The Tumor Bed Effect: Increased Metastatic Dissemination from Hypoxia-Induced Up-regulation of Metastasis-Promoting Gene Products

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Abstract

Cancer patients with recurrent local disease after radiation therapy have increased probability of developing regional and distant metastases. The mechanisms behind this observation were studied in the present work by using D-12 and R-18 human melanoma xenografts growing in preirradiated beds in BALB/c-\textit{nu/nu} mice as preclinical models of recurrent primary tumors in humans. D-12 tumors metastasize to the lungs, whereas R-18 tumors develop lymph node metastases. Based on earlier studies, we hypothesized that metastasis was governed primarily by the proangiogenic factor interleukin-8 (IL-8) in D-12 tumors and by the invasive growth-promoting receptor urokinase-type plasminogen activator receptor (uPAR) in R-18 tumors. Pimonidazole was used as a hypoxia marker, and hypoxia, microvascular hotspots, and the expression of IL-8 and uPAR were studied by immunohistochemistry. The metastatic frequency was significantly higher in tumors in preirradiated beds than in control tumors in unirradiated beds, and it increased with the preirradiation dose. D-12 tumors showed increased fraction of hypoxic cells, increased fraction of IL-8-positive cells, and increased density of microvascular hotspots in preirradiated beds, and R-18 tumors showed increased fraction of hypoxic cells and increased fraction of uPAR-positive cells in preirradiated beds. Strong correlations were found between these parameters and metastatic frequency. IL-8 was up-regulated in hypoxic regions of D-12 tumors, and uPAR was up-regulated in hypoxic regions of R-18 tumors. Daily treatment with anti–IL-8 antibody (D-12) or anti-uPAR antibody (R-18) suppressed metastasis significantly. Our preclinical study suggests that primary tumors recurring after inadequate radiation therapy may show increased metastatic propensity because of increased fraction of hypoxic cells and hypoxia-induced up-regulation of metastasis-promoting gene products. Two possible mechanisms were identified: hypoxia may enhance metastasis by inducing neoangiogenesis facilitating hematogenous spread and by promoting invasive growth facilitating lymphogenous spread. The aggressive behavior of postirradiation local recurrences suggests that they should be subjected to curative treatment as early as possible to prevent further metastatic dissemination. Moreover, the possibility that patients with a high probability of developing local recurrences after radiation therapy may benefit from postirradiation treatment with antiangiogenic and/or anti-invasive agents merits clinical investigation. (Cancer Res 2005; 65(6): 2387-96)

Introduction

The overall survival rates after radiation therapy are significantly better for patients achieving local tumor control than for patients with recurrent local disease, primarily because local treatment failure increases the probability of developing metastatic disease in distant organ sites (1, 2). Primary tumors recurring after unsuccessful radiation therapy commonly show aggressive growth, have exceptionally poor oxygen supply, are resistant to treatment, and may have elevated metastatic propensity (3–5). The treatment of patients with postirradiation local recurrences, therefore, represents a difficult and challenging problem (5–7).

Tumors growing in preirradiated beds in rodents are frequently being used as experimental models of recurrent primary tumors in humans (8). Most tumors show a prolonged latency period and a reduced volumetric growth rate when transplanted into preirradiated tissue (9–11). This effect is a classic phenomenon in radiation biology, which was discovered by Frankl and Kimball in 1914 (12) and named the tumor bed effect in 1955 (13). Radiation-induced injury to the host vasculature and connective tissue, resulting in impaired neovascularization, is considered the major cause of the tumor bed effect (14, 15). Compared with control tumors in unirradiated beds, tumors in preirradiated beds commonly show a hostile microenvironment characterized by reduced blood perfusion (16), low extracellular pH (16), and low oxygen tension (17), resulting in extensive necrosis (9, 11, 14, 15), elevated hypoxic fractions (18–22), and impaired curability after treatment with ionizing radiation (18, 20, 22) and cytotoxic agents (8, 23).

Moreover, the metastatic propensity has been shown to be higher in tumors in preirradiated beds than in control tumors in unirradiated beds (24–29). The mechanisms underlying this observation are poorly understood. It has been suggested that exposure to ionizing radiation may make the tumor stroma, particularly the wall of the blood vessels, more permeable to tumor cells, thus facilitating intravasation and metastasis (24, 25). Tumor necrosis has been shown to promote metastatic dissemination (30), and because tumors in preirradiated beds are more necrotic than tumors in unirradiated beds (11, 15), it has also been suggested that increased metastasis after preirradiation of the tumor bed may arise from metastasis-promoting compounds released from necrotic tumor regions (27). However, Milas et al. (26, 28) have done detailed studies involving several tumor lines that do not support any of these suggestions. Their experiments rather
suggested that the rate of release of tumor cells into the circulation was similar in tumors in preirradiated and unirradiated beds, and that the increased metastasis observed in tumors in preirradiated beds was merely a secondary effect of the longer time period these tumors needed to reach a given volume (26, 28). This interpretation is not in accordance with a more recent experimental study by Baker et al. (29), who showed that tumors in preirradiated beds developed lung metastases more frequently than tumors in unirradiated beds, even when the time from tumor transplantation to mouse euthanasia was similar.

It has been shown recently that tumor hypoxia may promote metastasis by exerting a microenvironmental pressure selecting for metastatic cell phenotypes and by up-regulating the expression of genes involved in the metastatic process (31, 32). Clinical studies have shown that invasive growth, the development of regional or distant metastases, or disease-free survival is associated with hypoxia in cervical carcinoma (33, 34), head and neck carcinoma (35), and soft tissue sarcoma (36). Experimental studies have shown that tumors with a high hypoxic fraction metastasize more frequently than tumors with a low hypoxic fraction, whether high hypoxic fractions are imposed by keeping the host mice in a low-oxygen atmosphere (37, 38) or are occurring naturally (39, 40).

Because tumors in preirradiated beds show higher hypoxic fractions than tumors in unirradiated beds (18–22), we hypothesized that increased metastasis after preirradiation of the tumor bed could arise as a consequence of hypoxia-induced up-regulation of metastasis-promoting gene products. To test this hypothesis, experiments were done with two human melanoma xenograft lines (D-12 and R-18) in which tumor hypoxia has been shown to promote distant metastasis (39–41). D-12 tumors develop pulmonary metastases, and hypoxia promotes metastasis of D-12 tumors by up-regulating the proangiogenic factor interleukin-8 (IL-8; ref. 39). R-18 tumors metastasize to lymph nodes, and hypoxia promotes metastasis of R-18 tumors by increasing the proteolytic activity near the tumor cell surface by up-regulating urokinase-type plasminogen activator receptor (uPAR; refs. 40, 41). The experiments gave results consistent with our hypothesis, demonstrating that tumors in preirradiated beds may have increased metastatic propensity because of hypoxia-induced up-regulation of gene products promoting metastatic dissemination.

Materials and Methods

Mice and Tumors. Adult (8–10 weeks of age) female BALB/c-nu/nu mice, maintained as described elsewhere (42), were used as host animals for xenografted tumors. Primary tumors were initiated from D-12 or R-18 monolayer cell cultures (42). Approximately 3.5 × 10^5 cells suspended in 10 μL of Ca^2+ and Mg^2+-free HBSS were inoculated intradermally into the left mouse flank (43). The inoculation sites were irradiated with 0 (unirradiated control), 10, or 20 Gy 24 hours before the inoculation by using a Siemens Stabilipan X-ray unit (Erlangen, Germany), operated at 220 kV, 19 to 20 mA, and with 0.5-mm Cu filtration (44). The radiation field was 20 × 20 mm, and a dose rate of 5.1 Gy/min was used. The mice were anesthetized with ketamine (33 mg/kg) and azaperone (25 mg/kg) before irradiation. Tumor volume (V) was calculated as V = π/6 × ab^2, where a is the longer and b is the shorter of two orthogonal diameters (43). The animal experiments were approved by the Institutional Committee on Research Animal Care and were done according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing, and Education (New York Academy of Sciences, New York, NY).

Metastasis Assays. The primary tumors were resected at predetermined times after initiation. The host mice were then examined daily for clinical signs of metastases (listlessness, weight loss, or hunched posture). They were euthanized 3 months after the primary tumor resection or when moribund. Mice having borne D-12 primary tumors were examined for pulmonary metastases; that is, the lungs were fixed in Bouin’s solution for 24 hours and inspected for macroscopic metastases by stereomicroscopy (39). Mice having borne R-18 primary tumors were examined for external lymph node metastases in the interscapular, submandibular, axillary, and inguinal regions and internal lymph node metastases in the abdomen and mediastinum (40). Metastases were always found in moribund mice. The presence of metastases was confirmed by histologic examinations. Mice were scored to be metastasis negative if pulmonary (D-12) or lymph node (R-18) metastases could not be detected by autopsy 3 months after the primary tumor was resected. Previous experiments have shown that mice appearing healthy at 3 months after primary tumor resection also are free from metastases 3 months later. Further details of the assays have been reported elsewhere (39, 40, 43).

Treatment with Neutralizing Antibody. Anti–IL-8 or anti-uPAR treatment was done by using an anti-human IL-8 mouse monoclonal antibody or an anti-human uPAR mouse monoclonal antibody (R&D Systems, Abingdon, United Kingdom). Antibodies showing no cross-reactivity with recombinant murine IL-8 or recombinant murine uPAR were used. The anti–IL-8 treatment was given to mice bearing D-12 tumors and consisted of 21 doses of 100 μg of antibody given in 24-hour intervals. The first dose was given at day 7 and the last dose at day 27 after the primary tumor was initiated. The D-12 primary tumors were resected at day 28 after initiation. The anti-uPAR treatment was given to mice bearing R-18 tumors and consisted of 28 doses of 25 μg of antibody given in 24-hour intervals. The first dose was given at day 24 and the last dose at day 51 after the primary tumor was initiated. The R-18 primary tumors were resected at day 52 after initiation. Control mice were treated at 24-hour intervals with 21 doses of 100 μg (D-12) or 28 doses of 25 μg (R-18) of an irrelevant anti-human mouse monoclonal antibody of the same isotype as the neutralizing antibodies. Antibody solutions were diluted in PBS and given in volumes of 0.25 mL by i.p. injection.

The primary tumors had developed a vascular network and were in exponential growth when the anti–IL-8 and anti-uPAR treatments were initiated. Previous studies have shown that D-12 tumors do not metastasize before day 7 after initiation (39), and R-18 tumors do not metastasize before day 24 after initiation (40). Antibody doses were determined on the basis of titration experiments, which showed that daily treatment with at least 100 μg of anti–IL-8 antibody or daily treatment with at least 25 μg of anti-uPAR antibody is necessary for suppressing metastasis efficiently in D-12 and R-18 tumors, respectively. The anti–IL-8 and anti-uPAR treatments had no detectable effect on tumor blood perfusion, as determined in separate experiments by using the 86Rb uptake method.

Immunohistochemical Detection of Tumor Hypoxia, IL-8 Expression, uPAR Expression, and Microvessels. CD31 was used as a marker of tumor endothelial cells, and pimonidazole [1-[2-hydroxy-3-piperidinyl]propyl]2-nitroimidazole, given as described previously (39, 40), was used as a marker of tumor hypoxia (44). Tumor slices were fixed in phosphate-buffered 4% paraformaldehyde or liquid nitrogen. Immunohistochemistry was done by using a peroxidase-based indirect staining method (43). An anti-pimonidazole rabbit polyclonal antibody (a gift from Prof. J.A. Raleigh, Department of Radiation Oncology, University of North Carolina School of Medicine, Chapel Hill, NC), an anti-human IL-8 rabbit polyclonal antibody (Endogen, Woburn, MA), an anti-human uPAR mouse monoclonal antibody (American Diagnostica, Greenwich, CT), or an anti-mouse CD31 rat monoclonal antibody (Research Diagnostics, Flanders, NJ) was used as primary antibody. Diaminobenzidine was used as chromogen, and hematoxylin was used for counterstaining. Controls included omission of the primary antibody, incubation with normal rabbit immunoglobulin or normal rabbit serum, and incubation with blocking peptides before staining. Quantitative studies of tumor hypoxia, IL-8 expression, uPAR expression, microvascular hotspots, or microvascular density were based on four cross sections of each tumor. Area fractions showing positive pimonidazole, IL-8, or uPAR staining were determined by image analysis (44) and used as parameters for fraction of hypoxic cells, fraction of IL-8–positive cells,

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and fraction of uPAR-positive cells, respectively. Microvessels and microvascular hotspots were defined and scored as described previously (39).

**Statistical Analysis.** Experimental data are presented as arithmetic mean ± SD unless otherwise stated. Statistical comparisons of data sets were done by using the Student’s t test (single comparisons) or by one-way ANOVA (multiple comparisons) when the data sets complied with the conditions of normality and equal variance. Under other conditions, comparisons were done by nonparametric analysis using the Mann-Whitney rank sum test (single comparisons) or the Kruskal-Wallis one-way ANOVA on ranks (multiple comparisons). The Bonferroni method (parametric tests) or the Dunnett method (nonparametric tests) was used to identify data sets that differed from the control data in multiple comparisons. Probability values of $P < 0.05$, determined from two-sided tests, were considered significant. The statistical analysis was done by using SigmaStat statistical software (Jandel Scientific GmbH, Erkrath, Germany).

**Results**

**D-12 and R-18 Tumors Show Decreased Growth and Increased Metastasis in Preirradiated Beds.** The tumor bed effect was studied by growing tumors in unirradiated beds and in beds preirradiated with 10 or 20 Gy. Tumors in preirradiated beds grew significantly slower than tumors in unirradiated beds (Fig. 1A and B). The volumetric growth rate was higher in unirradiated beds than in preirradiated beds by factors of $~1.3$ (10 Gy, $P < 0.000001$) and $~1.7$ (20 Gy, $P < 0.000001$) for D-12 tumors and by factors of $~1.3$ (10 Gy, $P < 0.000001$) and $~1.6$ (20 Gy, $P < 0.000001$) for R-18 tumors. In metastasis experiments, the primary tumors were resected at day 28 (D-12) or day 52 (R-18) after initiation, corresponding to the last measuring points in Fig. 1A and B. The tumor volumes at resection were $~2000$ mm$^3$ (0 Gy), $~700$ mm$^3$ (10 Gy), and $~250$ mm$^3$ (20 Gy) for both tumor lines.

The percentage of mice developing pulmonary (D-12) or lymph node (R-18) metastases was used as a parameter for metastatic frequency. Preirradiation of the tumor bed increased the metastatic frequency significantly (Fig. 1C and D). The metastatic frequency was higher in preirradiated beds than in unirradiated beds by factors of $~1.5$ (10 Gy, $P = 0.0020$) and $~1.9$ (20 Gy, $P = 0.000013$) for D-12 tumors and by factors of $~1.4$ (10 Gy, $P = 0.0052$) and $~1.7$ (20 Gy, $P = 0.000054$) for R-18 tumors. Moreover, tumors in beds preirradiated with 20 Gy showed a higher metastatic frequency than tumors in beds preirradiated with 10 Gy (D-12, $P = 0.0035$; R-18, $P = 0.012$).

**D-12 Tumors Show Hypoxia-Induced Up-regulation of IL-8, Increased Hypoxic Fraction, IL-8 Expression, and Density of Microvascular Hotspots in Preirradiated Beds, and Correlations between Pulmonary Metastasis and Hypoxic Fraction, IL-8 Expression, or Density of Microvascular Hotspots.** D-12 primary tumors showed highly heterogeneous staining for pimonidazole and IL-8. Necrotic regions were encompassed by a rim of hypoxic and IL-8–positive cells, two to four cell layers thick. Foci of hypoxic and IL-8–positive cells were scattered throughout the parenchyma in tumor regions without necrosis. The IL-8–positive foci were 1.4- to 1.8-fold larger than the hypoxic foci. The remaining tissue did not show detectable pimonidazole or IL-8 staining. Examinations of adjacent sections showed a high degree of colocalization of pimonidazole and IL-8 staining (Fig. 2A and B). Quantitative colocalization studies were done for 10 tumors from unirradiated beds, 10 tumors from beds preirradiated with 10 Gy, and 10 tumors from beds preirradiated with 20 Gy. When IL-8–positive foci were seen, hypoxic foci were always found in the same positions in the adjacent section. When hypoxic foci were seen, IL-8–positive foci were found in the same positions in the adjacent section in 85% to 95% of the cases. The IL-8–positive regions covered 90% to 100% of the pimonidazole-positive regions, and the pimonidazole-positive regions covered 55% to 65% of the IL-8–positive regions (Fig. 2C). These observations were independent of whether the tumors had grown in unirradiated or preirradiated beds and show that IL-8 is upregulated in hypoxic regions of D-12 tumors.

![Figure 1](https://www.aacrjournals.org/doi/figure/10.1158/0008-5472.CAN-04-2321-fig1.png)

**Figure 1.** Growth curves (A and B) and metastatic frequency (C and D) for D-12 and R-18 tumors in unirradiated or preirradiated beds in BALB/c- nu/nu mice. Approximately $3.5 \times 10^5$ tumor cells were inoculated intradermally in the mouse flank 24 hours after the inoculation sites had been irradiated with 0, 10, or 20 Gy. A and B, points, geometric means of 17 to 20 tumors; bars, SDs. C and D, the mice were euthanized and autopsied when moribund or 3 months after the primary tumor was resected. The percentage of mice with pulmonary (D-12) or lymph node (R-18) metastases was used as a parameter for metastatic frequency. Columns, means of four independent experiments involving 17 to 20 mice each. Bars, SDs.
The hypoxic fraction was higher in tumors in preirradiated beds than in tumors in unirradiated beds by factors of ~1.5 (10 Gy, \( P = 0.00069 \)) and ~2.0 (20 Gy, \( P < 0.000001 \)), and tumors in beds preirradiated with 20 Gy showed a higher hypoxic fraction than tumors in beds preirradiated with 10 Gy (\( P = 0.00045 \)). The IL-8–positive fraction was higher in tumors in preirradiated beds than in tumors in unirradiated beds by factors of ~1.6 (10 Gy, \( P = 0.000045 \)) and ~2.2 (20 Gy, \( P < 0.000001 \)), and tumors in beds preirradiated with 20 Gy showed a higher IL-8–positive fraction than tumors in beds preirradiated with 10 Gy (\( P = 0.000001 \)). The microvascular hotspot density was higher in tumors in preirradiated beds than in tumors in unirradiated beds by factors of ~1.6 (10 Gy, \( P < 0.000001 \)) and ~2.1 (20 Gy, \( P < 0.000001 \)), and tumors in beds preirradiated with 20 Gy showed a higher hotspot microvascular density than tumors in beds preirradiated with 10 Gy (\( P = 0.000002 \)). Consequently, metastatic frequency was strongly correlated to tumor hypoxia, IL-8 expression, and angiogenic activity in D-12 tumors (compare Figs. 1C and 3).

D-12 primary tumors also showed highly heterogeneous staining for CD31, consistent with staining of endothelial cells. Isolated microvessels as well as microvascular hotspots were seen in tumor regions without necrosis. The microvascular hotspots differed substantially in size and shape and were scattered throughout the tissue in a pattern similar to those of the hypoxic and IL-8–positive foci. High-power examination of microvascular hotspots revealed lumens with erythrocytes encircled by endothelial cells and individual endothelial cells separated by parenchymal melanoma cells, as illustrated elsewhere (39). CD31-positive mitotic figures were detected in some microvascular hotspots, indicating active endothelial cell proliferation. The microvessel density was 5- to 10-fold higher within microvascular hotspots than elsewhere in non-necrotic tissue and, consequently, microvascular hotspots were easily recognizable and their density could be scored with high degree of accuracy. Significant colocalization of microvascular hotspots and hypoxic or IL-8–positive foci could not be shown by examining adjacent tumor sections.

Correlations between metastatic frequency and tumor hypoxia, IL-8 expression, or angiogenic activity were searched for by measuring fraction of hypoxic cells, fraction of IL-8–positive cells, and density of microvascular hotspots in all primary tumors from one of the four metastasis experiments included in Fig. 1C. Tumors in preirradiated beds showed significantly higher hypoxic fraction (Fig. 3A), IL-8–positive fraction (Fig. 3B), and microvascular hotspot density (Fig. 3C) than tumors in unirradiated beds.

**Figure 2.** Immunohistochemical preparations of adjacent sections of a D-12 tumor stained with anti-pimonidazole antibody to visualize hypoxia (A) or anti–IL-8 antibody to visualize IL-8 expression (B). C, degree of colocalization of hypoxia and IL-8 expression in D-12 tumors in unirradiated or preirradiated beds in BALB/c-\( n u/n u \) mice. Approximately \( 3.5 \times 10^5 \) tumor cells were inoculated intradermally in the mouse flank 24 hours after the inoculation sites had been irradiated with 0, 10, or 20 Gy. The tumors were resected at day 28 after initiation and processed for histologic examinations. The percentage of pimonidazole-positive tumor area showing positive IL-8 staining (black columns) and the percentage of IL-8–positive tumor area showing positive pimonidazole staining (white columns) were used as parameters for degree of colocalization. Columns, means of 10 tumors; bars, SDs.

**Figure 3.** Hypoxic fraction (A), IL-8–positive fraction (B), and density of microvascular hotspots (C) in D-12 tumors in unirradiated or preirradiated beds in BALB/c-\( n u/n u \) mice. Approximately \( 3.5 \times 10^5 \) tumor cells were inoculated intradermally in the mouse flank 24 hours after the inoculation sites had been irradiated with 0, 10, or 20 Gy. The tumors were resected at day 28 after initiation and processed for histologic examinations. The percentage of tumor area showing positive pimonidazole staining was used as a parameter for hypoxic fraction, the percentage of tumor area showing positive IL-8 staining was used as a parameter for IL-8–positive fraction, and the number of microvascular hotspots per square millimeter of tumor tissue was used as a parameter for density of microvascular hotspots. Columns, means of 17 to 20 tumors; bars, SDs.
R-18 Tumors Show Hypoxia-Induced Up-Regulation of uPAR, Increased Hypoxic Fraction and uPAR Expression in Preirradiated Beds, and Correlations between Lymph Node Metastasis and Hypoxic Fraction or uPAR Expression. R-18 primary tumors did not develop macroscopic necrosis during growth. The staining for pimonidazole and uPAR was highly heterogeneous. Foci of hypoxic and uPAR-positive cells, differing substantially in size and shape, were seen throughout the tumor parenchyma. The uPAR-positive foci were 1.3- to 1.5-fold larger than the hypoxic foci. The remaining tissue showed no detectable pimonidazole staining and very weak uPAR staining; that is, the boundary line between stained and unstained cells was sharp for both pimonidazole and uPAR. Examinations of adjacent sections showed a high degree of colocalization of pimonidazole and uPAR staining (Fig. 4a and B). Ten tumors from unirradiated beds, 10 tumors from beds preirradiated with 10 Gy, and 10 tumors from beds preirradiated with 20 Gy were subjected to quantitative colocalization studies. The uPAR-positive regions covered 90% to 100% of the pimonidazole-positive regions, and the pimonidazole-positive regions covered 65% to 75% of the uPAR-positive regions (Fig. 4c), irrespective of whether the tumors had grown in unirradiated or preirradiated beds, demonstrating up-regulation of uPAR in hypoxic regions of R-18 tumors.

Highly heterogeneous CD31 staining consistent with staining of endothelial cells was seen also in R-18 primary tumors. Isolated microvessels were observed throughout the tumor tissue, but in contrast to D-12 tumors, R-18 tumors did not develop distinct microvascular hotspots. CD31-positive mitotic figures were detected in some microvessels, indicating active endothelial cell proliferation.

Fraction of hypoxic cells, fraction of uPAR-positive cells, and microvascular density were measured in all primary tumors from one of the four metastasis experiments included in Fig. 1D to search for correlations between metastatic frequency and tumor hypoxia, uPAR expression, or angiogenic activity. Tumors in preirradiated beds showed significantly higher hypoxic fraction (Fig. 5a) and uPAR-positive fraction (Fig. 5b) than tumors in unirradiated beds. The hypoxic fraction was higher in tumors in preirradiated beds than in tumors in unirradiated beds by factors of $\sim 1.6$ (10 Gy, $P = 0.00013$) and $\sim 2.3$ (20 Gy, $P < 0.000001$), and tumors in beds preirradiated with 20 Gy showed a higher hypoxic fraction than tumors in beds preirradiated with 10 Gy ($P = 0.000003$). The uPAR-positive fraction was higher in tumors in preirradiated beds than in tumors in unirradiated beds by factors of $\sim 1.6$ (10 Gy, $P = 0.00012$) and $\sim 2.5$ (20 Gy, $P < 0.000001$), and tumors in beds preirradiated with 20 Gy showed a higher uPAR-positive fraction than tumors in beds preirradiated with 10 Gy ($P < 0.000001$). In contrast, tumors in preirradiated beds showed significantly lower microvascular density than tumors in unirradiated beds (Fig. 5c; 10 Gy, $P = 0.017$; 20 Gy, $P = 0.0019$). Consequently, there were strong positive correlations between metastatic frequency and tumor hypoxia or uPAR expression (compare Figs. 1D and 5a and B) and no positive correlation between metastatic frequency and angiogenic activity (compare Figs. 1D and 5c) in R-18 tumors.

Anti–IL-8 Antibody Treatment Inhibits Pulmonary Metastasis in D-12 Tumors and Anti-uPAR Antibody Treatment Inhibits Lymph Node Metastasis in R-18 Tumors. The specific role of IL-8 in the development of metastases in D-12 tumors was investigated by treating tumor-bearing mice with neutralizing antibody against IL-8. The experiments involved six groups of mice.

Mice bearing tumors in unirradiated beds or beds preirradiated with 10 or 20 Gy were treated with anti–IL-8 antibody or control antibody for 3 weeks until the primary tumor was resected at day 28. The anti–IL-8 treatment reduced the growth rate of the primary tumor significantly, i.e., the tumor volumes at resection were lower in anti–IL-8–treated mice than in control mice (Fig. 6a; 0, 10, and 20 Gy, $P < 0.000001$). The density of microvascular hotspots in the primary tumor was also reduced significantly by the anti–IL-8 treatment (Fig. 6b; 0, 10, and 20 Gy, $P < 0.000001$). Moreover, the anti–IL-8 treatment inhibited the development of metastases significantly; that is, anti–IL-8–treated mice showed a lower metastatic frequency than control mice (Fig. 6c; 0 and 10 Gy, $P = 0.030$; 20 Gy, $P = 0.020$). It should be noticed, however, that the metastatic frequency was 20% to 30% in antibody-treated mice, implying that the anti–IL-8 treatment did not block metastasis completely.

The significance of uPAR in the development of metastases in R-18 tumors was studied by treating tumor-bearing mice with neutralizing antibody against uPAR. The experiments involved six groups of mice. Mice bearing tumors in unirradiated beds or beds preirradiated with 10 or 20 Gy were treated with anti-uPAR antibody or control antibody for four weeks until primary tumor resection at day 52. The growth rate of the primary tumor was not influenced significantly by the anti-uPAR treatment, i.e., the tumor volumes at resection did not differ between anti-uPAR–treated and control mice (Fig. 6d; 0, 10, and 20 Gy, $P > 0.05$). The anti-uPAR treatment had no significant effect on the microvascular density of the primary tumor either (Fig. 6e; 0, 10, and 20 Gy, $P > 0.05$). Moreover, the histologic appearance differed substantially between primary tumors from anti-uPAR–treated and control mice. Thus, tumors from control mice, whether the beds were preirradiated or not, showed regions with diffuse invasion and no distinct borderline against surrounding tissue. Cordlike invasion and invasion of subepidermal lymphatic vessels were also seen. In contrast, tumors from anti-uPAR–treated mice showed a well-defined borderline against adjacent tissue and regions where the tumor parenchyma was separated from the surrounding tissue by a thin capsule of stroma.

Moreover, the development of metastases was suppressed significantly by the anti-uPAR treatment. Anti-uPAR–treated mice showed a lower metastatic frequency than control mice (Fig. 6f; 0 Gy, $P = 0.0061$; 10 Gy, $P = 0.0041$; 20 Gy, $P = 0.010$) and fewer involved lymph nodes than control mice, i.e., metastatic disease was detected in up to six lymph nodes in control mice and in no more than two lymph nodes in antibody-treated mice. The metastatic frequency was only $\sim 10\%$ in all groups of antibody-treated mice, implying that metastasis was blocked almost completely by the anti-uPAR treatment.

Discussion

D-12 and R-18 tumors showed a clear tumor bed effect, i.e., tumors in beds preirradiated with 10 or 20 Gy grew significantly slower than tumors in unirradiated beds. Moreover, the metastatic frequency was higher in tumors in preirradiated beds than in tumors in unirradiated beds, and it was inversely correlated to the tumor volumetric growth rate in both lines. The increased metastatic frequency was not a secondary effect of a longer growth period or a larger volume of the tumors in preirradiated beds. The time from initiation to resection of the primary tumor was kept at 28 (D-12) or 52 (R-18) days in all groups, and the tumors resected from preirradiated beds were substantially smaller than those resected from unirradiated beds.
Tumors in preirradiated beds, therefore, gave rise to a higher number of metastases per unit time and tumor volume than tumors in unirradiated beds, possibly because of an increased release of metastatic cells. Consequently, our study showed unequivocally that tumors in preirradiated beds may have increased metastatic propensity. This observation is consistent with data reported by Baker et al. (29), but contrasts with those of Milas et al. (26, 28). The latter group studied size-matched tumors and concluded that the rate of metastatic cell dissemination was similar in tumors in preirradiated and unirradiated beds, and that tumors in preirradiated beds gave rise to an increased number of metastases merely because they needed a longer time period to grow to a given size (26, 28).

It has been suggested that increased metastasis of tumors in preirradiated beds may be the result of metastasis-promoting compounds released from necrotic tumor regions or radiation-induced injury to the vascular network, facilitating the entry of metastatic cells into the blood circulation (24, 25, 27, 30). Our study does not support these suggestions. R-18 tumors did not develop detectable necrotic regions even in beds preirradiated with 20 Gy, and D-12 tumors in preirradiated beds showed necrotic fractions that did not differ significantly from those of control tumors in unirradiated beds (data not shown). Moreover, metastatic deposits of R-18 tumors were found only in lymph nodes; that is, this tumor disseminated via lymphatic vessels and not via blood vessels.

Metastatic frequency was strongly correlated to fraction of hypoxic cells in both D-12 and R-18 tumors, and IL-8 was up-regulated in hypoxic regions of D-12 tumors and uPAR was up-regulated in hypoxic regions of R-18 tumors. We propose, therefore, that the metastatic frequency of D-12 and R-18 tumors was enhanced in preirradiated beds because growth in preirradiated beds increased the hypoxic fraction, and hypoxia induced up-regulation of metastasis-promoting gene products. This interpretation of our data is consistent with several recent experimental and clinical observations linking metastasis to hypoxia (31–41). It is also in agreement with conclusions from previous, comprehensive studies of D-12 (39) and R-18 (40) tumors.

These previous studies have shown unequivocally that hypoxia promotes pulmonary metastasis in D-12 tumors in untreated beds by the following main mechanism: hypoxic foci develop whenever the intercapillary distances exceed twice the diffusion distance of oxygen, hypoxia causes up-regulation and increased secretion of IL-8, IL-8 promotes neovascularization of the
hypoxic foci, the hypoxic foci subsequently develop into microvascular hotspots, and the microvascular hotspots facilitate tumor cell intravasation, dissemination, and the development of metastases (39). In the present study, we showed that fraction of hypoxic foci, fraction of IL-8–positive cells, and density of microvascular hotspots were positively correlated to metastatic frequency and thus were higher in tumors in preirradiated beds than in tumors in unirradiated beds, that IL-8 was up-regulated in hypoxic tumor regions, and that treatment with neutralizing antibody against IL-8 inhibited primary tumor growth, reduced the density of microvascular hotspots in the primary tumor, and suppressed metastasis significantly, whether the primary tumor was growing in preirradiated or unirradiated beds. Consequently, the study reported here and our previous study (39) thus strongly suggest that R-18 tumors in preirradiated beds increased the fraction of hypoxic cells, and hypoxia induced up-regulation of the proangiogenic factor IL-8.

Our studies cannot exclude the possibility that potent proangiogenic factors other than IL-8 also were up-regulated in hypoxic regions of D-12 tumors and hence contributed significantly to the increased metastatic frequency of tumors in preirradiated beds, because several proangiogenic factors have been shown to be up-regulated by hypoxia (45). However, immunohistochemical studies of D-12 tumors have revealed that staining indicating up-regulation of vascular endothelial growth factor A, platelet-derived endothelial cell growth factor, placental growth factor, fibroblast growth factor 3, angiogenin, or angiopeptin-2 does not colocalize with pimonidazole staining.

Previous studies in our laboratory have shown that hypoxia promotes lymph node metastasis in R-18 tumors in untreated beds by the following mechanism: hypoxic foci develop whenever the intercapillary distances exceed twice the diffusion distance of oxygen, hypoxia causes up-regulation of uPAR and increased binding of urokinase-type plasminogen activator (uPA), increased ligand binding results in increased proteolytic activity adjacent to the tumor cells, and increased proteolytic activity facilitates tumor cell migration toward lymphatic vessels (40). The present study showed that fraction of hypoxic cells and fraction of uPAR-positive cells were positively correlated to metastatic frequency and thus were higher in tumors in preirradiated beds than in tumors in unirradiated beds, that tumors in preirradiated and unirradiated beds showed up-regulation of uPAR in hypoxic regions, and that primary tumor invasiveness and lymph node metastasis were suppressed significantly by treatment with neutralizing antibody against uPAR. In contrast, microvascular density was not positively correlated to metastatic frequency and was not changed after anti-uPAR treatment. The data presented here together with those reported in our previous study (40) thus strongly suggest that R-18 tumors in preirradiated beds showed increased metastasis mainly because growth in preirradiated beds increased the fraction of hypoxic cells, and hypoxia induced up-regulation of the cell surface receptor uPAR.
Other components of the plasminogen activation system, particularly uPA and plasminogen activator inhibitor 1, have also been shown to play a critical role in tumor growth, invasion, and metastasis (46). However, it is unlikely that the hypoxia-induced metastasis reported here involved these components, because Western blot analysis and immunohistochemical studies have shown that hypoxia does not up-regulate tissue-type plasminogen activator, uPA, and plasminogen activator inhibitor 1 or 2 in R-18 tumors (40).

The possibility that cell surface receptors other than uPAR also were involved in the hypoxia-induced metastasis of R-18 tumors cannot be excluded, because several cell surface receptors have been shown to promote tumor cell migration, invasive growth, and the development of metastases (47). Autocrine motility factor receptor (AMFR) and hepatocyte growth factor receptor (HGFR; also known as Met) are particularly interesting candidates, because studies of tumor cells in vitro have shown that hypoxia may promote cell motility and invasion in collagen gels by up-regulating either of these two receptors (48, 49). However, the hypoxia-induced metastasis of R-18 tumors observed here did probably not involve AMFR or HGFR, because immunohistochemical preparations of R-18 tumors show only weak and homogeneous staining for AMFR and HGFR (i.e., neither is up-regulated in hypoxic tumor regions) and lymph node metastasis in R-18 tumors is not inhibited by treatment with neutralizing antibody against AMFR or HGFR (41).

Recent studies have shown that tumor-induced lymphangiogenesis may be an important mechanism in lymph node metastasis (50). However, it is not likely that tumor-induced lymphangiogenesis played an important role in the development of lymph node metastases in R-18 tumors because R-18 tumors do not show significant expression of the prolymphangiogenic factors vascular endothelial growth factor C and D, and functional lymphatic vessels cannot be detected within R-18 tumors by fluorescence or ferritin microlymphography.1

The IL-8-positive foci were 1.4- to 1.8-fold larger than the pimonidazole-positive foci in D-12 tumors, and the uPAR-positive foci were 1.3- to 1.5-fold larger than the pimonidazole-positive foci in R-18 tumors. Previous studies of D-12 and R-18 tumors have shown that pimonidazole staining appears only in cells having oxygen tensions <7 to 10 mm Hg (44). The threshold oxygen tension for hypoxia-induced up-regulation of IL-8 and uPAR may be higher than that for pimonidazole staining, causing the IL-8- and uPAR-positive foci to be larger than the corresponding pimonidazole-positive foci. An alternative explanation is that cells adjacent to pimonidazole-positive foci had experienced periods of transient hypoxia that were sufficiently severe to induce up-regulation of IL-8 and uPAR, but not sufficiently severe to result in detectable pimonidazole binding. It is also possible that some normoxic tumor cells adjacent to hypoxic regions showed strong IL-8 or uPAR expression because of low tissue pH or glucose starvation (31, 32).

Hypoxia-induced activation of hypoxia-inducible factor-1, nuclear factor yB, and other transcription factors may lead to increased expression of a large number of gene products (31, 45). Therefore, the possibility that cell surface proteins promoting invasive growth are up-regulated in hypoxic regions of D-12 tumors and secretory proteins stimulating angiogenesis are up-regulated in hypoxic regions of R-18 tumors has been investigated thoroughly by using immunohistochemical procedures similar to those described here. Interestingly, our studies showed no up-regulation of uPAR, AMFR, or HGFR in hypoxic regions of D-12 tumors, and no up-regulation of IL-8, vascular endothelial growth factor A, platelet-derived endothelial cell growth factor, placental growth factor, fibroblast growth factor 3, angiogenin, or angiopoietin-2 in hypoxic regions of R-18 tumors.1

A common feature of D-12 and R-18 tumors is that they develop hypoxic regions during growth and respond to hypoxia by up-regulating the expression of metastasis-promoting gene products, enabling hypoxic tumor cells to escape their hostile microenvironment and give rise to secondary tumors at distant organ sites where growth is not limited by the supply of oxygen. However, hypoxia activates different cellular programs in D-12 and R-18 tumors, which most likely results in utilization of different escape routes. D-12 tumors react to oxygen deprivation by attempting to restore a normal oxygen supply by inducing neoangiogenesis through increased secretion of proangiogenic molecules, enabling increased tumor cell intravasation and dissemination via immature blood vessels. R-18 tumors respond to hypoxia by activating a cell motility program involving degradation of extracellular matrix components, probably leading to invasive growth and tumor cell dissemination via lymphatic vessels. Consequently, the present study suggests that hypoxia may increase the malignant potential and promote metastatic spread of tumors by at least two parallel mechanisms using complementary strategies, that is, by inducing neoangiogenesis characterized by migration of endothelial cells toward hypoxic tumor cells and by activating a program for invasive growth facilitating migration of hypoxic tumor cells toward lymphatic vessels.

Clinical investigations have shown that primary tumors recurring after unsuccessful radiation therapy may have increased probability of developing regional and distant metastases (1, 2, 5). Increased metastatic propensity of recurrent tumors could in principle be the result of direct effects (i.e., radiation-induced genetic changes in parenchymal tumor cells) as well as indirect effects of the treatment (i.e., radiation-induced changes in the tumor microenvironment). We have shown previously that genetic changes that may be induced by subcurative radiation treatment do not increase the metastatic propensity of D-121 and R-18 (41) tumors. In these experiments, primary tumors were initiated from tumors that had regrown after having been irradiated with single doses of 10, 15, or 20 Gy. This second generation of primary tumors was similar in growth rate, hypoxic fraction, and metastatic frequency to the first generation of primary tumors initiated from monolayer cell cultures and the second generation of primary tumors initiated from unirradiated tumors. Consequently, taken together, these studies and those reported here suggest that the increased probability of metastases in patients with local recurrences after radiation therapy is a result of radiation-induced changes in the tumor microenvironment rather than radiation-induced genetic changes in parenchymal tumor cells. It should be noticed, however, that this suggestion is based only on studies of two experimental tumor lines of the same histologic type and is, therefore, not necessarily valid for every tumor type in humans.

Our studies may have significant implications for the treatment of patients with local recurrences after radiation therapy. Experimental tumors have been shown to express a similar tumor bed effect in beds pretreated with large single radiation doses and beds...
pretreated with clinically relevant fractionated irradiation (8, 10). D-12 and R-18 tumors growing in beds preirradiated with 10 or 20 Gy should, therefore, be relevant models of postirradiation local recurrences in humans. The observation that D-12 and R-18 tumors in preirradiated beds show increased propensity for developing pulmonary and lymph node metastases, respectively, suggests that patients given radiation therapy with curative intent should be examined frequently for postirradiation recurrences, and local recurrences should be subjected to curative treatment as early as possible to prevent metastatic dissemination. Moreover, it is possible that patients with a high probability of developing local recurrences may benefit from being treated up front with curative radiation therapy combined with postirradiation antimetastatic treatment, i.e., treatment involving antiangiogenic agents (e.g., agents blocking the action of proangiogenic factors such as IL-8) and/or anti-invasive agents (e.g., agents blocking cell surface receptors such as uPAR), depending on the metastatic pattern of the tumor type in question.

In summary, our preclinical study with human melanoma xenografts suggests that primary tumors recurring after unsuccessful radiation therapy may show increased metastatic propensity because of increased fraction of hypoxic cells and hypoxia-induced up-regulation of metastasis-promoting gene products such as secretory proteins promoting neangiogenesis and cell surface receptors promoting invasive growth. The possibility that patients with a high probability of developing local recurrences after curative radiation therapy may benefit from combined radiation therapy and postirradiation treatment with antiangiogenic and/or anti-invasive agents merits additional investigation in clinical studies.

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The Tumor Bed Effect: Increased Metastatic Dissemination from Hypoxia-Induced Up-regulation of Metastasis-Promoting Gene Products

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