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Advances in Brief

Irradiation of a Primary Tumor, Unlike Surgical Removal, Enhances Angiogenesis Suppression at a Distal Site: Potential Role of Host-Tumor Interaction[1]

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Abstract

Changes in distal angiogenesis in response to irradiation of primary tumors are not known. To this end, PC-3, a human prostate carcinoma, and FSA-II, a murine fibrosarcoma, were grown in the gastrocnemius muscles of male nude mice. Distal angiogenesis was measured in gel containing human recombinant basic fibroblast growth factor placed in the cranial windows of these mice. PC-3-bearing mice showed inhibition of distal angiogenesis, as compared with non-tumor-bearing controls. Surgical removal of tumors tended to accelerate distal angiogenesis; in comparison, after irradiation of the PC-3 primary tumor, rates of angiogenesis in the cranial window were retarded. Irradiation of the non-tumor-bearing leg or of non-tumor-bearing animals showed no measurable effect on rate of growth of vessels in the cranial window. Similar results were found with the FSA-II tumors, with slowed distal angiogenesis in tumor-bearing animals and further suppression in animals with irradiated tumors. These results demonstrate that the effect of irradiation of a primary tumor on angiogenesis at a distal site may differ from the effect of surgical removal of the primary tumor. Unlike surgery, irradiation of a tumor may enhance angiogenic suppression at a distal site, and this difference may involve host-tumor interaction.

Introduction

Beyond a diameter of 1–3 mm, the growth of a tumor, whether at a primary or metastatic site, is angiogenesis dependent. Recent work has identified several molecules, such as angiostatin, the production of which is associated with the presence of a primary tumor and that serve to inhibit the growth of distal endothelial cells (1–5). Although recent work has demonstrated an improved therapeutic effect of irradiation with the administration of angiostatin (6, 7), the effect of irradiating a primary tumor on the suppression of angiogenesis at a secondary (distal) site is not known. To this end, we measured angiogenesis suppression in a distal site (cranium) in animals bearing a human prostate carcinoma (PC-3) or a murine fibrosarcoma (FSA-II) in their hind limbs. We show here that, unlike surgical resection, irradiation of primary tumor enhances angiogenesis suppression in the cranium.

Materials and Methods

Tumor Models. PC-3, a human, hormone-independent adenocarcinoma of the prostate, suppresses angiogenesis at remote sites in a volume-dependent manner and is associated with angiostatin production (3, 8, 9). FSA-II, a murine fibrosarcoma spontaneously arising from a C3Hf/Sed mouse, has been well characterized in terms of its level of radiation sensitivity (TCD_{50}[3]), rapid growth, and pathological features (10). *In vivo* FSA-II represents a rapidly growing tumor, whereas PC-3 is a slowly growing tumor. For tumor implantation, a single cell suspension containing $1–5 \times 10^5$ tumor cells was injected i.m. in 10 μl of inocula into the middle to upper third of the right gastrocnemius muscle of the mouse.

Surgical Techniques. Cranial window preparation in the mouse was performed as previously described (11). Briefly, the preparation involved removal of a cranial bone flap, opening of dura and arachnoid membranes, and sealing with an 8-mm coverglass. Resection of the mouse leg involved dissection of muscle around the femoral head, ligation of all identified significant blood vessels, surgical amputation with a minimum 1-cm margin around palpable tumor in the gastrocnemius muscle, and closure with interrupted suture.

Angiogenesis Assay. Preparation of the angiogenic gel containing human recombinant basic fibroblast growth factor, its implantation into the cranial window, and its utility as a quantitative assay for angiogenesis have been detailed previously (12, 13). Briefly, newly mixed gel containing a solution of 60 ng of human recombinant basic fibroblast growth factor (Life Technologies, Inc., Gaithersburg, MD) was sandwiched between two pieces of nylon mesh and then placed on the pia mater inside the cranial window. Ensuing angiogenesis was monitored as vessels grew between the individual fibers of the mesh during subsequent days and quantified as the fraction of the number of squares of the nylon mesh that contained at least one newly formed vessel. This method is most sensitive in the range of 20 to 80% of filled mesh squares. Animals with any signs of brain bleeding, inflammation, or neurological deficits were sacrificed and excluded from the study.

Radiation Procedure. The mouse leg from the ankle to the hip was irradiated with parallel opposed 3-cm-diameter ^{137}Cs portals (14), at a dose rate of 5.8 Gy/min. With lead shielding, the dose at 1 cm from the edge of the treatment field was 0.3% of the dose at the field center. Local irradiation was administered at the time that the tumors exceeded 500 mm^3. Radiation was given in a single fraction of 100 Gy, the dose level chosen because of the high TCD_{50} required by a 250-mm^3 FSA-II tumor (10). For the PC-3 cell line, the TCD curve is not known, but unpublished data in our laboratory showed that >80% of PC-3 tumors 8 mm in diameter implanted in the leg achieved long term local control at the single-fraction 60-Gy dose level.

Experimental Protocol. All experiments were conducted in accordance with guidelines of the Massachusetts General Hospital's Subcommittee on Research Animal Care. Male nude mice, 6 to 10 weeks old, were bred and housed in our gnotobiotic animal colony. PC-3 tumors were allowed to grow for 6 to 8 weeks before gel implant, whereas FSA-II tumors were allowed to grow for 3–4 days before gel implant. Tumors measured 300–500 mm^3 at day 4 after gel implant, the minimum size (for PC-3) for angiogenesis suppression in the cranial window model (9). Procedures (surgery or irradiation) were performed when the tumors exceeded 500 mm^3: days 5–6 post-gel implantation in the case of FSA-II; and days 6 to 7 post-gel implantation in the case of PC-3. Tumor volumes were estimated by the formula $V = \pi/6 \times a^2 \times b$, where a was the short axis of the tumor, and b the long axis.

Statistical Analyses. Angiogenic responses are reported as the median (along with the 25th and 75th percentiles) of the fractions of nylon mesh that contained vessels on any given day after gel implant (12). All comparisons between groups for a given day were made using a two-sided Mann-Whitney U test (15). Also, to assess overall differences between groups in terms of their

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[3] The abbreviations used are: TCD_{50}, the radiation dose required to control 50% of tumors locally in a tumor model; PSA, prostate-specific antigen; APFM, average percentage of filled mesh.

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angiogenic responses over several days, a summary statistic was calculated for each mouse: the APFM. The APFM was the average of the percentages of mesh containing blood vessels across days 8 through 14 (days postimplantation) for a given mouse. This served as a summary statistic of the angiogenic growth rate in the cranial window; a fast growth rate yielded a high APFM. An ANOVA test was done comparing the APFMs of the different groups for the days subsequent to treatment (days 8 through 14). To control for possible variations in initial starting values at time of treatment and to maximize the rigor of this analysis, the ANOVA was done controlling for the values at day 6. Hence, differences ascribable to different starting values at day 6 would not incorrectly influence the outcome of the ANOVA test of the APFM statistic.

Results

PC-3 or FSA-II in Gastrocnemius Muscle Suppressed Angiogenesis in the Cranial Windows. Consistent with published work from our own laboratory as well as from others using several different angiogenic assays (3, 8, 9), we found suppression of angiogenesis in the cranial windows of mice bearing PC-3 tumors in the leg, as compared with control mice without tumor (Fig. 1). The difference in angiogenesis became significant by the sixth day after gel implantation ($P < 0.01$), with the median fraction of mesh with newly formed vessels being 0.39 (0.24–0.69, 25th through 75th percentiles) for the control group and 0.08 (0.0–0.20) for the group bearing PC-3. Differences continued ($P < 0.01$) until day 14, when the limits of the assay were reached with 100% of mesh squares filled with vessels. Similar suppression of angiogenesis was seen in cranial windows of mice bearing FSA-II growing in the leg compared with control mice without tumor (Fig. 2). The difference in angiogenesis became significant on the eighth day after gel implantation ($P < 0.01$), with the median fraction of mesh with newly formed vessels being 0.95 (0.90–1.00) for the control group and 0.80 (0.67–0.89) for the group bearing FSA-II. This difference was no longer statistically significant at the 11th day, at the limits of the assay. As a rigorous test of the summary measure of growth rates during the last week of the experiment, ANOVA analysis of the APFMs confirmed that PC-3-bearing mice (mean APFM, 0.74) had slower rates of distal angiogenesis from days 8 through 14 than did controls (mean APFM, 0.99; $P < 0.01$). Similarly, the ANOVA analysis of APFMs showed FSA-II-bearing mice (mean APFM, 0.93) to have slower distal angiogenesis than did non-tumor-bearing controls ($P < 0.05$).

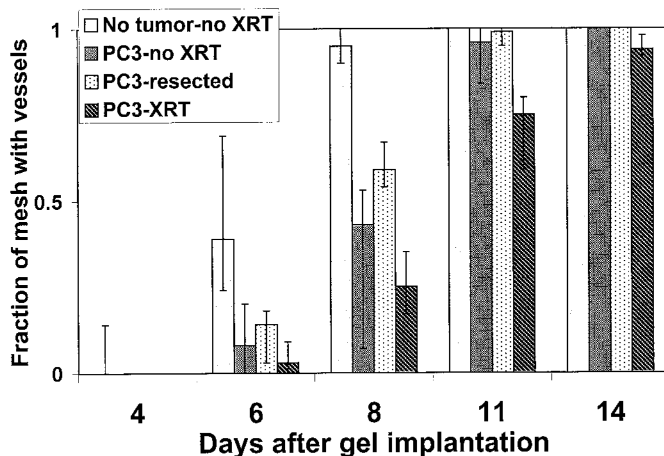

Fig. 1. PC-3 tumor growing in the leg slowed angiogenesis in the cranial window, and there was further slowing after irradiation of PC-3. Data are shown for unirradiated controls without tumor ($n = 15$), for unirradiated PC-3 growing in the leg ($n = 16$), for resected leg bearing PC-3 ($n = 5$), and for irradiated (*XRT*) leg bearing PC-3 ($n = 12$). Differences became statistically significant between control and unirradiated PC-3 groups by day 6 ($P < 0.01$) and remained significant through day 11 ($P < 0.05$), but were not significant on day 14 ($P > 0.05$). On day 6, no differences were significant ($P > 0.05$) among the unirradiated, the irradiated, and the resected PC-3 groups. Differences between unirradiated PC-3 and amputated PC-3 groups became significant by day 8 ($P < 0.05$) but were not significant by day 11 ($P > 0.05$). Differences between unirradiated and irradiated PC-3 groups became significant by day 11 ($P < 0.05$) and remained significant through day 14 ($P < 0.01$). Differences between amputated and irradiated PC-3 groups became significant by day 8 ($P < 0.05$) and remained significant through day 14 ($P < 0.05$). Bar graphs display median values for each group with lower and upper error bars representing the 25th and 75th percentiles, respectively.

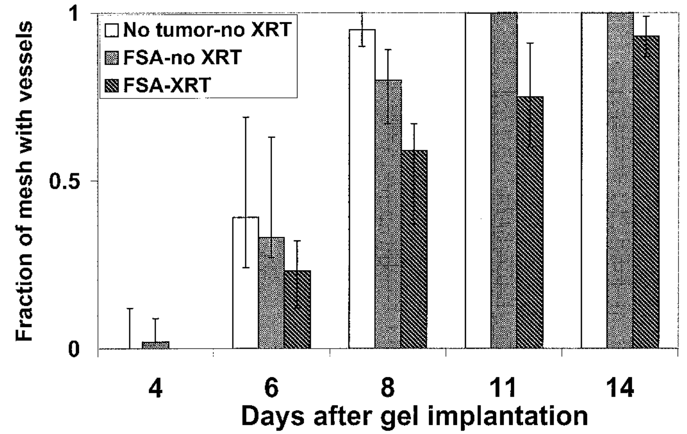

Fig. 2. FSA-II tumor growing in the leg slowed angiogenesis in the cranial window, and there was further slowing after irradiation of the tumor. Data are shown for unirradiated controls without tumor ($n = 15$), for unirradiated FSA-II growing in the leg ($n = 7$), and for irradiated (*XRT*) FSA-II in the leg ($n = 8$). Differences between control and unirradiated FSA-II groups became statistically significant by day 8 ($P < 0.01$), but were not significant by day 11 ($P > 0.05$). Differences between unirradiated and irradiated FSA-II groups became statistically significant by day 8 and remained so through day 14 ($P < 0.01$). Bar graphs display median values for each group with lower and upper error bars representing the 25th and 75th percentiles, respectively.

Effect of Surgical Resection of PC-3 Primary Tumor on Distal Angiogenesis. Also consistent with published work from several laboratories, including our own, distal angiogenesis tended to increase in five mice that underwent resection of legs bearing PC-3 primary tumors, as compared with control mice bearing PC-3 tumors not undergoing resection (Fig. 1) (3, 8, 9). This difference was significant by day 8 ($P < 0.05$), with 0.59 (0.54–0.67) as the median value for the mice undergoing amputation and 0.43 (0.07–0.53) for the PC-3-bearing control group. On day 11 and thereafter, at the limits of the assay, the difference tested insignificant ($P > 0.05$). Resection of the leg in six non-tumor-bearing controls (data not shown) had no measurable effect, but yielded results indistinguishable from non-tumor-bearing controls that did not undergo amputation ($P > 0.05$ all days). However, analysis of the APFMs did not show a significant overall difference between PC-3-bearing controls and those undergoing resection (mean APFM, 0.79; $P > 0.05$), consistent with the fact that day-by-day differences were observed for a relatively short time period.

Irradiation of PC-3 or FSA-II Primary Tumor Further Suppressed Distal Angiogenesis. There was an additional decrease in angiogenesis in the cranial window with irradiation of the PC-3-bearing leg (Fig. 1). By day 11, unirradiated PC-3-bearing mice had a median angiogenic fraction of 0.96 (0.84–1.00), whereas the irradiated PC-3-bearing mice had a median fraction of 0.75 (0.59–0.80; $P < 0.01$). Furthermore, irradiation of the PC-3 tumor-bearing leg yielded lower levels of angiogenesis than did its surgical removal. This difference was significant ($P < 0.01$) beginning on day 8, with a median of 0.63 (0.57–0.77) for the mice that had undergone amputation and a median of 0.25 (0.17–0.35) for mice that had undergone irradiation of PC-3 tumor. Differences between these two groups remained significant ($P < 0.05$) for the subsequent time points studied. ANOVA analysis of the summary APFMs yielded similar overall results for these PC-3-bearing mice. Although mice with irradiated PC-3 tumors (mean APFM, 0.60) tested not significantly different

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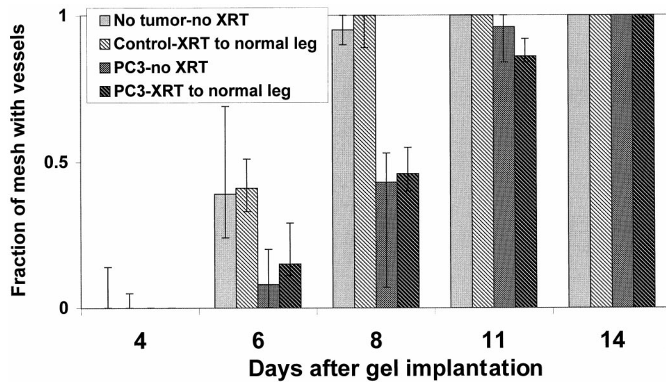

Fig. 3. Irradiation of non-tumor-bearing (normal) leg did not inhibit angiogenesis in the cranial window. Data are shown for unirradiated controls without tumor ($n = 15$), and for irradiated (*XRT*) controls without tumor ($n = 7$); differences between the two groups were not statistically significant ($P > 0.05$). Data are also shown for unirradiated controls bearing PC-3 tumor in the leg ($n = 16$), and for mice receiving irradiation to one leg and bearing PC-3 tumor in the contralateral leg ($n = 4$); differences between these two groups were not statistically significant ($P > 0.05$). Bar graphs display median values for each group with lower and upper error bars representing the 25th and 75th percentiles, respectively.

compared with unirradiated PC-3-bearing mice ($P > 0.05$), mice with irradiated PC-3 tumors did test significantly different from those mice undergoing surgical resection ($P < 0.05$).

Similarly, there was a further decrease seen in the rate of angiogenesis in the cranial window with irradiation of FSA-II growing in the mouse leg (Fig. 2). By day 8, differences in angiogenesis between unirradiated and irradiated FSA-II groups were statistically significant ($P < 0.01$), and they remained significant through day 14. ANOVA analyses of the APFM summary data were consistent with these findings, showing a significant difference between unirradiated and irradiated (mean APFM, 0.67) FSA-II bearing mice ($P < 0.01$).

The angiogenesis suppression observed with tumor irradiation did not correlate with an increase in tumor volume. There was no increase in the average tumor volume for irradiated tumors measurable up through day 15, whereas within 4 days unirradiated FSA-II tumors had grown 39% larger than irradiated ones. Irradiated PC-3 tumors showed a decline in average tumor volume of 14% by day 15, whereas unirradiated PC-3 tumors grew 43% over the same time period.

Irradiation of Non-Tumor-bearing Leg Did Not Slow Angiogenesis in the Cranial Window. Non-tumor-bearing mice underwent irradiation of their legs on day 6 after gel implantation. Compared with nonirradiated, non-tumor-bearing controls, there was no evidence of an inhibitory effect on angiogenesis [$P > 0.05$ for all days (Fig. 3)]. Similarly, PC-3-bearing mice underwent irradiation of their non-tumor-bearing legs on day 7 after gel implantation. These mice showed no additional inhibitory effect, as compared with unirradiated tumor-bearing controls [$P > 0.05$ for all days (Fig. 3)].

Discussion

As hypothesized by Folkman, some primary tumors can create systemic antiangiogenic environments, presumably because of the longer half-lives of angiostatic molecules compared with those of angiogenic molecules (1). We found that PC-3 primary tumor grown in the mouse leg exhibited a suppressive effect on angiogenesis at a secondary site, similar to results seen with PC-3 in other studies (3, 8, 9). We also found that FSA-II primary tumor grown in the mouse leg had a suppressive effect on distal angiogenesis.

Several laboratories have shown the suppressive effects of primary tumors to be related to tumor size and, thus, the surgical removal of primary tumors to be associated with a concomitant increase in distal angiogenesis and growth of metastases (1, 9, 16, 17). Extrapolating these data to the potential effects of irradiation of a primary tumor on angiogenesis at a secondary site, one would suspect that irradiation of a primary tumor to the point of long term local control should lead to an increase in distal angiogenesis. Unexpectedly, we found that local irradiation of a primary tumor had a measurable, inhibitory effect on angiogenesis at a distal site, for both FSA-II and PC-3, at least during the first several days after tumor irradiation, up to the limits of the assay. This finding, however, is consistent with the fact that irradiation of a tumor and its surrounding host tissues may not have the same immediate effects as surgical removal. The contrast between the two is clearly seen in the clinic. For example, there is elevation and then very gradual decline of PSA over several months in response to tumoricidal levels of irradiation of human primary prostate cancer, in contrast to the abrupt PSA nadir in response to radical prostatectomy (18). Interestingly, recent work has shown PSA to have antiangiogenic activity *in vitro* and antiproliferative effects *in vivo* (19). This is provocative, especially with other work demonstrating enzymatic activity of human prostate carcinoma cell lines such as PC-3 in the conversion of human plasminogen to angiostatin (3). However, there is no evidence based on survival data of prostate cancer patients, the most rigorous clinical criterion, that shows radiation therapy to provide a survival advantage over surgical intervention.

We found that irradiation of tumor, but not normal tissue, yielded enhanced inhibition of angiogenesis. The result was consistent across two very different cell lines, in terms of species (human *versus* mouse), tissue (prostate *versus* muscle), lineage (carcinoma *versus* sarcoma), and growth rates (slow *versus* fast). This suggests a potential interactive mechanism between tumor and host tissues. Tumor-host interactions can have important effects on angiogenesis. For example, host-tumor cell interactions via tumor-infiltrating macrophages may be an important component of angiostatin production (5). Differences in the microenvironment of the tumor can affect angiogenesis at the tumor site (20). Furthermore, tumor-host interactions can result in differences in cytokine production that affect distal angiogenesis (21). Several cytokines, including tumor necrosis factor α, transforming growth factor $\beta 1$, and various forms of angiostatin, have been shown to have apoptotic effects in endothelial cells (*e.g.,* Ref. 22). Levels of several cytokines have been shown to increase after irradiation of tumors and of normal tissues (23, 24).

Combination of angiostatin with radiation has been shown to augment the tumor response to radiation (6). Our study suggests that the endogenous production of such angiostatic factors may be a function of tumor-host interactions. Also, it demonstrates that the dynamic effects on distal angiogenesis of tumoricidal ionizing radiation, even in a single-fraction dose to the primary tumor, may be different from the effects seen with immediate tumor removal.

As a possible explanation of our findings, we propose that surgical resection of the primary tumor removes the sources of both pro- and antiangiogenic molecules, whether produced by the host or by the tumor, whereas irradiation leaves these sources in place. Further, we hypothesize that whereas irradiation may damage neoplastic cells and potentially host stromal cells, the sources of enzymes that convert various matrix molecules into angiostatic agents are still present in the host (*e.g.,* the conversion of collagen XVIII into endostatin). Our data suggest that the radiation dose applied to tumors in this series of experiments increases endostatin levels in the plasma (See "Note Added in Proof"). Whether other angiostatic molecules (*e.g.,* thrompospondin-1, angiostatin) are also involved remains to be investigated. Meanwhile, these results collectively suggest that angiostatic agents, whether endogenous or exogenous, may play a signifi-

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cant role in the inhibition and control of vessel growth in local as well as distant sites.

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Note Added in Proof

Two days after treatment, endostatin plasma levels measured by ELIZA (Accucyte assay; Cytimmune Sciences, Inc., College Park, MD) were twice as high in mice with irradiated FSA-II tumors (100.6 ± 21.8 ng/ml; $n = 7$) as compared with mice that underwent tumor resection (53.35 ± 7.0 ng/ml; $n = 6$). Vascular endothelial growth factor, basic fibroblast growth factor, and transforming growth factor-β plasma levels all measured by ELIZA (Quantikine assays; R&D Systems, Minneapolis, MN) were not significantly different between control groups and mice with irradiated tumors. These differences in endostatin levels are consistent with our own findings and those of another study on differences in growth rates and tumor cell kinetics seen in secondary tumors between amputated and irradiated FSA-II-bearing mice (J. Ramsay, *et al.*, Int. J. Radiat. Oncol. Biol. Phys., *17:* 809–813, 1989.)

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