Angiogenesis and tumor growth inhibition by a matrix metalloproteinase inhibitor targeting radiation-induced invasion

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Abstract
In this study, we have evaluated the interactions between ionizing radiation and a matrix metalloproteinase (MMP) inhibitor. Using Matrigel invasion assays, we show that ionizing radiation induced a dose-dependent increase in the invasive phenotype of cultured B16 melanoma cells and that conditioned medium from these irradiated B16 cells promoted endothelial cell [human microvascular endothelial cells (HMEC)] invasiveness. To determine whether the radiation-induced changes in invasive phenotype could be due to changes in MMP activation, we have tested the ability of the MMP inhibitor Metastat to modulate the ionizing radiation–induced invasive phenotype using both an in vitro melanoma model and a mouse s.c. tumor model. In these studies, Metastat inhibited the ionizing radiation–induced invasive phenotype in cultured B16 cells and similarly inhibited the increase in HMEC invasion induced by conditioned medium from irradiated B16 cells. Conversely, ionizing radiation increased B16 MMP-2 activity and the conditioned medium from irradiated B16 cells. Conversely, ionizing radiation increased B16 MMP-2 activity and the conditioned medium from irradiated B16 cells. Similarly, conditioned medium from irradiated B16 was also able to increase VEGF secretion in HMECs. Moreover, ionizing radiation–induced melanoma cell invasiveness was partially inhibited by an anti-VEGF monoclonal antibody. In vivo, ionizing radiation plus concomitant Metastat yielded the greatest growth inhibition of melanoma s.c. tumors and this effect correlated with inhibition of angiogenesis as measured by both Doppler ultrasonography and platelet/endothelial cell adhesion molecule-1 staining. Finally, ionizing radiation modulated MMP-2, VEGF, and VEGF receptor expression in these tumor samples using immunohistochemistry. Taken together, these results suggest that there is an ionizing radiation–induced tumor survival pathway and a possible paracrine ionizing radiation–induced stimulatory pathway emanating from tumor cells toward the endothelial bed that is impeded when Metastat is given simultaneously. This model could provide in vivo evidence of the antitumor efficacy of combining a MMP inhibitor with ionizing radiation to target radiation-induced invasion and angiogenesis.

Introduction
Because radiation therapy is a mainstay of cancer treatment, tumor resistance to ionizing radiation has been studied extensively. Many studies have focused on “intrinsic” tumor cell changes associated with radiation resistance, such as those involved in response to apoptosis, cell cycle regulation, DNA repair, hypoxia, or cell signaling pathways (1–10). However, tumor microenvironment and interactions between the tumor and the surrounding tumor (i.e., fibroblasts, pericytes, or the extracellular matrix) could also play an equally important role in tumor response to ionizing radiation (11, 12). Therefore, elucidating interactions between the tumor compartment and its microenvironment on exposure to ionizing radiation (12) could lead to a better understanding of overall cancer control with such treatment (13). This may unveil promising tools allowing the elaboration of strategies aimed at enhancing tumor suppression (14).

Matrix metalloproteinases (MMP) are zinc-dependent proteolytic endopeptidases (15) involved in cancer progression. They stimulate cancer cell growth, migration and invasion, and metastasis (16). During subsequent steps, these proteolytic enzymes can have multiple targets. They degrade basement membranes enabling cancer cell invasion and exposing cryptic sites within matrix molecules (17). Of major interest, they may even modulate the bioavailability of cytokines and growth factors (18). In highly selected models, it has been hypothesized that

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MMPs may play a role in changing tumor cell line motility and in the induction of an invasive phenotype after ionizing radiation. Moreover, the MMP proteolytic system may also modulate tumor angiogenesis by modulating the release of biologically active vascular endothelial growth factor (VEGF; ref. 19). Metastat, or COL-3, is a chemically modified tetracycline (20) that is able to inhibit neutrophil gelatinase and MMP expression in colon, breast, or melanoma cancer models. The potential clinical interest of this drug was first suggested following the promising results of phase I/II clinical trials for Kaposi’s sarcoma (21) and for patients with advanced solid malignancies (22, 23).

Although there is a strong biological background for the development of strategies targeting MMPs, the clinical response rates of MMP inhibitors as single-agent cancer therapy in subsequent trials have been modest at best (24, 25).

One possible explanation for these disappointing results might be that MMPs are involved at early stages of tumor progression, whereas clinical trials were mostly restricted to advanced-stage disease. Studies on tumor-bearing transgenic mice (e.g., the Rip-Tag model; ref. 26) suggested that the best potential of anti-MMPs might be displayed in regimens that combine MMP inhibitor with conventional cytotoxic strategies (27). Thus, in addition to possible MMP modulation by ionizing radiation mentioned above, this provided us with another rationale for testing Metastat in combination with ionizing radiation.

In this study, we have examined the action of Metastat on the possible interplay between irradiated melanoma tumor cells and the invasive endothelial cell phenotype. Ionizing radiation induced an increase in MMP and VEGF production in tumor cells, and Metastat inhibited ionizing radiation–induced changes in the VEGF level in vitro. We therefore evaluated the effect of Metastat given concomitantly with ionizing radiation in vivo in s.c. melanoma tumors. Using this approach, we have found that the MMP inhibitor interfered with the anti-tumor action of irradiation and that tumor response was strongly associated with marked changes in tumor vascularization.

Materials and Methods

Reagent and Cell Culture

Purified Metastat (6-demethyl, 6-deoxy, 4-dedimethylamino-tetracycline) was generously provided by CollaGenex Pharmaceuticals (Newton, PA). The drug was first reconstituted in DMSO to obtain a stock solution (1 mg/mL) and stored at −20°C.

The murine-specific neutralizing VEGF inhibitor was a monoclonal antibody (anti-VEGF-mAb) provided by R&D Systems (Minneapolis, MN). The drug was reconstituted in sterile PBS, aliquoted, and stored at −20°C.

Murine B16F10 melanoma cells were grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 0.1% L-glutamine, and 1% penicillin-streptomycin. Human microvascular endothelial cells (HMEC) were provided by Dr V. Randrianarison (Gustave Roussy Institute, Villejuif, France) and cultured in MCDB 131 medium (Life Technologies) supplemented with 10% FCS, 0.1% L-glutamine, 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, and 0.2% penicillin-streptomycin. The cell lines were maintained at 37°C in an atmosphere of 5% CO2. Cells were irradiated with a 137Cs source at a dose rate of 1 Gy/min.

Clonogenic Survival and Proliferation Assays

For colony formation assays, 200 to 1,000 cells per flask were seeded in triplicate according to treatment conditions and cell lines. After allowing cells to attach to the dishes, a single dose of irradiation and/or Metastat was given. Metastat was always added immediately before ionizing radiation when the two treatments were combined. Cells were cultured up to 6 days for B16F10 and 12 days for HMEC. Colonies were then fixed, stained with crystal violet, and counted. The surviving fraction was estimated as follows: (number of colonies formed) / (number of cells seeded) × [plating efficiency for the Metastat alone group (control group)]. Cell proliferation was assessed by a trypan blue dye exclusion assay. Cells (n = 35,000) were plated in duplicate in six-well dishes. Irradiation and/or Metastat (0.1 mg/L) was then delivered. Cells were collected at the indicated time and counted using a hemocytometer under a light microscope. Experiments were repeated twice.

B16F10 Conditioned Medium, HMEC Conditioned Medium, and Measurement of VEGF Level

B16F10 cells were grown in complete DMEM to subconfluent monolayers. Thereafter, culture medium was replaced (after washing cells twice with sterile PBS) by serum-free DMEM before irradiating them at 8 Gy. Conditioned medium was collected 24, 48, and 72 hours after irradiation, centrifuged, and used for Matrigel invasion assays or stored frozen at −80°C until analysis of VEGF content. VEGF was measured by two ELISA tests (Quantikine; R&D Systems) according to the manufacturer’s instructions. Each sample was analyzed in duplicate and experiments were repeated twice. Briefly, B16 cells were plated in six-well plates at 25% confluence, allowed to attach overnight, and then irradiated at 8 Gy with or without Metastat, which was added just before ionizing radiation. Conditioned medium was collected every 24 hours; most importantly, VEGF levels were normalized to the viable number of cells assessed by trypan blue. VEGF secretion was evaluated over time (at 24, 48, and 72 hours).

Conditioned medium was also collected from HMECs and analyzed according to the same procedures. In addition to the conditions mentioned above (0, 4, and 8 Gy), unirradiated HMECs were also incubated in B16F10 conditioned medium (B16CM) for 6 hours. For control, we used unirradiated HMECs after preincubation in conditioned medium from unirradiated B16 cells. Most importantly, they were then carefully washed twice with sterile PBS. Then, serum-free DMEM was added for...
zymographies. Those unirradiated Bi6CM-preincubated cells could also be used for Matrigel assays as described below.

**Matrigel Invasion Assay**

Invasion of B16F10 cells was measured by the invasion of cells through Matrigel-coated Transwell inserts (Becton Dickinson, Franklin Lakes, NJ) as reported previously. Briefly, Transwell inserts with an 8-μm pore size precoated with Matrigel were used (Becton Dickinson, Bedford, MA) and 2.5 × 10⁴ cells were added to the upper chambers. Cells were then irradiated at 8 Gy and/or treated with 0.1 mg/L Metastat (noncytotoxic concentration, with a surviving fraction at 0 Gy = 100 ± 3%; P < 0.01) or the anti-VEGF-mAb, which were added to the upper chamber just after irradiation. The sequence and timing of each experiment was identical: Cells were then incubated at 37°C in an atmosphere of 5% CO₂ for 24 hours after treatments. The same chemotactrant gradient was imposed using the appropriate completed medium with 10% FCS in the upper chambers and 30% FCS in the lower chambers. Cells that had invaded the lower surface of the Matrigel-coated membrane after 24 hours were fixed with 70% ethanol, stained with crystal violet, and counted (on the entire membrane surface) under a light microscope to ensure reproducibility. Each experiment was repeated thrice. Statistical analyses were done using ANOVA and the unpaired Student’s t test. All statistical analyses were done as two-sided tests.

To measure HMEC cell invasion, 5 × 10⁴ cells were added to the upper chamber and the same treatment conditions were applied, except, in addition, the evaluation of the unirradiated Bi6CM-preincubated HMECs. We used unirradiated HMECs after preincubation in conditioned medium from unirradiated B16 cells as controls in these experiments. Similarly, HMECs were treated with a non-cytotoxic concentration of Metastat (0.1 mg/L with a surviving fraction at 0 Gy = 100 ± 3%; P < 0.01).

**Gelatin Zymography**

Conditioned medium from either the sham control or irradiated B16F10 and HMECs was concentrated 10-fold with Centricon-10 (Amico, Beverly, MA). Samples were added to each lane and subjected to 10% SDS-PAGE using 10% polyacrylamide gel containing 1 mg/mL gelatin. After electrophoresis, the gel was washed in 2.5% Triton X-100 and incubated in 50 mmol/L Tris-Cl buffer (pH 8.0) containing 0.5 mmol/L CaCl₂ and 1 mmol/L ZnCl₂ for 20 hours at 37°C. The gel was stained with 1% Coomassie brilliant blue R-250 and then incubated in destain buffer (5% acetic acid and 10% methanol). Pro-MMP-2, MMP-2, and pro-MMP-9 standards were used at a concentration of 0.1 mg/mL (human or murine; R&D Systems). p-Aminophenylmercuric acetate concentrate in DMSO was added to pro-MMP-2 to obtain active MMP-2. MMP-2 and MMP-9 activity was therefore visualized in the gelatin-containing zymograms as clear bands against a blue background.

Of note, an additional condition was used for HMEC zymographies: the endothelial cells were preincubated in both conditioned medium from unirradiated B16 cells (data not shown) and Bi6CM (irradiated B16 cells).

**Gelatin Zymography on Tissue Samples**

In each treated group, three animals were randomly sacrificed at day 7 and each tumor was carefully separated from its surrounding tissue so that it could be analyzed independently. Then, frozen tissue samples were crushed to powder in liquid nitrogen and homogenized in a 50 mmol/L Tris-HCl buffer (pH 7.6) containing 150 mmol/L NaCl, 10 mmol/L CaCl₂, 1% Triton X-100, and protease inhibitors (Sigma-Aldrich, St. Louis, MO). Supernatants were collected and protein concentrations determined using the Lowry method. Gelatin zymographies were then done as mentioned above.

**Histopathology and Immunohistochemistry**

Animals were sacrificed and tumors resected at day 7, immediately fixed, embedded in paraffin, cut into 4-μm sections, and stained with HES. Platelet/endothelial cell adhesion molecule (PECAM) immunohistochemistry on endothelial cells required fixation in absolute ethanol and quenching with 3% H₂O₂ for 10 minutes. Sections were placed in coverplates (Shandon Life Sciences Technology, Pittsburgh, PA) and incubated with a mixture of two rat primary antibodies raised against mouse PECAM-1 (1:50; PharMingen, San Diego, CA; Mec13.3 and 390 clones) for 1 hour. Slides were then incubated with a goat anti-rat biotin-conjugated antibody 1:50 (PharMingen) followed by streptavidin peroxidase (1:100; DAKO, Carpinteria, CA) for 30 minutes and with the 3,3′-diaminobenzidine PowerVision kit (Immunovision Technologies, Microm, Franchesville, France) for 10 minutes before counterstaining with Mayer’s hematoxylin and mounted in aqueous medium.

For anti-VEGF (sc-152; 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLK1 (sc-315; 1:100) and anti-MMP-2 (1:100; NeoMarkers, Fremont, CA) immunohistochemistry, samples were fixed in Glyoxal (Glyoffix, Shandon Life Sciences Technology). After quenching of endogenous peroxidase activity, sections were incubated with primary antibodies for 1 hour (rabbit polyclonal antibodies) and processed with the rabbit Powervision alkaline phosphatase kit (30 minutes). Slides were treated with Fast Red chromogenic substrate (DAKO) over 20 minutes, counterstained with Mayer’s hematoxylin, and mounted.

Stained cells from tumors obtained from the three mice randomly sacrificed at day 7 were quantified in each treatment group. In each group, the number of CD31-positive, VEGF receptor (VEGFR)–positive, or VEGF-positive cells was counted in 10 to 30 fields at a magnification of ×100 or ×400 in a blinded fashion. The columns on the graphs show the percentage of total counts in each tumor from mice treated with one of the four treatments.

**Tumor Model and Treatment Conditions**

*In vivo* experiments were conducted in conformity with the institutional guidelines under applicable international laws and policies (EEC Council Directive 86/609, OJ L 358. 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Female athymic nude mice (6–8 weeks old), purchased from CER (Le Genest St Isle, France), were fed with a diet of animal chow and water *ad libitum*. B16F10 s.c. tumors were inoculated into the left hind footpad of each animal.
were obtained by s.c. injection of 3 × 10⁶ cells into the right flank of animals. Before initiating the study, 48 mice were assigned to four groups (12 mice per group, as summarized below) and the entire experiment was done in triplicate. Each group contained an equal number of large and intermediate-sized tumors and mice were stratified into groups so that the mean tumor volume in each group was comparable.

(a) Control group (no irradiation, sterile PBS injections),
(b) Radiation alone group (locally irradiated at a dose of 2 × 12.5 Gy),
(c) Drug alone group (continuous treatment with Metastat 5 days at 2.5 μg/kg/d), and
(d) Combined treatment group [concomitant ionizing radiation plus Metastat scheduling: Metastat 5 days at 2.5 μg/kg/d started 24 hours before 2 × 12.5 Gy ionizing radiation].

For irradiation treatment, mice were immobilized in a customized harness allowing exposure of the right flank, whereas the remainder of the body was shielded by lead. A 25-Gy dose of irradiation was delivered locally in two fractions of 12.5 Gy on day 3 by using a 250 kV RT Phillips X-ray at a dose rate of 0.69 Gy/min (20 mA and 0.2-mm Cu filter). Intratumoral injection of Metastat (0.1 mg/L) or anti-VEGF-mAb, added just before ionizing radiation, before starting the assay. Invasion in untreated controls (blue), after adding Metastat (red) or anti-VEGF-mAb (green). Columns, mean; bars, SE. D, invasion of HMECs in Matrigel: invading unirradiated HMECs after preincubation in conditioned medium from unirradiated B16 cells (blue) or after preincubation in B16CM from irradiated B16 cells (orange); their reaction to Metastat (MET; dark green) or to the anti-VEGF-mAb (purple). HMECs were also irradiated at 8 Gy (pink). Metastat (red) and the anti-VEGF-mAb (green) were also added to nonpreincubated unirradiated control cells. Results in triplicate (n = 3 in subsequent experiments). Columns, mean; bars, SD.

Figure 1. A, clonogenic survival of B16 melanoma cells with (●) or without (○) 0.1 mg/L Metastat: cells were cultured for 6 d before colony formation. Representative experiments (for assays done in triplicate). Points, mean; bars, SE (see Materials and Methods). B, clonogenic survival of HMECs with (●) or without (○) 0.1 mg/L Metastat: cells were cultured for 12 d until colony formation. Representative experiments (for assays done in triplicate). Points, mean; bars, SE (see Materials and Methods). C, invasion of melanoma cancer cells in Matrigel: influence of irradiation (ionizing radiation) was evaluated at various doses in vitro. B16 cells were irradiated at 0, 4, and 8 Gy, with Metastat (0.1 mg/L) or anti-VEGF-mAb, added just before ionizing radiation, before starting the assay. Invasion in untreated controls (blue), after adding Metastat (red) or anti-VEGF-mAb (green). Columns, mean; bars, SE. D, invasion of HMECs in Matrigel: invading unirradiated HMECs after preincubation in conditioned medium from unirradiated B16 cells (blue) or after preincubation in B16CM from irradiated B16 cells (orange); their reaction to Metastat (MET; dark green) or to the anti-VEGF-mAb (purple). HMECs were also irradiated at 8 Gy (pink). Metastat (red) and the anti-VEGF-mAb (green) were also added to nonpreincubated unirradiated control cells. Results in triplicate (n = 3 in subsequent experiments). Columns, mean; bars, SD.
percentage of treated versus control values was calculated from the mean of the relative tumor volume of the treated versus control group on each day of tumor measurement. The lowest treated versus control value within 4 weeks after treatment corresponded to the optimal treated versus control value. The optimal growth inhibition percentage was calculated as 100 minus the optimal treated versus control percentage value. The nonparametric Mann-Whitney t test (Statview software) was used to determine the statistical significance of the relative tumor volumes and for comparisons between treatment groups.

To meet the above-mentioned criteria (Animal Care License C94-076-11), mice in the control and Metastat alone groups had to be sacrificed before day 13.

**Doppler Ultrasonography Monitoring**

Animals were anaesthetized before all color Doppler high-frequency ultrasonography procedures by using an electric isofluorane chamber delivering nontoxic and continuous flow to avoid motion artifacts from living animals (which were under surveillance until full recovery). An ATL-HDI 5000 ultrasonograph provided by ATL/Phillips (Suresnes, France) with a 12 MHz high-frequency linear electronic probe was used. The theoretical depth explored was 40 mm and vessels as small as 80 μm in diameter could be visualized. Sonographic examinations of the complete tumor were done (12 mice per group) twice weekly. The number of peritumor and intratumor vessels was estimated from the Doppler ultrasonography data using HDI-Lab software (29).

**Results**

**Clonogenic Survival and Cell Proliferation Were Not Affected by Metastat**

The effect of Metastat on radiosensitivity was evaluated through colony formation assays on B16 and HMEC cell lines. No significant differences were observed in surviving fractions when a noncytotoxic concentration of Metastat (0.1 mg/L) was combined with irradiation exposure in the B16 tumor cell line (Fig. 1A) and in the HMEC endothelial cell line (Fig. 1B). Metastat did not enhance the antiproliferative effect of ionizing radiation and the combination of Metastat plus ionizing radiation did not decrease B16 and HMEC cell viability at 24, 48, and 72 hours after ionizing radiation. These experiments show that the combination of Metastat plus ionizing radiation should not have a direct cytotoxic effect in any of the subsequent planned experiments.

![Figure 2. A, MMP-2 and MMP-9 activity in vitro of B16 melanoma cells on gelatin zymography: MMP-2/MMP-9 activities were studied over time (data not shown) and at various doses of ionizing radiation: the most significant results were seen at 48 h for 0, 4, and 8 Gy for pro-MMP-2 (~72 kDa), active MMP-2 (~64 kDa), and pro-MMP-9 (~110 kDa). Lane 1, standard pro-MMP-9, pro-MMP-2, and active MMP-2 (see Materials and Methods). B, MMP-2 and MMP-9 activity in vitro of HMEC endothelial cells on gelatin zymography: MMP-2/MMP-9 activities were studied over time (data not shown) and at various doses of ionizing radiation: the most significant results were seen at 48 h for 0 and 8 Gy and for unirradiated cells preincubated in B16CM. Lane 1, standard pro-MMP-9 (~110 kDa), pro-MMP-2 (~72 kDa), and active MMP-2 (~64 kDa; see Materials and Methods). C, VEGF protein levels in B16CM measured by ELISA after exposure to ionizing radiation: VEGF level was measured after 8 Gy exposure with (orange) or without (pink) Metastat (added just before ionizing radiation). Levels were also examined for unirradiated controls (blue) and with the addition of Metastat (red). Columns, mean; bars, SE. D, VEGF protein levels measured by ELISA in HMEC conditioned medium: VEGF level was measured after 8 Gy exposure with (orange) or without (pink) Metastat (added just before ionizing radiation). Results for unirradiated controls after preincubation in conditioned medium from unirradiated B16 cells (blue), addition of Metastat alone (red), or unirradiated cells preincubated in B16CM with (purple) or without (green) Metastat. Columns, mean; bars, SE.](http://mct.aacrjournals.org/content/mct/4/11/1721)
Irradiation or Conditioned Medium Enhanced Invasion

In vitro

Irradiation increased B16 cell invasiveness (Fig. 1C) in a dose-dependent manner. The highest rate (3-fold greater compared with unirradiated controls) was seen after exposure to 8 Gy. Unlike the melanoma cells, HMECs (Fig. 1D) irradiated at 4 or 8 Gy did not exhibit a significant change in their invasive phenotype compared with unirradiated control-HMECs (preincubated in conditioned medium from unirradiated B16 cells). To determine whether a soluble factor was released from irradiated B16 cells that could affect HMEC cell invasiveness, HMECs were preincubated in B16CM (from irradiated B16 cells) and washed and the same Matrigel invasion assay was then done (see Materials and Methods). The average number of invading cells was increased by >2-fold \(^{P}<0.05\) for those B16CM-preincubated cells compared with HMECs preincubated in conditioned medium from unirradiated B16 cells \((n = 3, \text{ assay done in triplicate})\), showing that a soluble factor released from irradiated B16 cells increased the invasive potential HMECs.

Metastat Inhibited Ionizing Radiation–Induced Invasion but not B16CM-Induced Invasion

As shown in Fig. 1C, the differences in melanoma cell invasiveness reach statistical significance for two irradiation doses \([4 \text{ Gy} \ (P < 0.05) \text{ and } 8 \text{ Gy} \ (P < 0.005)]\). However, when Metastat was added (0.1 mg/L), the invasion rate dropped below the basal control level for unirradiated cells. As shown in Fig. 1D, Metastat (0.1 mg/L) had no effect on the invasiveness of the unirradiated endothelial cells or those treated with B16CM.

A Specific Neutralizing Anti-VEGF-mAb Inhibited Ionizing Radiation–Induced Melanoma Cell Invasiveness but Was Ineffective on B16CM-Induced HMEC Invasiveness

A specific blocking anti-VEGF-mAb was added to both B16 and HMEC Matrigel assays: as shown in Fig. 1C, inhibition of ionizing radiation–induced invasion of B16 cells was achieved. Interestingly, the degree of inhibition provided by the anti-VEGF-mAb (2-fold decrease at 8 Gy) in irradiated melanoma cells did not attain that achieved by Metastat (>5-fold decrease at 8 Gy). In unirradiated B16CM-preincubated HMECs (Fig. 1D), the degree of inhibition provided by the anti-VEGF-mAb was not significant. Similarly, the anti-VEGF-mAb did not inhibit the invasiveness of unirradiated control HMECs.

Irradiation or B16CM Modulated In vitro MMP-2 Activity

The ability of MMP inhibitor to decrease the ionizing radiation–induced invasiveness suggests that MMP activity is involved in the expression of this phenotype. To more directly determine the effect of ionizing radiation on MMP activation, zymography assays were done. As shown in Fig. 2A, MMP-2 was significantly increased (both active form and proform of MMP-2) in B16 melanoma cells, attaining a maximum at 48 hours after 8 Gy. However, as shown in Fig. 2B, HMEC cell MMP-2 activity was reduced by irradiation in a dose-dependent manner, declining in intensity (both active form and proform of MMP-2) with a minimum reached at 8 Gy. Significantly, when unirradiated HMECs were preincubated in B16CM (see Materials and Methods), MMP-2 activity was increased, again with a maximum reached after 48 hours for both the active form and the proform (Fig. 2B, lane 4). These results correlate well with the previously obtained MMP inhibitor data and suggest that MMP-2 is an important effector of ionizing radiation–induced invasive phenotype. Indeed, the specificity of MMP-2 activation in this response is underscored by the lack of MMP-9 modulation by ionizing radiation (Fig. 2A and B). Under all the conditions tested \([0, 4, \text{ and } 8 \text{ Gy and preincubation in conditioned medium from unirradiated B16 cells (data not shown)]\} and at each incubation time after ionizing radiation (24, 48, and 72 hours), a certain amount of MMP-9 activity was detected, but this activity was unchanged by ionizing radiation.

Irradiation Increased VEGF Expression in Melanoma Cells and B16CM Increased It in HMECs

As shown in Fig. 2C, conditioned medium from melanoma cells irradiated at 8 Gy showed a significant increase \((P < 0.05)\) relative to VEGF secretion. This increase reached a maximum of 3-fold compared with control (normalized to the viable number of cells as assessed by trypan blue staining) at 48 hours and declined to <2-fold by 72 hours. HMECs did not display an increase in VEGF secretion after ionizing radiation but displayed an increased VEGF secretion after preincubation in B16CM (>3.5-fold increase; Fig. 2D). VEGF levels were neither significantly induced after ionizing radiation compared with unirradiated controls (preincubated in conditioned medium from nonirradiated B16 cells) nor inhibited by Metastat (when added to those unirradiated non-B16CM preincubated cells).

Metastat Inhibited VEGF Expression in Melanoma Cells

As shown in Fig. 2C, adding Metastat (0.1 mg/L) almost completely inhibited the ionizing radiation–induced increase in VEGF secretion: a 2-fold decrease was noted at 48 hours and the decline dropped to below the control...
Figure 4. A, effect of Metastat alone or combined with ionizing radiation on s.c. melanoma tumor volumes (cm³) over time (days). Mice were treated with PBS (●), ionizing radiation (□), Metastat (▲), or ionizing radiation + Metastat (▲) and tumor volumes were monitored over time. Some mice were sacrificed earlier (x). Points, mean; bars, SE. B, color Doppler high-frequency ultrasonography procedures on living animals. Triangles, neovessels; T, tumor. Representative images for each treated group. C, prospective and dynamic evaluation of tumor vessels (absolute number of vessels per group) with Doppler ultrasonography over time (days). Results for each treatment group: control (PBS; ●), Metastat alone (▲), ionizing radiation alone (□), and ionizing radiation + Metastat (▲). Points, mean; bars, SE. D, right, evaluation of angiogenesis in representative microscopic fields (x50) by PECAM immuno-histochemistry analysis showing the distribution of CD31 staining (▲) on tumor (T) samples at day 7 in four treatment groups (control, Metastat, ionizing radiation alone, and ionizing radiation + Metastat); left, quantification of blood vessels in the skin: relative numbers of CD31-positive blood vessels were counted in each group of mice. Columns, mean; bars, SE.
basal expression level (control medium) at 24 and 72 hours. Interestingly (Fig. 2D), Metastat had no effect on the level of VEGF detected in unirradiated HMECs preincubated in B16CM.

**Irradiation Increased Tumor Tissue MMP-2, VEGF, and VEGFR Expression In vivo**

As illustrated in Fig. 3A, in vivo zymography assays on ex vivo tumor tissue showed that MMP-2 activity was highly induced in the radiation group when compared with controls. We did not observe relevant differences when samples from the other treatment groups were compared with controls. As in the in vitro studies, ionizing radiation did not seem to alter MMP-9 activity. To verify that the melanoma cells were indeed displaying increased MMP-2 after ionizing radiation, immunohistochemistry was used. In these studies, immunohistochemistry was done on tumors from all mice sacrificed at day 7 in each treatment group. Marked differences were observed when the control and the concomitant Metastat plus ionizing radiation groups (with poor and very poor MMP-2 expression, respectively) were compared with the ionizing radiation alone group ($P < 0.05$; Fig. 3B). Importantly, MMP-2 was markedly expressed on viable melanoma cells (or stromal fibroblasts mostly in close vicinity to the tumor). As illustrated in Fig. 3C and D, VEGF and VEGFR were expressed at low levels in the control and Metastat groups but not detected in the concomitant group. Conversely, VEGF and VEGFR levels were detectable ($P < 0.05$) in the ionizing radiation group. VEGFR and VEGF were expressed inside the tumor among viable melanoma cells.

**Metastat Combined with Irradiation Inhibited Tumor Growth and Angiogenesis In vivo**

As shown in Fig. 4A, simultaneous administration of Metastat markedly improved the mean specific growth delay of B16 s.c. tumors: the concomitant group had the highest mean specific growth delay, faring better than the ionizing radiation alone, Metastat alone, and control groups (PBS). In terms of tumor volume doubling time, the Metastat alone group showed no difference compared with the control group (2 versus 2.1 days, respectively). In contrast, combining Metastat with ionizing radiation significantly increased the antitumor effect of ionizing radiation. Optimal growth inhibition documented on day 18 for the concomitant treatment group was 88% compared with 80% for the ionizing radiation alone group ($P = 0.01$; day 18, concomitant versus ionizing radiation). According to Langdon et al. (see Materials and Methods), efficacy scoring was estimated as “moderately active” for ionizing radiation alone and “very active” for the combined treatment, suggesting a possible synergistic effect of this schedule. Those results also remained significant over time when combined treatment was compared with ionizing radiation alone at day 22 ($P = 0.02$).

To assess the effect of ionizing radiation on tumor angiogenesis, we prospectively and dynamically evaluated tumor vessels with Doppler ultrasonography (Fig. 4B). In these experiments, ionizing radiation antiangiogenic effects were enhanced by the simultaneous administration of Metastat and this observation correlates well with the growth inhibition shown in Fig. 4A. Indeed, the concomitant use of ionizing radiation plus Metastat led to marked inhibition of intratumor and peritumor neovascularization: the number of neovessels in this group did not increase significantly during the observation period with Doppler ultrasonography. Of interest (Fig. 4A and C), it was possible to measure a mean delay of 3.7 days between the increase in Doppler ultrasonography–depicted vasculature and the tumor burden in each treated group, suggesting, as described previously (30), the usefulness of Doppler ultrasonography in predicting treatment response or tumor outcome. In summary, as of day 5, inhibition of tumor neovascularization was strongly associated with tumor growth delay in all the four treated groups.

As shown in Fig. 4D, a marked decrease in endothelial cell staining on immunohistochemistry slides was associated with growth inhibition. Immunohistochemistry also showed a correlation between endothelial cell PECAM-1 staining and tumor growth inhibition: the graphs show that at day 7 both the concomitant (ionizing radiation plus MET) and the ionizing radiation alone group exhibited such inhibition compared with the control and Metastat groups.

**Discussion**

The results obtained with Metastat, for both melanoma and endothelial cells, showed that this drug did not alter clonogenic cell survival in the dose range used either when given alone or when combined with ionizing radiation. This was a prerequisite for studying invasive properties on Matrigel and to ensure that the subsequent findings could not be attributed to increased cell death due to Metastat. The in vitro results showed that ionizing radiation induced a dose-dependent invasive phenotype in cultured B16 melanoma cells and that this effect could be induced in unirradiated HMEC endothelial cells by preincubation in B16CM for a short period.

Although this is unusual and unexpected in this model, the ionizing radiation–induced effects on melanoma cells could, to a certain extent, correspond to the induction of specific pathways for tumor cells. This has been suggested in selected models (31, 32). However, the etiology of such events remains unclear as MMPs exert multiple effects downstream (16). This prompted us to test Metastat at a noncytotoxic concentration: the ionizing radiation–induced, dose-dependent invasive phenotype of cultured melanoma cells was indeed inhibited (Fig. 1C).

We therefore examined the possible causative role of MMPs and downstream molecules, such as VEGF, to attempt to explain the mechanism(s) underlying promoted invasion. MMP-2 activation has been associated with invasive phenotype in cancer cells (15, 33). In our experiments, ionizing radiation induced a marked increase in MMP-2 activity in the B16 melanoma cells (Fig. 2A). Given the marked effect of the MMP-inhibitor (known to be
specific for MMP-2), these observations could, to a certain extent, account for the exceeding invasiveness after ionizing radiation. Likewise, the increase in MMP-2 activity in unirradiated HMECs preincubated in B16CM could (Fig. 2B), to a certain extent, partially explain enhanced endothelial cell invasiveness. These data are supported by previous observations that suggest that the tumor cell compartment might play a role in stimulating its rather radiosensitive endothelial bed (34).

We next attempted to study the MMP system further downstream to try to identify any other specific underlying mechanisms distinct from the well-known effects of MMPs on the extracellular matrix structure (16). The marked increase in VEGF secretion after irradiation of melanoma cells (Fig. 2C) was again almost completely inhibited by the MMP inhibitor. This finding is supported by other authors who suggested a link between MMPs and the release of VEGF (19, 35). Furthermore, it is compatible with other observations of ionizing radiation–induced interplay between the tumor cell compartment and its endothelial environment leading to increased tumor radioresistance (34–37).

The inhibitory effect of a specific blocking anti-VEGF-mAb on our Matrigel assays in melanoma cells (Fig. 1C) suggests a potential link between MMP-2 up-regulation, release of VEGF, and induction of proinvasive phenotypes. As shown in various cancer models (35, 38), these observations could indicate that VEGF was biologically active when released after ionizing radiation on melanoma cells and might be partly implicated in promoting invasion. This would be in agreement with the previously postulated existence of a loop involving MMPs and VEGF (39, 40). Hence, a rather simple explanation of our results (Fig. 1C and D) could be that Metastat is active against melanoma invasion through a dual action: (a) it would counteract ionizing radiation–induced MMP-2 (known to promote invasion directly) and (b) it would thereby indirectly impede VEGF release (given the MMP-2 role in promoting VEGF). However, further investigation using an in vitro model is needed to determine for example which VEGF isoforms are involved (38) and to evaluate the level and specificity of VEGF-R on melanoma cells (41).

Greater in vitro MMP-2 activity (zymographies) after ionizing radiation was seen in dissected tumor tissues. Hence, as suggested in vitro, immunohistochemistry seems to point to ionizing radiation–induced signals (Fig. 3B–D), because the highest expression levels of MMP-2, VEGF, and VEGFR were seen among mice treated with ionizing radiation alone. Conversely, virtually no staining was observed for those markers when Metastat was added (concomitant group, ionizing radiation plus MET).

In vivo, the concomitant administration of ionizing radiation plus MMP inhibitor led to a marked increased tumor growth delay (Fig. 4A) in s.c. melanoma tumors compared with ionizing radiation alone (P = 0.01). These results were strongly associated with tumor angiogenesis evaluated by two independent techniques [Doppler ultrasonography (Fig. 4B and C) and PECAM staining (Fig. 4D)].

Many studies have shown that Doppler ultrasonography was accurate and reliable in measuring tumor vascularity changes in various models. These results were independently confirmed by qualitative methods relying on direct visualization of the microvasculature (PECAM staining; refs. 42, 43).

Again, in this respect, there would be a dual rationale for using Metastat in combination with ionizing radiation. First, it could theoretically impede ionizing radiation–induced autocrine proinvasive tumor behavior. Secondly, it could counteract a possible ionizing radiation–induced paracrine stimulatory pathway proceeding from the radiated tumor cells toward the vascular endothelium.

All of these observations require some background clarification. First, the rationale for using Matrigel assays to show ionizing radiation–induced promoted events (i.e., occurring with a totally artificial membrane) should be analyzed cautiously: The use of an in vitro model or at least using a human tumor cell line could be more appropriate to correlate these findings with data in the literature (44–47) so that we may differentiate the human VEGF isoforms that diffuse through the extracellular matrix (VEGF165 and VEGF121) rather than remain bonded to some cryptic sites.

Our objective in this study was to emphasize how local tumor control could be influenced by the action of ionizing radiation on the tumor and its microenvironment and that, to a certain degree, this action could be correlated with tumor angiogenesis evaluated by complementary techniques. Taken together, these results suggest that agents targeting extracellular matrix components are potentially useful. The clinical implication of these findings based on a radioresistant melanoma model is already of interest given the numerous radioresistant models encountered in many patients. However, although compatible with former studies (48–50), our findings may also reflect a relative overrepresentation of extrinsic tumor radiosensitivity (i.e., its endothelium), thus indicating the need for less radioresistant models than our melanoma cells.

Nonetheless, our data, combined with the known specificity of Metastat for MMP-2 and MMP-9, provide insights into the suggested interplay between ionizing radiation, MMP-2, and downstream molecules, such as VEGF. Our study is also in agreement with other studies showing that the tumor microenvironment, including the stimulation of tumor-associated endothelial cells, can contribute to tumor blood vessel resistance to therapy (47). It is also noteworthy that MMP inhibitors have yielded some interesting results combined with photodynamic therapy (51) and that recent findings have shown links between ionizing radiation, MMPs, and VEGFR (52).

In conclusion, combining a MMP inhibitor with ionizing radiation in this model provided in vivo evidence of antitumor efficacy through targeting of ionizing radiation–induced invasion and angiogenesis and suggests the possible use of Metastat with radiation therapy in early clinical trials.
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References


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