Interleukin 1 Receptor Antagonist Inhibits the Augmentation of Metastasis Induced by Interleukin 1 or Lipopolysaccharide in a Human Melanoma/Nude Mouse System

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ABSTRACT
This study examined the ability of the recombinant human interleukin 1 receptor antagonist (IL-1ra) to block interleukin 1 (IL-1)-mediated experimental metastases from the A375M human melanoma. In vivo, IL-1ra administered at concentrations ≥200 times IL-1 significantly inhibited the increase in lung colonies induced by IL-1 in nude mice. The response to IL-1 was significantly inhibited when IL-1ra was administered simultaneously with or 1 to 3 h before IL-1. In vitro, the incubation of IL-1-activated endothelial cells with IL-1ra prevented the increase in adhesion of A375M melanoma cells. At the same experimental conditions, IL-1ra inhibited the augmented expression of the intracellular and vascular cellular adhesion molecules 1 and E-selectin induced by IL-1 on endothelial cells. Lipopolysaccharide, an IL-1 inducer, increased the number of lung colonies in nude mice. IL-1ra injected with or 1 h after lipopolysaccharide inhibited this augmentation, suggesting a role for host-produced IL-1 in metastasis formation.

INTRODUCTION
Altered production and response to cytokines have often been described in relation to malignancy. Clinical and experimental reports have described tumor cell arrest and growth at sites of injury, healing, and inflammation (1, 2). The injection of nonspecific inflammatory stimuli may increase experimental metastases associated with the intravascular release of inflammatory mediators (3). We and others have shown that the injection of inflammatory cytokines, such as IL-1β and TNF, increased the number of metastases in different murine and human tumor models (4–9).

A naturally occurring IL-1ra has recently been identified and cloned (10–12). IL-1ra binds to cellular IL-1 receptors and blocks several IL-1-mediated responses in vitro. In animal models IL-1ra provides protection against several pathological processes associated with inflammation. For review, see Refs. 13 and 14.

The present study investigated the effect of IL-1ra on the augmentation of experimental metastases by IL-1. To assess the role of endogenous IL-1 in metastasis formation, the effect of IL-1ra on lung colonies induced by LPS was investigated.

MATERIALS AND METHODS
Animals. Female NC/nu/nu mice were obtained from the National Cancer Institute Animal Program, Frederick, MD, and used when 6 to 8 wk old. Throughout the experiments mice were housed in a laminar flow cabinet and manipulated under aseptic conditions. 1

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: IL-1, interleukin 1; IL-1ra, interleukin 1 receptor antagonist; EC, endothelial cells; LPS, lipopolysaccharide; ICAM-1, intracellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; HSA, human serum albumin.

4 Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987, and NIH guide for the care and use of laboratory animals. NIH Publication 85-23, 1985).

Cell Culture. The A375M human melanoma metastatic to the lungs of nude mice was maintained on plastic in Eagle’s minimal essential medium supplemented with 10% fetal calf serum (4). Tumor cells were harvested by brief exposure to 0.25% trypsin-0.02% EDTA, washed twice, and resuspended as indicated in the respective assays.

EC isolated from human umbilical vein as previously described (15) were maintained in Medium 199 supplemented with 10% fetal calf serum and 10% newborn calf serum, 5 μg/ml endothelial cell growth supplement, and 100 μg/ml of heparin. All culture reagents were from Gibco, Paisley, Scotland.

Reagents. Human recombinant IL-1β (specific activity, 107 units/mg) was obtained through the courtesy of T. Barlozzari (BASF, Bioresearch Corp., Cambridge, MA), and IL-1ra was generously supplied by E. Tracey of The Upjohn Company, Kalamazoo, MI. Aliquots of cytokines were stored at −80°C until injection. LPS (Escherichia coli endotoxin strain 055: B5) was from Sigma Chemical Co., St. Louis, MO. Monoclonal antibodies to VCAM-1 (4B2), E-selectin (13D5), and ICAM-1 (11C8/1) were obtained through the courtesy of A. Gearing, (British Biotechnology, Ltd., Oxford, England). For EC treatment, cytokines were diluted in Medium 199. The vehicle for injections was endotoxin-free PBS. All treatments were given at the doses and schedules indicated in “Results.”

Lung Colonization Assay. Single cell suspensions resuspended in Ca2+-

Mg2+-free Hanks’ balanced salt solution at the concentration of 5 × 104/ml were injected i.v. in the lateral tail vein of nude mice (4). At autopsy, 6 wk later, lungs were fixed in Bouin’s solution, and the number of tumor colonies was counted under a dissecting microscope. Differences in the numbers of colonies were analyzed using the Mann-Whitney U test. All the results on experimental metastases are representative of at least two independent experiments.

Adhesion Assay. EC grown to confluent monolayers in 96-well plates were washed twice and then activated for 4 h with IL-1, IL-1ra, or the combination of the two at the concentrations indicated in “Results.” After incubation monolayers were washed twice and refilled with 50 μl of Medium 199 containing 1% bovine serum albumin (test medium). Tumor cells were radiolabeled with [3H]thymidine (Amersham International, Amersham, United Kingdom) as described previously (15). Fifty μl of cell suspension (3 × 104 cells) were added to each well containing EC previously treated with cytokines or medium alone and incubated for 30 min at 37°C. After incubation, nonadherent cells were removed by careful aspiration and three washes with test medium. Wells were incubated for 10 min with 200 μl of 0.1 M NaOH, and the lysate was counted in a gamma counter. Results are expressed as the percentage of attached cells relative to total cells added. Differences were analyzed by multiple comparisons using the Dunnett test.

Flow Cytometric Analysis. EC activated with cytokines as described above were harvested by brief exposure to 0.25% trypsin-0.02% EDTA, washed twice, and resuspended in PBS with 2.5% HSA. The expression of adhesion proteins was determined by indirect immunofluorescence using a FACs-STAR-Plus (Beckton-Dickinson) (16). Briefly, after incubation with the appropriate primary antibody (1:50 dilution) in PBS with 2.5% HSA for 30 min at 4°C, cells were washed and incubated with an affinity-purified fluorochrome isothiocyanate-labeled goat anti-mouse immunoglobulin antiserum for 30 min at 4°C (Teco Genetics, Milan, Italy). Results were expressed as the percentage of positive cells and the mean channel fluorescence intensity.

RESULTS
Effect of IL-1ra on IL-1-induced Increase in Lung Colonies. We have reported that IL-1 (from 0.1 to 10 μg/mouse i.v.), given before the injection of A375M tumor cells, significantly increased the number of lung tumor colonies in nude mice (4). Using the working concentration of 0.1 μg of IL-1 i.v. 4 h before tumor cells, we
investigated the concentrations and schedules of IL-1ra that counteracted this increase (Figs. 1 and 2).

Lung colonies were significantly more numerous (median number, 268) in mice receiving IL-1 than in vehicle-treated mice (median number, 62). IL-1ra administered simultaneously with IL-1 prevented this increase in a dose-dependent manner (Fig. 1). The inhibition was significant when a ≥200-fold excess of IL-1ra (≥20 μg/injection) over IL-1 was administered (median number of lung colonies, 83 and 29 in mice receiving, respectively, 20 and 100 μg of IL-1ra). IL-1ra alone (from 2 to 200 μg/mouse i.v.) before tumor cells did not significantly influence the number of lung colonies from the A375 melanoma in nude mice (data not shown).

Complete inhibition of lung colonies was achieved when IL-1ra was injected together with IL-1 (Fig. 1) or 1 to 3 h before it (with tumor cells injected 4 h after IL-1) (Fig. 2). The median numbers of lung colonies were 23 and 6 in mice treated with IL-1ra 3 h and 1 h before IL-1, compared to 270 in IL-1-treated mice, while no significant inhibition was seen when IL-1ra was given 24 h before IL-1 (median number of lung colonies, 224) (Fig. 2). IL-1ra had some effect even when given 1 or 3 h after IL-1 (tumor cells injected 4 h after IL-1) (median lung colonies, respectively, 113 and 84) (Fig. 2).

**Inhibition of Tumor Cell Adhesion to IL-1-activated EC by IL-1ra.**

Fig. 3 shows that the adhesion of A375M melanoma cells was significantly increased on IL-1-activated EC. IL-1ra added to IL-1 during EC activation inhibits the increased adhesion of A375M melanoma cells (Fig. 3). In accordance with the in vivo results, a ≥200-fold excess of IL-1ra was needed to completely block the increment of tumor cell adhesion on IL-1-activated EC. IL-1ra added 1 and 4 h after IL-1 did not prevent the increase of tumor cell adhesion on EC (data not shown). Resting EC incubated for 4 h with the same concentrations of IL-1ra alone did not change their adhesivity for A375M cells (data not shown).

To evaluate the relevance of adhesion molecules on the inhibition of tumor cell adhesion on activated EC, the effect of IL-1ra on their expression was studied. Treatment of EC with IL-1 induced the expression of VCAM-1 and E-selectin and increased the expression of ICAM-1 on EC (Fig. 4). The addition of IL-1ra inhibited IL-1-induced expression of VCAM-1 and E-selectin and IL-1-augmented expression of ICAM-1 on EC (Fig. 4). The treatment of EC with IL-1ra alone did not influence the expression of adhesion molecules on EC (data not shown).

**Endothelium treatment**

Fig. 3. Inhibition by IL-1ra of tumor cell adhesion on IL-1-activated endothelial cells. EC were treated with medium (C), IL-1 alone (2 ng/ml), or IL-1 plus IL-1ra for 4 h at 37°C before the adhesion assay. Cell adhesion was evaluated after 30 min. Columns, mean of four replicates and representative of one experiment of three done; bars, SD. *, P ≤ 0.01; **, P < 0.05 compared to IL-1-treated EC.
IL-1ra INHIBITS IL-1-INDUCED METASTASIS

![Graph showing effect of IL-1ra on lung colonies.](image)

**DISCUSSION**

Our previous work showed that IL-1 treatment induced the increase of lung metastases in different tumor systems (4, 5). The study described here shows that this augmentation can be inhibited by IL-1ra. Since treatment with IL-1ra inhibits LPS-increased experimental metastasis formation, these results also indicate that IL-1ra inhibits metastasis augmentation induced by host-produced IL-1.

The inhibition of lung colonies was dose dependent, showing a significant effect when IL-1ra was given at least 200-fold in excess over IL-1. In other experimental models a large excess of IL-1ra (10² to 10⁴-fold) is generally required to reduce by 50% or block the biological response to IL-1 (13, 14). It has been suggested that, although IL-1 and IL-1ra bind to receptors with approximately the same affinity (10), probably only a few IL-1 receptors are necessary to elicit the biological response to IL-1 and, therefore, higher concentrations of IL-1ra are needed to saturate the IL-1 receptors and achieve antagonism (18). The effect of IL-1ra was time dependent and, consistently with the kinetics of IL-1 lung colony induction (4), the inhibitory effect was achieved when IL-1ra was injected together or shortly before or after IL-1, but not 24 h before.

Several mechanisms associated with response to cytokines may be responsible for metastasis formation (19). Metastasis is a complex multistep process that requires circulating tumor cells to extravasate into specific metastatic sites. The interaction of metastatic cells with the microvasculature is a crucial step in tumor cell arrest at the capillary bed of secondary organs (20, 21). Inflammatory cytokines such as IL-1 and TNF activate specific adhesive mechanisms on EC, resulting in increased adhesiveness for different human tumor cells (15, 22, 23). In this study we show that IL-1ra inhibits the increased adhesion of A375 melanoma cells on IL-1-activated EC, further supporting that IL-1-mediated tumor/EC interaction is involved in the metastatic process. Moreover, in agreement with recent findings obtained using the intracellular form of IL-1ra (24), we show inhibition of adhesion protein expression on EC surface by IL-1ra. Specific adhesion proteins have been shown to mediate tumor cell adhesion on activated EC. Among the others VCAM-1 appears to play an important role in melanoma adhesion on activated EC (25, 26) including A375 melanoma cells. ⁵ Our results showing inhibition of VCAM-1 and other adhesion protein expression on EC surface by IL-1ra suggest that IL-1ra inhibits tumor cell adhesion by preventing augmented expression of adhesion molecules on EC surface.

IL-1ra alone did not significantly influence the number of lung colonies by the A375 melanoma in nude mice; it affects neither A375M melanoma cell adhesion nor the expression of adhesion molecules on EC surface (data not shown). Thus, IL-1ra alone has no agonist effect on metastasis formation even at a high concentration, supporting its function as a pure antagonist (10). However, it is worth noting that a large excess of IL-1ra further inhibits the number of colonies in IL-1-treated mice compared to control mice (Fig. 1) and tumor cell adhesion on activated EC. Among the others VCAM-1 appears to play an important role in melanoma adhesion on activated EC (25, 26) including A375 melanoma cells. ⁵ Our results showing inhibition of VCAM-1 and other adhesion protein expression on EC surface by IL-1ra suggest that IL-1ra inhibits tumor cell adhesion by preventing augmented expression of adhesion molecules on EC surface.

![Graph showing effect of IL-1ra on lung colonies.](image)

**Fig. 4.** Effect of IL-1ra on IL-1-induced expression of adhesion molecules on EC. EC were treated with medium, (C), IL-1 alone (2 ng/ml), or IL-1 plus IL-1ra for 4 h at 37°C before cytometric analysis. Results are the percentage of positive cells and the mean channel of fluorescence intensity.

**Fig. 5.** Inhibition by IL-1ra of the LPS-induced increase in lung colonies. Nude mice were treated with LPS (1 μg/mouse i.v.) 4 h before the injection of 5 × 10⁶ A375M cells i.v. IL-1ra (100 μg/mouse) was injected together with LPS or 1 h after it in a final volume of 0.2 ml. Control mice (C) received the same volume of vehicle. Autopsies were performed 6 wk after tumor cell injection. Results are the median number of lung colonies per group (n = 8). All mice presented lung colonies at autopsy. * P < 0.001 compared to LPS-treated mice.

**TABLE 1.**

<table>
<thead>
<tr>
<th>Mouse treatment</th>
<th>Median lung colonies (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>122 (11-273)</td>
</tr>
<tr>
<td>IL-1 ra (with)</td>
<td>165 (16-79)</td>
</tr>
<tr>
<td>IL-1 ra (+1h)</td>
<td>241 (20-165)</td>
</tr>
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</table>

**Fig. 6.** Median number of lung colonies. * P < 0.001 compared to LPS-treated mice.
producing IL-1 have been described (27, 28), and IL-1-producing melanomas may induce the expression of adhesive molecules on EC and increase tumor EC adhesion (29).

Increased tumor cell retention and more metastases after IL-1 and TNF treatment have been described in different animal tumor models (4–6, 8, 9, 22). However, all these studies were conducted with exogenous cytokine administrated to mice, so the significance of these findings in the pathophysiology of cancer metastasis is questionable.

While recent reports showed a contribution of tumor-induced TNF in tumor malignancy (7, 8), there is no information on the role of endogenous IL-1 in metastasis. LPS is a nonspecific inflammatory stimulus that triggers a cascade of host-derived mediators responsible for its proinflammatory effects. IL-1ra appears to improve endotoxin-toxic effects due to IL-1-mediated acute inflammation (30–32). Since IL-1ra specifically binds IL-1 receptors, our results showing that IL-1ra inhibits the LPS-induced increase of lung colonies indicate that host-produced IL-1 plays a pivotal role in this increase.

In conclusion, we have shown that IL-1ra blocks two aspects of the metastatic process modulated by IL-1: the increased tumor cell adhesion on EC and lung colony formation. The inhibitory effect of IL-1ra on the increase in artificial metastases induced by LPS suggests that endogenous IL-1 plays a role in promoting malignancy. Increased synthesis and release of cytokines, including IL-1, have been reported in neoplastic diseases (33), but their contribution to the malignant behavior of tumor cells is not clear. The effects of IL-1 antagonists on the malignant behavior of IL-1-inducing tumor cells merit further investigation.

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REFERENCES

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