Interleukin 1-induced Augmentation of Experimental Metastases from a Human Melanoma in Nude Mice

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ABSTRACT

This study has examined the effect of the cytokine interleukin 1 (IL-1) on metastasis formation by the human melanoma A375M in nude mice. We have found that human recombinant IL-1β (a single injection >0.01 μg per mouse i.v. given before tumor cells) induced an augmentation of experimental lung metastases from the A375M tumor cells in nude mice. This effect was rapidly induced and reversible within 24 h after IL-1 injection. A similar effect was induced by human recombinant IL-1α and human recombinant tumor necrosis factor, but not by human recombinant interleukin 6. Some IL-1β-induced diodeoxyuridine-radioiodinated A375M tumor cells injected i.v. remained at a higher level in the lungs of nude mice receiving IL-1 than in control mice. In addition, IL-1 injected 1 h, but not 24 h, after tumor cells enhanced lung colonization as well, thus suggesting an effect of IL-1 on the vascular transit of tumor cells. These findings may explain the observation of enhanced secondary localization of tumor cells at inflammatory sites and suggest that modulation of secondary spread should be carefully considered when assessing the ability of this cytokine to complement cytoreductive therapies.

INTRODUCTION

IL-1 is a pleiotropic cytokine involved in inflammatory and immunological circuits and in the generation of acute phase responses (1). It induces a wide spectrum of biological responses with systemic and local tissue changes (2). Recently it has been shown that IL-1 stimulates hematopoietic precursors, has radioprotective activity, and counteracts the myelotoxicity of cancer chemotherapy agents (3–6). These findings suggest that this cytokine may have potential clinical application during radiotherapy and chemotherapy or in the context of bone marrow transplantation.

In addition it has been suggested that IL-1 can have antitumor activity in vitro (7) and in vivo (8), dependent on the experimental model used. However, in a recent work no therapeutic activity was observed when IL-1 was given as a single agent for the treatment of metastatic disease (6). A major target of the action of IL-1 is the endothelial lining of blood vessels, whose functional status is reprogrammed by this cytokine (9, 10). In the context of studies on the effect of IL-1 on EC function recently, our group and others have found that IL-1 treatment of human umbilical vein endothelial cultures increased their adhesivity to different human tumor cells (11, 12). To investigate the relevance of this phenomenon in vivo, we examined the effect of IL-1 on experimental metastasis formation in the lungs of nude mice given a human melanoma cell line.

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3 The abbreviations used are: IL-1, interleukin 1; hu rIL-1, human recombinant interleukin 1; IL-6, interleukin 6; hu rIL-6, human recombinant interleukin 6; TNF, tumor necrosis factor; hu rTNF, human recombinant tumor necrosis factor; EC, endothelial cells; HBSS, Ca2+- and Mg2+-free Hank's balanced salt solution.

MATERIALS AND METHODS

Animals. Six- to 8-wk-old male NCr-nu/nu mice were obtained from the National Cancer Institute Animal Program, Frederick, MD. Mice were housed throughout the experiments in a laminar flow cabinet under specific-pathogen-free conditions. In accordance with institutional guidelines mice did not suffer unnecessary discomfort, pain, or injury and received proper care and maintenance.

Tumor Line. A375M, a human melanoma line selected in vivo for high lung colonization capacity (13), was maintained on plastic in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold vitamin solution (Gibco, Paisley, Scotland). Cultures were routinely verified as Mycoplasma free. The human origin of the tumor line was confirmed by the human β-lactamase isoenzyme pattern (ISO-LAD; Chemetron Chimica, Milan, Italy). A375M tumor cells from mid-log phase culture were harvested by exposure to 0.25% trypsin-0.02% EDTA solution, washed twice, and resuspended in HBSS at the concentration indicated for injection.

Cytokines. Highly purified hu rIL-1β (Escherichia coli 117-269) was from Slavo, Siena, and De Bi Division, Cassina Dei Pecchi, Italy. The hu rIL-1β preparation used had a specific activity of 105 units/mg of protein, and it contained <0.5 pg of endotoxin/μg (LAL chromogenic assay; Whittaker Bioproducts, Walkersville, MD). Lyophilized hu rIL-1β was reconstituted to 100 μg/ml in pyrogen-free, sterile, phosphate-buffered saline solution; sterilized by microfiltration in 0.2 μm; and stored frozen in 10-μg aliquots.

Hu rIL-1α (specific activity, 3 × 106 units/mg) was provided by Hoffman-La Roche, Nutley, NJ. Hu rIL-6 (50 × 105 units/ml; less cell line) was kindly supplied by Dr. S. Gillis (Immunex Corporation, Seattle, WA), and hu rTNF-α (specific activity, 8.1 × 106 units/mg) was from Dr. E. Schlick (Basf-Knoll, West Germany). Working concentrations of cytokines were diluted in 0.9% NaCl solution before injection and given i.v. 1 h before tumor cells unless otherwise indicated. Control mice received vehicle alone.

Experimental Metastasis Assay. Single cell suspensions (>95% viability by trypan blue exclusion) at concentrations from 5 × 105 to 10 × 105 in 0.2 ml of HBSS, as detailed in “Results,” were injected in the lateral tail vein of nude mice. Mice were autopsied 8 wk later, lungs were removed and fixed in Bouin’s solution, and the number of lung colonies was counted under a dissecting microscope according to the standard technique. Extrapulmonary metastases were checked for all animals. Differences in the numbers of lung colonies were analyzed using the Mann-Whitney U test. Results are representative of at least two different experiments.

Organ Distribution Analysis. Cultures of A375M melanoma cells in mid-log phase were prelabeled with 5[3H]iodo-2'-deoxyuridine (American International, Buckinghamshire, United Kingdom) (specific activity, 5 Ci/mg) at 0.3 μCi per ml of medium for 24 h (14). The cell monolayer was then rinsed twice to remove nonbound radiodine and harvested as described above. The cell suspension was washed and resuspended at a final concentration of 5 × 105 cells/0.2 ml of HBSS for i.v. injection. Labeled tumor cells were injected into nude mice treated 1 h earlier with hu rIL-1β (1 μg/0.2 ml) or 0.2 ml of 0.9% NaCl solution. At the time indicated after tumor cell injection, nude mice were autopsied; lungs, liver, spleen, and kidneys were collected from each mouse and washed in three changes of 70% ethanol over a 72-h period to remove free label (14). Radioactivity in all organs was then counted in a gamma counter.

Electron Microscopy Analysis. Lungs from nude mice were removed
AUGMENTATION OF EXPERIMENTAL METASTASES BY IL-1

at different times after hu rIL-1/3 injection (3 lungs/time group from 1 to 24 h). Pieces of lungs were randomly chosen and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in ethanol, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss Model EM 109 electron microscope.

RESULTS

Effect of IL-1 on Lung Colony Formation by A375M. The i.v. injection of 1 µg of hu rIL-1/3 into nude mice 1 h before A375M human melanoma cells given i.v. results in a dramatic increase of tumor colonies in the lungs of mice autopsied 8 wk later (Fig. 1). Table 1 shows the results representative of several experiments run in the course of this study. A similar effect was observed when hu rIL-1/3-pretreated nude mice were given injections of a tumor cell number ranging from 5 × 10⁵ to 10 × 10⁶ (Table 1).

The number of lung colonies was slightly higher in animals receiving 0.01 µg of hu rIL-1/3 (median number of lung colonies, 17), and the increase was significant in mice receiving higher doses (median number of lung colonies, respectively, 61, 68, and 95 in nude mice receiving 0.1, 1, or 10 µg) compared with mice receiving vehicle (median number of lung colonies, 6.5) (Table 2). The kinetics of IL-1 augmentation of experimental metastases was examined by injecting hu rIL-1/3 i.v. at a dose of 1 µg per mouse at various times before and after tumor cell injection and counting the number of lung colonies 8 wk later (Fig. 2). A significant augmentation (P < 0.005) of lung colonies was observed when hu rIL-1/3 was given from 5 min to 4 h before A375M tumor cells (median number of lung colonies, 199, 187, and 126 in mice pretreated at 5 min, 1 h, and 4 h). Hu rIL-1/3 given 1 h after tumor cells increased the number of lung colonies as well (median number of lung colonies, respectively, 28 and 10) compared with control mice (median number of lung colonies, 25) (Fig. 2).

To investigate whether the observed phenomenon was due to a direct effect on tumor cells, A375M tumor cells were harvested from tissue culture, incubated for 1 h at 37°C with hu rIL-1/3 (1 × 10⁶ cells with 1 µg of hu rIL-1/3 in 0.2 ml), washed repeatedly, and then injected i.v. into nude mice. At autopsy, 8 wk later, the number of lung colonies from mice given injections of tumor cells exposed to hu rIL-1/3 was not significantly different (median, 25; range, 6 to 62) from mice that were given control tumor cells (median number of lung colonies, 31; range, 2 to 57).

Specificity of IL-1-induced Lung Colony Augmentation. We examined whether the augmentation of lung tumor colonies was, in fact, because of the IL-1 molecule itself and whether functionally related cytokines showed the same property. First of all, heating the hu rIL-1/3 preparation (100°C for 15 min) completely abolished its ability to increase metastases, thus excluding a role of possible endotoxin contamination of the recombinant cytokine (Table 3). Similarly preincubation (20 h at 4°C) with appropriate specific antibodies abolished the stimulatory effect of hu rIL-1/3 on metastasis formation (data not shown). Table 3 shows that under the same experimental condition, similar results were obtained with the α and β species of IL-1. We then examined the effect of the cytokines IL-6 and TNF which, though molecularly distinct and acting through distinct receptors, share several biological properties with IL-1 (2, 15). Interestingly, hu rIL-6 did not affect metastasis formation by A375M melanoma in nude mice, whereas hu rTNFα significantly augmented lung colony formation compared with control mice (Table 3).

Organ Distribution and Arrest of A375M Cells. To investigate the mechanisms responsible for the IL-1-mediated increase of experimental metastases, we examined the organ distribution and arrest of radiolabeled A375M tumor cells in hu rIL-1/3- or 0.9% NaCl solution-pretreated nude mice from 10 min to 72 h (Fig. 3). After i.v. injection, radiolabeled tumor cells rapidly localized in the lung, and no difference was observed between the two groups of animals in the first hour. The level of retention was higher 4 h after tumor cell injection in hu rIL-1/3-treated mice (52% versus 35%). This ratio increased pro-

Fig. 1. Increase of experimental lung metastasis in nude mice given injections of IL-1. Ten × 10⁶ A375M tumor cells were injected i.v. into nude mice treated 1 h earlier with 1 µg of hu rIL-1/3, and lung colonies were counted 8 wk later. Lungs from nude mice pretreated with hu rIL-1/3 (bottom) show an extensive tumor burden, while those from mice pretreated with 0.9% NaCl solution (top) show fewer tumor colonies.
gressively over the 72-h observation period (Fig. 3). The loss of cell-associated cpm from the lungs was rapid between 4 and 24 h, as described (14), but at 24 h the retention was significantly higher in the lungs of treated mice than in control mice (5% versus 0.4%). By 72 h, less than 0.2% of the injected cells were detectable in the lungs of control mice, while hu rIL-1β-treated mice showed 3% tumor cell retention. Cell-associated radioactivity in the visceral organs (spleen, kidneys, liver) did not differ in the two groups (data not shown). This distribution pattern suggests that IL-1 does not interfere with the ability of tumor cells to reach the lung, but with their retention in the secondary organ.

Lungs from nude mice were collected at 1, 4, and 24 h after hu rIL-1β treatment, and sections were examined by electron microscopy. Although at this stage we did not perform a quantative morphometric assessment of enhanced retention of tumor cells in the lungs, no obvious alteration of EC was observed at any time in nude mice receiving hu rIL-1β compared with mice receiving 0.9% NaCl solution (*).

Table 1 Effect of IL-1 on lung colony formation by A375 M melanoma injected into nude mice

<table>
<thead>
<tr>
<th>No. of tumor cells</th>
<th>Treatment</th>
<th>No. of mice with lung colonies/no. receiving injection</th>
<th>Median no. of lung colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^5</td>
<td>Vehicle</td>
<td>6/8</td>
<td>3 (0-25)°</td>
</tr>
<tr>
<td></td>
<td>Hu rIL-1β</td>
<td>7/8</td>
<td>45 (0-125)°</td>
</tr>
<tr>
<td>7.5 x 10^5</td>
<td>Vehicle</td>
<td>8/8</td>
<td>16.5 (1-45)°</td>
</tr>
<tr>
<td></td>
<td>Hu rIL-1β</td>
<td>8/8</td>
<td>85 (28-133)°</td>
</tr>
<tr>
<td>10 x 10^5</td>
<td>Vehicle</td>
<td>9/9</td>
<td>30 (14-60)°</td>
</tr>
<tr>
<td></td>
<td>Hu rIL-1β</td>
<td>9/9</td>
<td>225 (162-293)°</td>
</tr>
</tbody>
</table>

° Nude mice were given injections i.v. of 1 μg of hu rIL-1β or vehicle 1 h before tumor cells.
° Numbers in parentheses, range.
° P < 0.01 compared with mice receiving vehicle.
° P ≤ 0.001.

Table 2 Lung colony formation by A375 M melanoma in nude mice given injections of different concentrations of IL-1

<table>
<thead>
<tr>
<th>Hu rIL-1β (μg/mouse)</th>
<th>Median no. of lung colonies</th>
<th>Range</th>
<th>P°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6.5</td>
<td>1-12</td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>5</td>
<td>2-34</td>
<td>0.5</td>
</tr>
<tr>
<td>0.01</td>
<td>17</td>
<td>1-93</td>
<td>0.11</td>
</tr>
<tr>
<td>0.1</td>
<td>61</td>
<td>1-115</td>
<td>0.01</td>
</tr>
<tr>
<td>1</td>
<td>68</td>
<td>3-120</td>
<td>0.01</td>
</tr>
<tr>
<td>10</td>
<td>95</td>
<td>58-98</td>
<td>0.002</td>
</tr>
</tbody>
</table>

° Nude mice were given injections i.v. of different concentrations of hu rIL-1β 1 h before 5 x 10^5 tumor cells. Control mice received the same volume of vehicle.
° n = 8. All mice presented lung colonies at autopsy.
° Probability of no difference from control mouse.

DISCUSSION

In this study we found that hu rIL-1β increased the formation of tumor colonies from the human A375M melanoma in the lungs of nude mice. The effect was concentration dependent, time dependent, and short lived. The increase in experimental metastases was observed within the range of IL-1 concentrations that have many biological activities, including protection against myelosuppression by radiation and chemotherapy (3-6). The enhancement of lung colonization was seen with hu rIL-1β administered before tumor cell injection, thus implying an indirect host-mediated effect. In further support of this hypothesis, we found the exposure of A375M melanoma cells to hu rIL-1β before injection (followed by washing) did not change their lung-colonizing ability.

TNF possesses many of the activities described for IL-1, including EC activation (2, 9, 10), so it is not surprising that hu rTNFα showed an effect similar to that observed with hu rIL-1β. The possibility that TNF acts by inducing IL-1 needs to be taken into consideration (16).
IL-1 has been suggested to exert several of its activities through induction of IL-6 (17); the lack of activity of hu rIL-6 suggests that IL-1 affects metastasis formation either per se or through intermediate cytokines other than IL-6. It is of interest that IL-6, unlike IL-1 and TNF, does not affect other endothelial functions (18), including adhesive properties,* thus further suggesting that the effects of cytokines on endothelial linings are an important component in the modulation of the metastatic process.

A variety of mechanisms can be postulated to explain IL-1's promoting lung colonization. Metastasis formation is a complex process involving several sequential steps that tumor cells have to complete after their release from the primary tumor (19). Physiological systemic or local alterations can influence the tumor cells' ability to disseminate and metastasize. The organ distribution and arrest of radiolabeled tumor cells (14) have been often used as an experimental tool for following the dissemination of tumor cells. The distribution pattern of A375M cells (Fig. 3), showing higher retention in the lungs of hu rIL-1α-injected mice, suggests a more specific effect at the level of the target organ. Morphological studies on the intravascular arrest of circulating tumor cells indicate an initial arrest of tumor cells characterized by a tumor-EC contact during the first hour, followed by penetration of the tumor cells through vascular endothelium after 4 h and the complete extravasation by about 24 h (20). We found that hu rIL-1α given 1 h, but not 24 h, after tumor cells induced an augmentation of lung colonies as well. Moreover higher levels of radiolabeled tumor cells were found in the lungs of hu rIL-1α-treated mice from 4 to 72 h after their inoculation. These results support the hypothesis that the IL-1 treatment interferes with the retention and/or initial extravasation of circulating tumor cells in the secondary organs but does not affect the tumor cells' intrinsic capability to grow and form metastases.

The intravascular transit of malignant cells may perhaps be one of the most important steps in metastasis (21). IL-1 induces a cascade of cellular and biochemical events on EC (2, 9, 10) that leads to vascular congestion, clot formation, and cellular infiltration (22, 23), which could then affect tumor cell arrest and extravasation. However, in our study we found that the effect of hu rIL-1α-induced augmentation of lung colonies was not prevented in nude mice under warfarin-anticoagulant treatment (data not shown). Moreover, treatment with hu rIL-1β at the concentrations and schedules used in our study did not cause a reduction of circulating platelets (data not shown). Finally, we did not observe fibrin deposition or accumulation of neutrophils and platelets on the surface of EC that appeared to be intact by electron microscopy analysis. In addition the treatment of nude mice with ibuprofen, a nonsteroid antiinflammatory inhibitor of cyclooxygenase, described to protect against hemodynamic effects induced by IL-1, including vascular damage (24), did not prevent IL-1's lung colony augmentation (data not shown). In this regard it is possible that others' inflammatory mediators produced via the lipoxigenase pathway of the arachidonic acid metabolism (i.e., leukotrienes), which specifically bound to EC, can regulate tumor cell-endothelial cell interaction (25). Taken together these observations suggest that mechanism(s) other than microvascular injury or intravascular clotting are responsible for metastasis augmentation.

Several reports have shown that IL-1 stimulates leukocyte adhesion to EC and that this process is mediated by the induction of adhesive molecules on the EC membrane (26–28). Our group and others have shown that different tumor cell lines, including A375M melanoma, adhere more efficiently to IL-1-activated EC (11, 12). Recently Rice and Bevilacqua (29) have identified a novel adhesion structure responsible for IL-1-augmented adhesion of melanoma cells, thus making the hypothesis that IL-1-induced augmentation of lung tumor colonies results from a modification of EC surface properties quite attractive. IL-1's effect in vivo is already present when this cytokine is injected 1 h or even less before tumor cells. In vitro, the IL-1-induced increase in tumor cell adhesion to EC requires at least 2 h of incubation (11, 12). This discrepancy can be explained by the observation mentioned earlier that tumor cells can take a few hours to interact with and penetrate vascular endothelium (20), a time compatible with the modulation of EC properties by IL-1. In addition, it is worth noting the other effects by IL-1, like prostaglandin-mediated fever response that appears as early as 30 min after IL-1 injection, while in vitro the production of prostaglandin E requires several hours (30). Further and more direct studies are under way to define the role of IL-1-induced EC activation in the increased incidence of lung colonies.

The observations reported here are of interest in the context of the pathogenesis of metastasis and considering that IL-1 is proposed in combination with antineoplastic therapy. Macrophages, the major producers of IL-1, can exert a complex dual influence in the regulation of tumor progression and metastasis (31). Augmented secondary localization of cancer has been documented at sites infiltrated by inflammatory macrophages (32), and IL-1 could be a major mediator under these circumstances. Moreover, nonhematopoietic tumor lines, including melanomas, have been shown to produce IL-1 (33, 34). In light of the present observations, IL-1 production could represent a contributing element in the malignant behavior of tumors, by favoring the implantation of neoplastic cells at secondary sites.

IL-1 stimulates hematopoietic precursors, has radioprotective activity, and counteracts or ameliorates myelosuppression induced by cancer chemotherapy (3–6). The concerns raised by the observation reported here should not prevent trials in animals and humans designed to assess the efficacy of IL-1 in combination with cytoreductive therapy. These results should, however, serve to heighten awareness that an overall assessment of the potential of this cytokine and proposed agonists should include careful evaluation of their influence on the secondary spread of neoplasia.

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* E. Dejana, unpublished observation.


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