Radiation Therapy to a Primary Tumor Accelerates Metastatic Growth in Mice

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ABSTRACT

The surgical removal of a primary tumor can result in the rapid growth of metastases. The production of angiogenesis inhibitors by the primary tumor is one mechanism for the inhibition of metastatic tumor growth. The effect of curative radiotherapy to a primary tumor known to make an inhibitor of angiogenesis and the effects on distant metastases has not been studied. We here show that the eradication of a primary Lewis lung carcinoma (LLC-LM), which is known to generate angiostatin, is followed by the rapid growth of metastases that kill the animal within 18 days after the completion of radiation therapy. The right thighs of C57BL/6 mice (n = 25) were injected s.c. with 1 × 10⁶ LLC-LM cells. Animals were randomized to one of five groups: no irradiation, 40 Gy in one fraction, 30 Gy in one fraction, 40 Gy in two 20 Gy fractions, or 50 Gy in five 10 Gy fractions. Tumors were clinically eradicated in each treatment group. All of the surviving animals became dyspneic and were killed within 14–18 days after the completion of radiation therapy. Examination of their lungs revealed >46 (range, 46–62) surface metastases in the treated animals compared with 5 (range, 2–8) in the untreated animals. The lung weights had increased from 0.2 g (range, 0.19–0.22 g) in the controls to 0.58 g (range 0.44–0.84) in the experimental animals. The most effective dose regimen was 10 Gy per fraction for five fractions, and serial experiments were conducted with this fractionation scheme. Complete response of the primary tumor was seen in 25 of 35 (71%) mice. The average weight of the primary tumor was 0.66 g (range, 0.61–0.70 g). The average number of surface metastases increased from five per lung (range, 2–13) in the control animals to 53 per lung (range, 46–62) in the irradiated animals. Both differences were statistically significant with P < 0.001. If the nontumor-bearing leg was irradiated or the animals were sham-irradiated, no difference in the number of surface metastases or lung weights was observed between the control group and the treated group. Urinary levels of matrix metalloproteinase 2, the enzyme responsible for angiostatin processing in this tumor model, were measured and correlated with the viability and size of the primary tumor. Administration of recombinant angiostatin prevented the growth of the metastases after the treatment of the primary tumor. In this model, the use of radiation to eradicate a primary LLC-LM tumor results in the growth of previously dormant lung metastases and suggests that combining angiogenesis inhibitors with radiation therapy may control distant metastases.

INTRODUCTION

As the fields of surgical, radiation, and medical oncology have become more effective at treating primary tumors, distant metastases have become an increasingly important limiting factor in patient survival. The treatment of the clinically observable tumor alone is no longer adequate, and potential interactions that occur between host-tumor, host-metastases, and tumor-metastases must be considered. The endothelium is the common interface between host, tumor, and metastases and functions as a molecular gatekeeper (1). Angiogenesis has been shown to be essential for the growth and survival of solid tumors and their metastases (2). Moreover, tumors can also mobilize antiangiogenic fragments from larger molecules. For example, fragments of prolactin (3), platelet factor 4 (4), thrombospondin (5), epidermal growth factor (6), and laminin (7), as well as the tumor-derived fragments angiostatin (8), endostatin (9), and antiangiogenic antithrombin (10), have been shown to be inhibitors of endothelial cell proliferation. The balance between these pro- and antiangiogenic factors determines the growth and survival of the tumor and its metastases.

An estimated 50% of all cancer patients will develop metastases (11). There are five typical patterns of presentation based on the growth of the metastases in relation to the primary tumor (12). The first pattern is the rapid growth of previously undetectable metastases after the treatment of a known primary tumor. We developed a model of LLC-LM in which the surgical removal of the primary tumor was followed by the explosive growth of previously dormant lung metastases (8). This led to the discovery of angiostatin, a potent angiogenesis inhibitor. The second pattern is the simultaneous presentation of the primary tumor and its metastases. This pattern is seen in the laboratory with a strain of LLC that does not make angiostatin, and the primary tumor and the metastases grow at equal rates. The third pattern is an occult primary tumor presenting with known metastases. This pattern was noted in up to 5% of head and neck cancer patients seen in a typical radiation oncology clinic (13). The fourth pattern is the treatment of a primary tumor without detectable metastases, followed by the growth of the metastases years after the initial primary treatment. This pattern is seen in a laboratory model of B16 melanoma in which the primary tumor sheds micrometastases that remain dormant after removal of the primary tumor. They can be reactivated to grow years later from this dormant state. The fifth pattern is the simultaneous presentation of a primary tumor and metastases in which treatment of the primary tumor is followed by the regression of these metastases. This is seen in rare cases of renal cell cancer after surgical removal of the primary tumor.

In this study, we show that radiation therapy to a primary LLC-LM tumor, analogous to its surgical removal, is also followed by the explosive growth of the previously dormant metastases. We also show that exogenous angiostatin administration can prevent the growth of the metastases and that urinary MMPs may act as a surrogate marker for angiostatin production. These data suggest that a subset of patients may be at increased risk of metastatic growth after the surgical or radiotherapeutic treatment of their primary tumors and could benefit from combination treatment with the addition of an angiogenesis inhibitor.
RADIOThERAPY CAN STIMULATE METASTATIC GROWTH

MATERIALS AND METHODS

Cell Lines and Cell Culture. LLC-LM and T241 cells were grown at 37°C in 10% CO₂ in DMEM with 10% heat-inactivated fetal bovine serum plus 1% glutamine-penicillin-streptomycin under sterile tissue culture conditions.

Animals and Tumor Model. Male, 4–6-week-old C57BL/6 mice (Jackson Labs, Bar Harbor, ME) were used. Mice were caged in groups of five or less, and their backs and hind limbs shaved. All of the animals were fed a diet of animal chow and water ad libitum. Animals were anesthetized in an isofluorane chamber prior to all procedures and were observed until fully recovered. Animals were killed by lethal inhalation of carbon monoxide.

Peptide production, for each experimental group 0.5% Coomassie Blue R-250 in acetic acid, isopropyl alcohol, and water were selected in 100 mM methotrexate-containing growth medium. Concentrated medium was loaded on a Protein A-Sepharose column. The mFc-muratine angiostatin was eluted with 100 mM citric acid and was then neutralized with 20% 2.5% Triton X-100 for 30 min. The gels were rinsed and soaked overnight in substrate buffer (50 mM Tris-HCl, 0.05 M EDTA, and 0.001% bromphenol blue]. Samples were loaded into wells of a 4% acrylamide gel containing 0.1% (w/v) gelatin. Gels were run at 15 mA during stacking and 20 mA during the resolving phase. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 min. The gels were rinsed and soaked overnight in substrate buffer (50 mM Tris-HCl buffer, 5 mM CaCl₂, and 0.02% NaN₃). Gels were then stained for 15 min in 0.5% Coomassie Blue R-250 in acetic acid, isopropyl alcohol, and water (1:3:6), destained in acetic acid, ethanol, and water (1:3:6), photographed, and dried for permanent storage.

Data Analysis. Mean lung weights and number of surface metastases, counted with a hand-held magnifier at 5× power, for each experimental group with SE were calculated. Differences between pairs of treatment groups were tested using a Student’s t test, with a significance level of P < 0.05.

RESULTS

Effect of Irradiation on a Primary LLC and its Metastases. LLC-LM cells were injected s.c. into the right thighs of C57BL/6 mice (n = 25). When the tumors had reached 500 mm³, 8 days after implantation, the animals were randomized into one of five treatment groups: no irradiation, 40 Gy in one fraction, 30 Gy in one fraction, 20 Gy per fraction in two fractions, or 10 Gy per fraction in five fractions. The control animals that received no irradiation were killed when their tumors grew to >6000 mm³. Representative lungs from the treated animals were photographed and are shown in Fig. 1A. Between 18 and 21 days after the completion of irradiation, all of the surviving animals became dyspneic and were killed. Fig. 1B demonstrates representative lungs from animals whose primary tumors were untreated. Normal lung weight in a mouse is 0.2 g (data not shown). The primary site in a treated mouse showed no evidence of measurable tumor and small areas of denuded tissue. Further examination of the lungs revealed at least 46 (range, 46–62) large surface metastases in the irradiated animals compared with 5 (range, 2–8) pinpoint metastases in the unirradiated animals. Lung weight, which correlates with tumor burden, had increased from 0.2 g (range, 0.19–0.22 g) in the controls to 0.58 g (range, 0.44–0.84) in the experimental animals. Repeated experiments using 30 Gy in one fraction, 20 Gy per fraction for two fractions, and 10 Gy per fraction for five fractions were conducted with similar results. Ten Gy per fraction on 5 consecutive days, when the tumors averaged 500 mm³, was the most effective dose for producing a clinical cure of the primary tumor, with reasonable amounts of animal handling.

Multiple experiments were conducted with this dosage, and all animals with regressed primary tumors succumbed to lung metastases. Complete response of the primary tumor was seen in 25 (71%) of 35 mice with this fractionation scheme. The lungs from mice in which the primary tumor only partially regressed demonstrated little growth of the metastases. The average weight of the lungs in the nonirradiated animals was 0.22 g (range, 0.19–0.24 g) and in the irradiated animals was 0.66 g (range, 0.61–0.70 g). The average number of surface metastases increased from 5 per lung (range, 2–13) in the control animals to 53 per lung (range, 46–62) in the irradiated animals. Both comparisons were statistically significant (P < 0.001). Fig. 2 demonstrates the average number of surface metastases and lung weights in the control animals versus irradiated animals. If the nontumor-bearing leg was irradiated or the animals were sham-irradiated, then no difference in the number of surface metastases or lung weights was observed between the control group and the treated group (data not shown).

Effect of Irradiation on Fibrosarcoma T241 and Its Metastases. To test whether the growth of previously dormant metastases after the regression of the primary tumor was exclusive to LLC-LM, T241 fibrosarcoma, which is known to suppress the growth of its metastases, was tested. T241 cells were implanted in the right thighs of C57BL/6 mice. Tumors were allowed to grow to an average size of 350 mm³, when mice were randomized to no-radiation or 10 Gy per fraction for five fractions. Controls that received no irradiation were killed when their tumors were >5000 mm³ and irradiated animals were killed when they became dyspneic. The total number of surface metastases in the controls was 3 (range, 2–6) compared with 42 (range, 32–51) in the irradiated animals. Likewise, the average lung weights increased from 0.33 g (range, 0.28–0.38) in the controls to 0.67 g (range, 0.55–0.78) in the treated mice. This tumor line also demonstrated suppression of lung metastases by the primary tumor, but the size of the lung metastases in the control mice were larger than in the control mice of LLC-LM. This may explain the higher lung weights but fewer total number of metastases found in the T241 control group.

MMP Activity Is Increased in the Urine of Tumor-bearing Mice. MMP-2 in the culture media of LLC cells has been shown to process angiostatin from its parent molecule plasminogen (15). In addition, MMP levels have also been detected in the urine of cancer patients and have been shown to be predictive of disease status (16). We, therefore, sequentially collected urine from individual mice to assay the level of MMP production to determine whether MMP-2 levels might act as a surrogate marker for angiostatin production. Urine was collected on the day of tumor implantation (day 1), postim-
plantation (day 4), the first day of radiotherapy (day 8), and 6 days postirradiation (day 15). Gelatin zymography of these urine samples showed that the level of MMP-2 increased from day 1 to day 4 and again from day 4 to day 8. (Fig. 3) However, at day 15, which is 6 days after the completion of 20 Gy per fraction for two fractions, very little MMP-2 activity could be detected. This decrease in the level of MMP-2 in the irradiated mice is consistent with the regression of their tumors by irradiation, as well as with a decreased production of angiostatin. Likewise the increased level of MMP in the urine of untreated mice is consistent with continued tumor growth, production of MMPs, and release of angiostatin that maintains the lung metastases in a dormant state.

**Exogenous Angiostatin Suppresses the Growth of Metastases in LLC-LC.** Sixteen mice were injected with LLC-LM on the right thigh. When the tumors were 500 mm³, the mice were randomized into two groups. Both groups were irradiated with 10-Gy fractions for five doses. One group was treated with mFc-mAS at a dose of 20

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Fig. 1. Local control of primary LLC-LM tumor with radiotherapy leads to the growth of lung metastases. A, three representative sets of lungs from mice whose primary tumor was treated by local radiotherapy. A large number of surface metastases are visible. On the left, the dose given to the primary tumor. B, two sets of lungs removed from mice whose primary tumor was not irradiated. Small micrometastases are visible.

Fig. 2. Increased metastatic burden after local radiotherapy to a LLC-LM tumor. Twenty-four mice whose tumors were 750 mm³ were separated into two groups, one of which received radiation to the primary tumor. Within 18–21 days, the number of surface metastases (A) and the lung weights (B) had increased significantly in the irradiated mice compared with the nonirradiated mice.

Fig. 3. Representative gelatin zymogram of urinary MMPs. Increased production of MMP-2 in the urine of mice implanted with LLC-LM as measured by substrate gel electrophoresis. An increasing level of enzymatic activity was detected in the urine of mice from day 1 to day 4 and again from day 4 to day 8. However, urine collected on day 15, 6 days postradiotherapy, contained significantly lower levels of MMP-2 concomitant with the tumor regression observed at this same time point.
mg/kg/day for 21 days beginning on the first day of irradiation. The group that received irradiation alone became dyspneic 18–21 days after irradiation; they were killed and their lungs were weighed. The mice treated with mFc-mAS were also killed at this time. The weight of the lungs from the mice treated with irradiation alone weighed 0.67 g (range, 0.51–0.77) and the lungs from the animals treated with mFc-mAS plus irradiation weighed 0.30 g (range, 0.26–0.39). These data demonstrate that in the case that a primary tumor producing an endogenous inhibitor of angiogenesis is regressed by radiotherapy, replacement therapy of the angiogenesis inhibitor can potentially prevent metastatic growth.

**DISCUSSION**

The goal of cancer treatment is to cure the primary tumor and any underlying metastases. However, numerous researchers have observed that the treatment of the primary tumor with either surgery or radiotherapy can have unpredictable effects on metastases. The first major experiment examining the effect of local irradiation and metastases frequency was performed by Kaplan and Murphy in 1949 (17). Their work was based on the clinical observation that several patients with epidermoid carcinoma of the lip who had received prior radiotherapy presented to them with widely metastatic disease. In this study, the focus was to determine whether localized radiotherapy could stimulate metastatic disease. A Bagg-Jackson mammary tumor was implanted s.c. over the rear leg in 185 C57 black mice. Ninety-six mice were subjected to local irradiation and 85 were kept as controls. The results showed that 43.5% of the treated animals developed lung metastases *versus* 9.6% of untreated controls (*P* < 0.0001). These results are similar to our results when comparing the metastases, but the primary tumors in Kaplan and Murphy’s (17) experiment only had a growth delay of 2 weeks and were not cured. The authors could propose no explanation for this finding other than that perhaps the longer survival after treatment allowed the development of a greater metastatic burden. In a similar study, Suit *et al.*, in 1970 (18), observed that animals with recurrent tumor at the primary site after radiotherapy had a higher likelihood of metastatic disease.

In a recent review by Von Essen in 1991 (19), the data from 41 different experiments was gathered to examine the effect of local irradiation on distant metastases; however, because of the wide variety of different animal species, tumor lines, fraction schemes, and experimental techniques, no generalizations regarding the effect that local radiotherapy had on the growth of distant metastases could be made. Some of the experiments demonstrated a greater metastatic load after irradiation, whereas others showed a decreased load. These findings are consistent with our prior findings that of 10 tumors screened, only 1 suppressed its own metastases (8).

We have previously proposed that a tumor’s metastatic growth pattern is dictated by the intensity of angiogenesis in the vascular bed of the metastasis (1). The ratio of positive-negative angiogenic factors within this vascular bed determines whether the metastasis will remain dormant or begin to grow. The question that remains is: what is the effect of local radiotherapy on this balance within the vasculature of the metastasis at a remote site? Canney and Dean showed that after local radiotherapy, which included the liver in the treated field, the level of TGF-β in liver biopsies from these irradiated patients was elevated, compared with biopsies from the livers of nonirradiated patients (20). Roberts *et al.* (21) demonstrated in 1986 that TGF-β was a potent stimulator of angiogenesis *in vivo*. Likewise, Gorski *et al.* (22) demonstrated that the VEGF mRNA levels detected within tumors, by immunohistochemistry or Northern analysis, were increased after local irradiation. It was also found that the increased VEGF levels decreased the efficacy of the radiation against the primary tumor. However, their study did not demonstrate detectable increases in the plasma VEGF levels after radiotherapy. These are examples in which the byproducts of local irradiation could have proangiogenic effects at a remote site at which VEGF or TGF-β could stimulate angiogenesis within the metastases vascular beds. Conversely, in the present model, the removal of inhibition of angiogenesis at a remote site by decreasing production of angiostatin shifts the balance in the metastatic bed to a proangiogenesis status, thereby facilitating metastatic growth. Therefore, the delicate balance controlling metastatic growth can be shifted to proangiogenesis by increasing the level of the stimulators, *e.g.*, VEGF or TGF-β, or by decreasing the level of inhibitors, for example, angiostatin.

The combination of radiotherapy and angiogenesis inhibition was first demonstrated in 1992 by Teicher *et al.* (23), who showed a synergistic effect of combining radiotherapy, TNP-470, and minocycline, a weak inhibitor of MMP activity. This work was expanded on by Mauzer *et al.* (24), who demonstrated that when angiostatin was administered in conjunction with radiation therapy, there was a synergistic effect against the primary tumor. Gorski *et al.* (25) noted that the combination worked best when the angiostatin was given simultaneously with the radiotherapy. Gorski *et al.* (22) also showed that radiotherapy and antibodies against VEGF had a synergistic effect against primary tumors. This growing body of work is important to consider as the role of radiotherapy and antiangiogenic molecules against the primary tumor is further examined. This combination was historically thought not to be effective, because radiotherapy was thought to work best against well-oxygenated tumors, and, if antiangiogenic molecules caused a decrease in oxygenation, then the radiotherapy would be less effective. However, the previous studies above have shown that the opposite is true and that these two modalities work best against the primary tumor when given simultaneously.

The effect of local irradiation on distant angiogenic sites is more complicated. In a recent paper by Hartford *et al.* (26), the irradiation of a primary tumor decreased the amount of angiogenesis seen in a cranial window at a distant site. This result, using radiotherapy, contrasted the effect seen after surgery in which the net result was stimulation of angiogenesis in the cranial window. An interesting finding was that the plasma levels of endostatin, an endogenous angiogenesis inhibitor, in the irradiated mice were twice those found in the nonirradiated mice. Taken together, these results suggest an obvious synergy between radiotherapy and antiangiogenic molecules on the primary tumor that can lead to an increased cure rate. However, the effects of this local treatment on the endothelium at a distant site is a much more complicated interaction in which small imbalances can lead to the quick growth of previously dormant metastases. We speculate that radiation therapy may have a complex effect on angiogenesis at a local site as well as systemically.

In this study, through close monitoring, the production of MMPs in the urine of the implanted mice was shown to be correlated with the production of angiostatin. When the primary tumor is cured by irradiation, a net imbalance of proangiogenic over antiangiogenic factors occurs within the metastatic vascular bed, and rapid expansion follows. This has been shown previously with surgical removal of the primary tumor, but the current study is the first to demonstrate this phenomenon after radiotherapy in the setting of a tumor known to produce angiostatin. Notably, after irradiation of the primary tumor, the net imbalance created within the metastatic vascular bed can be shifted back to the inhibitory side by the exogenous administration of angiostatin. Potential future clinical studies could include examination of patients’ urine or serum for markers of an inhibitor of angiogenesis produced by their tumors, which may be keeping undetected metastases dormant prior to the beginning of their local treatment. Screening of a patient’s urine for MMP activity as a surrogate marker for
production of endogenous inhibitors of angiogenesis is the first step. If the tumor is found to produce one of these markers, then concurrent radiotherapy and the administration of an angiogenesis inhibitor followed by maintenance therapy with the angiogenesis inhibitor might be recommended. The length of time for this maintenance therapy is unknown, but as patients are enrolled on antiangiogenic clinical trials and our understanding of angiogenesis continues to grow, these questions will hopefully be answered.

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REFERENCES

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