor can be determined. Given the availability and safety profile of IL-1Ra and the growing amount of data showing growth inhibition of tumor growth and metastases in IL-1-producing tumors in murine models, further evaluation of the use of IL-1Ra for the treatment of human cancers is warranted.

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