Prolonged Treatment with Angiostatin Reduces Metastatic Burden during Radiation Therapy

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Abstract

Ionizing radiation (IR) and concomitant angiostatin (AS) produce greater than additive local antitumor effects. We examined whether prolonged AS treatment added to IR reduces proliferation of lung metastases from LLC primary tumors. Flank tumors were treated with 40 Gy with or without AS (25 mg/kg/day). IR plus a 14-day course of AS improved local tumor control and blocked the increase in lung weights observed in the group receiving IR plus a 2-day course of AS group. Animals treated with prolonged AS exhibited no increase in lung weight and no macrometastases. These findings suggest that long-term treatment with antiangiogenic compounds may be effective in preventing metastases from IR-treated tumors as well as increasing the local antitumor effects of radiotherapy.

Introduction

Patients with cancer often have subclinical metastatic disease at the time of diagnosis, which can result in death from metastases after apparently successful local therapy of the primary tumor. Local therapy may include either surgery alone, radiation therapy alone, or, commonly, a combination of the two. For some tumors, the use of adjuvant systemic therapy, including hormonal manipulation or chemotherapy, has been shown to be effective in preventing or delaying the growth of metastases (1, 2). Recently, the inhibition of tumor-induced angiogenesis has emerged as a promising new treatment modality that can be used to inhibit the growth of microscopic disease (3–7), either alone (3, 6, 7) or in combination with conventional therapies (5, 8, 9). Key advantages of this approach are that it targets normal endothelial cells rather than genetically unstable tumor cells, making the development of drug resistance less likely (3), and that antiangiogenic therapy can exert a powerful antitumor effect with little or no toxicity (3, 4, 6, 7). We have previously investigated combining antiangiogenic agents with IR3 and demonstrated a local synergistic antitumor interaction between the two modalities (10–13). This synergistic effect requires only brief concomitant exposure to the antiangiogenic agents at the time IR is administered. Thus far, we have shown such a synergistic interaction with IR for AS (10), anti-VEGF antibody (11), and endostatin (12). Importantly, a prolonged course of antiangiogenic therapy is not required to produce this synergistic effect (10–12). One potential drawback to the synergistic effect of short-course AS and IR is that the more potent observed antitumor effects of this combination might remove the inhibition of the growth of metastases by tumors that generate AS (6, 7). In the present study, because there is evidence that the treatment of primary tumors by radiation or surgery can also result in increased secretion of proangiogenic factors (6, 11, 14, 15), which could potentially have a deleterious effect by stimulating the growth of micrometastases (15), we examine whether extending the course of AS beyond the completion of IR can prevent the accelerated growth of micrometastases after IR. We report here that extending the course of AS treatment reduces the metastatic burden to the lung compared with IR alone or IR combined with short-course AS. These findings suggest a potentially important use for antiangiogenic therapy to suppress the growth of micrometastases after treatment of a primary tumor with IR.

Materials and Methods

AS Production and Dosage. Human AS was a gift of G. Soff (Northwestern University, Chicago, IL) and was generated from human plasminogen as described previously (16). AS was suspended in PBS and administered as i.p. injections twice daily at a total dose of 25 mg/kg/day (0.5 mg/day/mouse) or 50 mg/kg/day (1 mg/day/mouse).

Tumor Model. Eight-week-old female C57BL/6 mice (Frederick Cancer Research Institute, Frederick, MD) were injected with 5 × 103 to 2 × 104 LLC cells (low metastatic strain, LM), a gift of J. Folkman (Harvard University, Boston, MA); (10, 11, 13, 17). Tumor volume was estimated three times a week by direct measurement and calculated as described previously (10, 11, 13, 17). A total of 6–10 mice were assigned to each experimental group on day 0. At various time points, mice were anesthetized using Metafane inhalation and euthanized by cervical dislocation.

Measurement of Lung Metastases. Lung weights are a good estimate of total metastatic burden to the lung and are frequently used as such in this model (6). For comparison, the normal combined lung weights were determined as i.p. injections twice daily at a total dose of 25 mg/kg/day (0.5 mg/day/mouse) or 50 mg/kg/day (1 mg/day/mouse).

Tumor Irradiation. Mice were irradiated using a General Electric Maxitron X-ray generator operating at 150 kV, 30 mA, using a 1-mm aluminum filter at a dose rate of 188 cGy/min, as described previously (10, 11, 13). Mice were shielded with lead except for the tumor-bearing right hind limb.

Data Analysis. Mean tumor volumes for each experimental group ± the SE were calculated. Differences in lung weights, tumor sizes, and number of lung metastases between multiple treatment groups were determined overall by nonparametric methods, the Kruskal-Wallis test with Dunn’s multiple comparison test as the post-test for pairwise comparisons, using the Prism 3.0 statistics software package (GraphPad). Differences between treatment groups were considered statistically significant if P ≤ 0.05.

Results and Discussion

Effect of AS on the Growth of Primary LLC Tumors. To characterize the effects of treatment with AS alone on LLC tumor growth and metastasis, we tested two different doses of human AS, 25 mg/kg/day and 50 mg/kg/day, divided into two equal doses/day. Untreated controls received an equivalent volume of PBS. Neither
dose of AS produced shrinkage or growth arrest of these primary tumors, although a small growth delay that did not reach statistical significance (P = 0.06) was observed (Table 1). However, there was a significant difference in lung weights (P = 0.003), with AS decreasing the metastatic burden to the lung. Lung weights were not statistically different from lung weights in non-tumor-bearing mice (147 ± 12 mg). Furthermore, there was no significant difference in lung weights between the doses of 25 mg/kg/day and 50 mg/kg/day AS. Because we wanted to evaluate an interactive effect between AS and IR, we chose the lower dose of AS for subsequent studies.

**Effect of IR Schedule on Primary Tumor Growth and Metastases.** We hypothesized that IR would behave similarly to surgical extirpation of the primary tumor (6) and result in increased metastases to the lung because of a decrease in AS production by the primary tumor. We, therefore, studied the effects of different IR schedules on LLC tumor growth and lung metastases. LLC tumors (s.c.) were grown to ~500 mm³, at which point treatment was begun. Because LLC is a rapidly growing tumor that can increase in volume 10-fold within 14 days (5, 10–13), we chose IR treatment regimens that included small numbers of relatively large fractions. For both doses of IR, using more fractions resulted in a smaller antitumor effect than using two large fractions (Table 2). Two fractions of IR produced a statistically significant (P < 0.001) decrease in tumor volume as compared with the effect of untreated controls. For all of the groups, there was a significant increase in lung weights compared with non-tumor-bearing mice (P < 0.01). However, there was no significant difference in lung weights between IR-treated animals and untreated controls, with the exception of mice treated with 40 Gy given as 2 fractions (Table 2). These findings imply that causing rapid shrinkage of LLC primary tumors is similar to surgical excision in terms of the release of inhibition of micrometastases growth. It is possible that this is a result of the death of LLC cells, which results in the decreased generation of AS, as observed when tumors are surgically excised (6, 7). An alternate explanation is the increased secretion of proangiogenic factors, such as VEGF, by irradiated tumors, as we have reported previously (11). Therefore, to determine the interactive effects of IR and AS on LLC lung metastases, we selected an IR schedule that produces moderate primary tumor growth delay and few fractions (Refs. 10, 13; Fig. 1).

**Effect of Combined Therapy with IR and AS on Primary Tumor Growth.** Tumor-bearing mice (mean volume, 510 ± 151 mm³) were treated with IR alone (40 Gy, two fractions, on days 0 and 1) with and without AS (25 mg/kg/day) as follows: IR (20 Gy, days 0 and 1) plus a 2-day concomitant course of AS (days 0 and 1); or IR (20 Gy, days 0 and 1) plus a 14-day course of AS (days 0 through 13); or IR (20 Gy, days 0 and 1) followed by a 12-day course of AS (days 2 through 13). These groups were compared with untreated controls and with mice treated with 40 Gy given in two fractions or in five fractions. The combination of IR and short-course (2-day) AS produced a greater than additive antitumor effect (Fig. 1A), consistent with our previous observations (10, 11). By day 14, the tumors in the untreated control group had achieved a mean volume of 6110 ± 582 mm³. Experimental groups with statistically significant differences in fractional tumor volume at day 14 included: IR alone (P < 0.05 relative to untreated control); AS alone (P < 0.05 relative to untreated control); IR plus short-course AS (P < 0.001 relative to IR alone); IR plus long-course AS (P = 0.04 relative to IR alone). There was no significant difference in tumor volume at 14 days between IR alone and IR followed by AS (P > 0.05). There were no significant differences in mean tumor sizes among any of the groups combining AS + IR.

**AS Decreases Pulmonary Metastatic Burden after Treatment with IR or IR and Short-Course AS.** Lungs were weighed as an estimate of total metastatic burden after treatment with combinations of IR and AS (Fig. 1B). Differences between the mean lung weights of the groups as measured by the Kruskal-Wallis test were significant (P = 0.0002). Lung weights in tumor-bearing animals, including untreated controls, IR alone, and IR plus AS (2-day course), were elevated compared with those observed in non-tumor-bearing animals (P < 0.05). In contrast, the lung weights observed in the groups treated with prolonged courses of AS (IR + 14-day course AS, AS alone, and IR followed by AS) were not significantly different from the lung weights of non-tumor-bearing animals (P > 0.05) but were significantly different from those of the group receiving IR + AS (2-day course; P = 0.0068). There was no significant difference between lung weights of untreated controls (248 ± 18 mg), IR-treated mice (two fractions, 276 ± 36 mg; five fractions, 240 ± 32 mg) or mice treated with AS alone for 2 days (279 ± 56 mg). These results suggest that a prolonged course of AS can result in a decrease in metastatic burden such that only micrometastases are present, and lung weights do not increase significantly above those of non-tumor-bearing animals.

Next, to investigate possible reasons for the difference in lung weights among treatment groups, we counted the number of pulmonary metastases by examining H&E-stained slides of lung tissue sections. There was a significant difference between the means of all of the treatment groups by the Kruskal-Wallis test (P = 0.0004). In the untreated controls, there were numerous micrometastases (Figs. 1C and 2A), which resulted in a higher total number of metastases compared with all treatment groups (P < 0.05), consistent with the previously reported inhibition of micrometastases growth because of tumor-generated AS (6). The total number of metastases in the two groups treated with IR alone was smaller (P < 0.01), but the individual metastases were larger in size than those in the untreated control group (Fig. 2). This was also true for the treatment group in which AS was given for 2 days concurrently with IR (Fig. 2), a regimen that produced greater primary tumor shrinkage than IR treatment alone (Fig. 1A). Lungs from this group were indistinguishable from lungs from mice treated with IR alone (Fig. 2) in that there were multiple large metastases, sometimes nearly replacing the entire lung parenchyma (not shown). However, for mice treated with IR + AS (14-day course) or IR followed by AS (12-day course), metastases tended to be much smaller in size than those in groups receiving only

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**Table 1. Effect of different doses of AS alone on LLC growth and metastases**

<table>
<thead>
<tr>
<th>Experimental group (n = 5-10 per group)</th>
<th>Primary tumor volume at day 14 (mm³)</th>
<th>Lung weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>5092 ± 368</td>
<td>250 ± 14</td>
</tr>
<tr>
<td>AS, 25 mg/kg/day</td>
<td>3666 ± 453</td>
<td>158 ± 19</td>
</tr>
<tr>
<td>AS, 50 mg/kg/day</td>
<td>4062 ± 498†</td>
<td>160 ± 8*</td>
</tr>
</tbody>
</table>

*No statistically significant difference compared with control (P > 0.05).
† P < 0.05 when compared with control. (Mean lung weight in non-tumor-bearing animals, 147 ± 12 mg).

**Table 2. Effect of differing schedules of IR on lung metastases**

<table>
<thead>
<tr>
<th>Experimental group (n = 5-10/group)</th>
<th>Primary tumor volume at day 14 (mm³)</th>
<th>Lung weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>5092 ± 368</td>
<td>250 ± 14</td>
</tr>
<tr>
<td>40 Gy (5 fractions)</td>
<td>3772 ± 395*</td>
<td>240 ± 32</td>
</tr>
<tr>
<td>50 Gy (2 fractions)</td>
<td>1855 ± 359*</td>
<td>210 ± 44</td>
</tr>
<tr>
<td>50 Gy (5 fractions)</td>
<td>3541 ± 395*</td>
<td>229 ± 35</td>
</tr>
</tbody>
</table>

*There is no statistically significant difference between the different courses of radiation for lung weights (P = 0.29). However, all are statistically significantly higher than lung weights observed in non-tumor-bearing animals, whose mean lung weight was 147 ± 12 mg.

*Statistically significant difference compared with control animals (P < 0.05).
*No statistically significant difference compared with control (P > 0.05).
Fig. 1. Growth and metastasis of LLC primary tumors subjected to IR and AS. LLC cells \(10^6\) were implanted in the hind limbs of C57BL/6 mice and grown to a volume of 500 mm\(^3\), at which point treatment was begun with either 40 Gy or 50 Gy. A, effect of adding short-course and long-course AS to IR. Treatment was begun either with IR alone or IR plus short- or long-course AS. A, untreated controls; B, 40 Gy (2 × 20 Gy); C, AS (14-d course); D, 40 Gy (5 × 8 Gy) + AS (14-d course); E, 40 Gy (2 × 20 Gy) + short-course AS (2-d course); F, 40 Gy (2 × 20 Gy) + plus long-course AS (14-d course); G, 40 Gy (2 × 20 Gy) followed by AS (12-d course). B, total metastatic burden to the lung in mice bearing LLC primary tumors treated with different schedules of concurrent AS. Lung weights were harvested from mice in the experiment described in Fig. 2 and weights determined immediately. Differences between the means as measured by the Kruskal-Wallis test were significant \((P = 0.0002)\). Lung weights in tumor-bearing groups treated with IR alone or IR plus AS (2-d course) were elevated compared with those in non-tumor-bearing animals \((P < 0.05)\), with the exception of the groups treated with prolonged courses of AS (IR +14-d course AS, AS alone, and IR followed by AS), whose lung weights were not significantly different from those of non-tumor-bearing animals \((P > 0.05)\). Lung weights in mice treated with IR alone and IR + AS (2-d course) were greater than those in untreated controls, but the difference did not achieve statistical significance \((P = 0.059)\). C, number of lung metastases in mice bearing LLC primary tumors treated with different schedules of concurrent AS. After weighing, lungs were fixed in neutral buffered formalin, the entire lung sectioned, and then the sections stained with H&E. Lung metastases were then counted by an observer blinded to treatment groups. Overall, there was a statistically significant difference between the means of the experimental groups \((P = 0.0004)\). See Text for discussion.

The objective of these experiments was to determine whether using antiangiogenic therapy as an adjuvant to IR could decrease metastatic burden after IR. We have previously shown that brief concomitant courses of AS (10) and other antiangiogenic therapies, such as anti-VEGF antibody (11) and endostatin (12), synergize with IR to inhibit the growth of primary tumors. However, it was unknown whether such short-term synergistic treatments might have a deleterious effect on metastatic spread of tumor. We hypothesized that adding a prolonged course of AS to treatment with IR, although not providing any additional benefit in controlling local disease when compared with using a short course of AS with IR (10), might provide a benefit in suppressing the growth of metastases. LLC was chosen as an experimental model because this tumor type has a rapid doubling time (10, 11, 13), is relatively resistant to chemotherapy and radiation therapy (18), and reliably produces lung metastases in a short period of time (6, 18). In this tumor model, combined treatment with angiogenic agents and IR result in synergistic antitumor activity (10–13) and enhanced toxicity to tumor blood vessels (11–13, 19).

Although a brief concomitant course of AS boosted the local antitumor efficacy of IR in a synergistic fashion at least as effectively as IR plus a longer course (14d) of AS (Fig. 1A), such a short course of AS failed to suppress the growth of lung metastases and, therefore, the metastatic burden to the lung as measured in this study by lung weight (Fig. 1B). The trend toward increased metastatic burden in the lung in the IR + AS (2-day course) group compared with IR alone \((P = 0.08)\) may be attributable to the more rapid decrease in the primary tumor volume that reduces the suppressive effect of the primary tumor on metastasis growth, or it may be caused by increased VEGF secretion by the primary tumor (11, 15). However, continuing AS treatment after the completion of IR administration did suppress the proliferation of these lung metastases \((P = 0.0068)\) compared with using a short-course AS. These data suggest that, although IR plus IR (Fig. 2 and not shown). They were similar in size to the micrometastases observed in the untreated control group or the group treated with AS alone but were decreased in number compared with untreated control \((P < 0.05)\). Given that there is no significant difference in raw lung weights between non-tumor-bearing mice and mice treated with IR plus prolonged courses of AS, either during or after IR, these results further suggest that treatment with AS for extended periods inhibits the growth of micrometastases: the overall lung weight remains equivalent to that in tumor-free animals (Fig. 1B), and the overall number of metastases decreases compared with the number for untreated controls (Fig. 1C).

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short-term concomitant AS therapy results in markedly improved local tumor control equivalent to that observed in mice treated with IR plus longer courses of AS, continued AS therapy may be necessary to suppress metastases from irradiated tumors. In human tumors, there is evidence that treatment of primary tumors with IR results in an increase in systemic VEGF levels, which may encourage the growth of subclinical metastatic disease (15). These studies, therefore, suggest the use of AS and other antiangiogenic compounds during and after treatment of primary tumors with conventional local therapies as an adjuvant therapy to suppress the growth of micrometastases. Moreover, because AS has minimal, if any, toxicity, it would be particularly appealing as a means of preventing the increase in pulmonary metastases that might result after treatment of the primary tumor with IR, secondary either to the release of additional VEGF by the primary tumor (11) in response to radiation or to the decrease in AS production. The outcome of such a strategy could ultimately be to improve relapse-free and long-term survival in cancer patients.

References

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