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Radiotherapy Decreases Vascular Density and Causes Hypoxia with Macrophage Aggregation in TRAMP-C1 Prostate Tumors

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Abstract

Purpose—To investigate how single or fractionated doses of radiation change the microenvironment in transgenic adenocarcinoma of the mouse prostate (TRAMP)-C1 tumors with respect to vascularity, hypoxia, and macrophage infiltrates.

Experimental Design—Murine prostate TRAMP-C1 tumors were grown in C57BL/6J mice to 4 mm tumor diameter and were irradiated with either 25 Gy in a single dose or 60 Gy in 15 fractions. Changes in vascularity, hypoxia, and macrophage infiltrates were assessed by immunohistochemistry and molecular assays.

Results—Tumor growth was delayed for 1 week after both radiation schedules. Tumor microvascular density (MVD) progressively decreased over a 3-week period to nadirs of 25% and 40% of unirradiated tumors for single or fractionated treatment, respectively. In accord with the decrease in MVDs, mRNA levels of endothelial markers, such as *CD31*, *endoglin*, and *TIE*, decreased over the same time period after irradiation. Central dilated vessels developed surrounded by avascularized hypoxic regions that became infiltrated with aggregates of CD68+ tumor-associated macrophages, reaching a maximum at 3 weeks after irradiation. Necrotic regions decreased and were more dispersed.

Conclusion—Irradiation of TRAMP-C1 tumors with either single or fractionated doses decreases MVD, leading to the development of disperse chronic hypoxic regions, which are infiltrated with CD68+ tumor-associated macrophages. Approaches to interfere in the development of these effects are promising strategies to enhance the efficacy of cancer radiotherapy.

The probability of local tumor control following radiation therapy (RT) is determined primarily by the number of clonogens (1,2), their intrinsic cellular radioresistance, and microenvironmental factors, such as hypoxia, which are in turn dependent on tumor

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microvasculature (3). It has been reported that RT decreases the number of capillary-like structures formed by endothelial cells *in vitro* (4) and induces endothelial cell apoptosis in tumors *in vivo* (5). These cellular effects are thought to result in morphologic and functional changes in tumor vasculature after RT, which include degradation of the vascular wall, disruption of the endothelial lining, perivascular fibrosis, interstitial edema, and vasodilatation (6).

The link between vascular damage and effectiveness of tumor RT has been illustrated by animal models with the use of acid sphingomyelinase-deficient and Bax-deficient mice (7), in which tumors show less endothelial apoptosis after large single doses of irradiation and faster growth than in wild-type mice. There is, however, controversy about the importance of vascular damage in tumors receiving fractionated RT (8,9).

Vascular function seems largely under the control of cytokines in the tumor microenvironment. The major sources of such cytokines are tumor-associated macrophages (TAM; ref. 10). Two distinct macrophage phenotypes, M1 or M2, have been described with the abilities to inhibit or promote tumor growth, respectively. The M1 phenotype is proinflammatory with high levels of inducible nitric oxide synthase production. The M2 phenotype (11) is anti-inflammatory, proangiogenic (12,13), and promotes metastasis (14,15). M1 and M2 TAMs seem to segregate to different areas of the tumor, with M2 TAMs migrating to and aggregating in avascular or hypoxia regions (16,17). Surface markers that can distinguish M1 from M2 TAMs are still poorly defined, but F4/80 (18) or CD68 (17,19) primarily identify M2 cells, with the two classes often being additionally defined by a composite of differences in scavenger receptor expression, effector function, and cytokine or chemokine production (11,20).

Translational Relevance

Although reoxygenation is believed to confer a therapeutic advantage for fractionated radiotherapy (RT) of tumors, our animal model suggests that both single and fractionated RT may cause chronic and persistent hypoxia. Indeed, there seems to be a shift from transient to chronic hypoxia during the later times of a fractionated course of RT that is associated with aggregation of CD68+ tumor-associated macrophages in hypoxic regions. These phenomena are important for our understanding of how the tumor microenvironment changes during and after RT, and provide clues about potential targets for intervention to enhance the effectiveness of RT or to treat recurrent tumors.

The potential of RT to alter TAM phenotype and function has rarely been studied. In our previous study, TAMs isolated from irradiated tumors expressed higher arginase I, cyclooxygenase-2, and inducible nitric oxide synthase levels than those from unirradiated tumors and were more effective at promoting tumor growth (21). In this study, we further analyze the dynamic relationships among tumor vascularity, hypoxia, and TAMs in tumors regrowing during or after multifraction (MF) or single-dose (SD) RT, respectively.

Materials and Methods

Tumor model and tumor irradiation

All experiments were done with the use of 7-to-8-wk-old male C57BL/6J mice. The transgenic adenocarcinoma of the mouse prostate (TRAMP)-C1 prostate cancer cell line was derived from transgenic mice with adenocarcinoma of the mouse prostate (22) and was purchased from the ATCC (CRL-2730). Tumors were generated by i.m. inoculation of 3×10^6 viable cells into the thigh. Mice with tumors 4 mm in diameter were selected and randomly allocated to groups for experimentation [tumor diameter was defined as $(a+b)/2$, where a and b are the width of two dimensions of the mouse thigh] that contained at least 5 mice per time point.

The irradiation protocol was previously described (21), and tumors were irradiated with either a single dose of 25 Gy (SD) or 60 Gy in 15 fractions, 4 Gy per day and 5 d per week. The tumors were removed at indicated times after irradiation.

For detecting hypoxia, pimonidazole hydrochloride (Hypoxyprobe-1 Kit; Chemicon) was administered as 4 mg in 0.1 mL by i.v. injection. One hour after administration of the hypoxia marker, perfusion levels were determined by injecting mice i.v. with a 0.05-mL solution of Hoechst 33342 (Invitrogen) in PBS (pH 7.4) at a dose of 15 mg/kg. One minute after Hoechst injection, the mice were sacrificed, and the tumors were removed and stored in optimal cutting temperature compound at -80°C. During the experiments, all mice were cared for in accordance with the guidelines approved by the Commission of Chinese National Laboratory Animal (approval number CGU03-59).

RNA isolation and RNase protection assay

The technique has been described in detail elsewhere (23). A set of mAngio-1 Multi-Probe Template (BD Biosciences) was used for RNase protection assay. The gel of the RNase protection assay samples in the same plate was exposed to film for various times to acquire adequate signal intensity appropriate for each gene, and the optical density of each band was determined by the free software ImageJ (<http://rsb.info.nih.gov/ij/>). The expression level of mRNA of each gene was normalized to the housekeeping gene, L32, and the ratio of expression to the control at 0 h was calculated.

Immunohistochemistry

Ten-micrometer cryostat sections were fixed in methanol at -20°C for 10 min and then rehydrated in PBS. Nonspecific binding was blocked by incubating sections in 1% bovine serum albumin in PBS for 30 min. Tumor sections were double stained for pimonidazole in combination with *CD31* or CD68. Pimonidazole was detected with mouse antibody (Chemicon) and goat anti-mouse IgG1 Alexa 488 (Invitrogen). For verification of chronic hypoxic cells, the section adjacent to that used for pimonidazole staining was stained with goat anti-carbonic anhydrase IX antibody (R&D Systems Inc.) and donkey anti-goat IgG Cy3 antibody (Chemicon). For endothelial cells, rat anti-*CD31* antibody (BD Biosciences) was used, followed by goat anti-rat Alexa 594 (Invitrogen). For macrophages, rat anti-CD68 (Serotec) was used, followed by goat anti-rat Alexa 594 (Invitrogen). Slides were rinsed in PBS and mounted with ProLong Gold antifade reagent (P-36931; Invitrogen). Sections that had been stained by standard H&E (Merck) were examined to identify necrotic areas.

Image acquisition, processing, and analysis

Immunofluorescent images from each tumor section were captured with the use of an external digital camera (DXM-1200C; Nikon) on a Nikon fluorescence microscope (Nikon Eclipse TE 2000-S). Scanning at $\times 100$ magnification yielded composite images of endothelial cells with the perfusion marker and pimonidazole with TAMs. Composite images were obtained at $\times 200$ or $\times 630$ magnification.

The images were analyzed by Image-Pro version 4 software (MediaCybernetics). Microvascular density (MVD) was determined as the number of pixels positive for *CD31* divided by the total tumor area. Regions of gross necrosis were visually selected on the H&E images and were outlined with a multiple-area-of-interest tool to determine the percentage in total tumor area. The hypoxia fraction was defined as the area positive for pimonidazole divided by the total tumor area (necrosis excluded). The density of macrophages in the hypoxic region was defined as the fraction of pixels positive for CD68 in the pimonidazole-positive tumor area divided by the fraction of total CD68+ pixels within the selected field.

Statistics

Data are presented as means with standard deviation. Statistical analyses used GraphPad Prism version 3 (GraphPad Software). For all comparisons, assessment of statistical significance was by unpaired *t* tests with significance set at $P \leq 0.05$.

Results

Reduction of tumor MVD after SD and fractionated-dose RT

Large SD (25 Gy) and MF (4 Gy per fraction $\times$ 5 days per week $\times$ 3 weeks) doses were chosen because both caused $\sim$ 1-week tumor growth delay. The effect on tumor MVD was compared with control tumors, with 1 day before irradiation as the reference point. As shown in Fig. 1, MVD remained relatively constant in control tumors until the tumors were larger than 10.6 mm. For SD irradiation, the MVD decreased to $71\% \pm 9\%$ of reference levels at 1 day ($P > 0.05$) and progressively thereafter to $25\% \pm 9\%$ at 3 weeks ($P < 0.05$). For MF, the MVD similarly decreased. Surprisingly, this was the case even 1 day after the first dose of 4 Gy (MVD, $79\% \pm 20\%$). Further decreases to $54\% \pm 7\%$ at day 3 ($P < 0.05$) and around 40% from day 5 to day 19 ($P < 0.05$) were observed.

Alterations in vascular morphology and expression of angiogenesis-associated markers in irradiated tumors

Staining for endothelial cells showed that the microvasculature in control tumors was organized in a network. (Fig. 2A-D) As tumors grew, the distance between vessels and the diameter of vessels increased, which may explain why the MVD did not change too much with time (Fig. 1). As early as 1 day after either SD or MF irradiation, there was loss of capillary-like vessels (Fig. 2E and I) that became more obvious with time (Fig. 2E-L) so that at 3 weeks, most of the surviving vessels were relatively large (Fig. 2H and L). Subjectively, the vessels in MF-irradiated tumors seemed more dilated than those receiving SD. Interestingly, vessel perfusion assessed by the presence of Hoechst 33342 dye was less in irradiated than in control tumors (Supplementary Fig. S1).

To verify if these histologic changes were reflected in the expression of angiogenesis-associated factors, RNase protection assay was done (Fig. 3A-C). The TRAMP-C1 cell line cultured *in vitro*, used as control, had high levels of vascular endothelial growth factor (VEGF) and thrombin receptor, and moderate levels of VEGF-C (Fig. 3A, column 1). Thrombin receptors and VEGF have been associated with angiogenesis, and VEGF-C is required for the initial steps in lymphatic development (24). TRAMP-C1 tumors *in vivo*, in addition to what was found *in vitro*, expressed *flt-1*, *flt-4*, *Tie*, *CD31*, and *endoglin* (Fig. 3A), which were presumed to be host-cell derived. Although mRNA levels varied slightly between tumors at the same time point, VEGF and thrombin receptor expression in control tumors remained relatively constant with tumor growth although VEGF-C may have initially been high and decreased slightly with time (Supplementary Fig. S2), and this did not change after SD or MF RT (Supplementary Fig. S2). On the other hand *tie*, *CD31*, and *endoglin* mRNA levels were significantly decreased 1 day after SD or MF RT (Fig. 3D; Supplementary Fig. S2). The same trend was noticed for *flt-4* although statistical significance was not reached. These results, put together, suggest that both RT schemes decrease the relative expression level of host-derived angiogenic factors, which is consistent with the decrease in MVD in irradiated tumors.

Irradiated tumors have less necrosis in a different distribution pattern

Although irradiated tumors had a progressive decrease in MVD, they continued to grow after a 1-week delay, raising the question of whether necrosis was increasing. Unexpectedly, less necrosis was evident in irradiated tumors (Fig. 4A). One week after RT, the percentage of

necrosis decreased to $2\% \pm 2.8\%$ and $2.8\% \pm 2.0\%$ for SD-treated and MF-treated tumors, respectively, compared with $31\% \pm 6.8\%$ for control. The corresponding figures were $14.7\% \pm 6.3\%$, $6.4\% \pm 5.7\%$, and $51.2\% \pm 16\%$, respectively, for tumors at 2 weeks after RT. The percentage increased slightly to $25.7\% \pm 8.3\%$ (SD) and $11\% \pm 5.6\%$ (MF) at 3 weeks after RT but was still lower than for control tumors at 2 weeks ($51.2\% \pm 16\%$). Furthermore, the distribution of necrosis differed after RT. As shown in Fig. 4B, necrosis (delineated by the yellow line) in control tumors was in large continuous zones whereas in irradiated tumors it was in small, randomly distributed sites.

The relationship between hypoxia and vasculature

Because hypoxia is possibly one cause of necrosis, the relationship between these phenomena was examined. Pimonidazole staining for hypoxic regions in control tumors showed that the microvasculature was not perfused with Hoescht dye unlike the vasculature in nonhypoxic areas (Fig. 5A-a; Supplementary Fig. S1). Three weeks after RT, the vasculature in the hypoxic areas had disappeared (Fig. 5A-b and c) and areas of central necrosis were evident. We interpret this as RT eliminating the microvasculature associated with acutely hypoxic regions, making such regions chronically hypoxic. The change from transient to chronic hypoxia after RT was further supported by staining for carbonic anhydrase IX (Supplementary Fig. S1), which is a long-lived hypoxia-inducible factor-inducible endogenous protein. Carbonic anhydrase IX was highly expressed after RT but not in control tumors.

The percent of tumor hypoxia (Fig. 5B) did not significantly change at 1 day after SD or MF RT but was decreased at later time points. There did seem to be differences between SD and MF treatments. The latter remained fairly constant with time whereas the former increased late in the response. This presumably reflects continuing reoxygenation during the MF course.

TAMs aggregate in chronic hypoxic regions after RT

TAMs were shown to be attracted to and anchored at hypoxic regions by responding to expressed chemokines and cytokines (17,25). Because they could be a major source of cytokines, we investigated their spatial location after RT. In contrast to Lewis' report on breast and cervical cancer models (10,19), we did not see any particular spatial relation between CD68 + TAMs and hypoxia in control tumors, in which they were randomly distributed throughout hypoxic and nonhypoxic regions (Fig. 6A). This pattern did not change over the 1st week after RT. However, at 2 weeks CD68+ TAMs started to aggregate in the developing chronic hypoxic regions. This was even more obvious at 3 weeks after both SD and MF RT (Fig. 6B and C). The degree of colocalization of CD68+ TAM with pimonidazole staining at 3 weeks after RT was calculated and plotted in Fig. 6D. The densities of CD68+ TAMs were significantly increased in hypoxic regions within both SD-irradiated and MF-irradiated tumors (both $P < 0.05$ by unpaired t test) but was more prominent in the former ($P < 0.05$).

Discussion

This study describes important microenvironmental changes after SD or MF RT. After either schedule, the MVD decreased from day 1 to week 3 to ~ 30% to 40% of that in control tumors. RT has been shown to induce apoptosis in endothelial cells *in vitro* (4) and *in vivo* (5) as early as 24 hours after exposure. We also detected apoptosis in endothelial cells in TRAMP-C1 tumors at 24 hours after SD RT (data not shown), and this can explain the rapid drop in MVD. What was more surprising was the persistence of this loss even when tumor growth resumed ~ 7 days after RT. A similar decrease has been reported in MVD at 14 days, but not 1 day, after a single dose of 12 Gy RT or an equivalent dose of fractionated RT to K1735 melanoma tumors (5) even though the authors did see endothelial cell apoptosis at day 1. The authors suggested that the late decrease in MVD was caused by ineffective angiogenesis (5). Our

mRNA data suggest that decreased expression of host proangiogenic, endothelial-associated factors may be the cause of persistently low MVD levels and also supports the concept that the loss in vasculature is the result of impotent attempts at angiogenesis by a radiation-compromised endothelium.

The increase in diameter of the remaining vessels after RT has also been observed before (5). The reason is unknown, but our previous study showed progressive increases of mRNA levels of inducible nitric oxide synthase in irradiated tumors (21), and low levels of inducible nitric oxide synthase may produce sufficient nitric oxide to maintain the dilator tone and increase blood flow in tumor vessels (26,27).

Pericytes are another principal cell in the blood vessel walls. These embed within the vascular basement membrane to form an outer sheath surrounding the endothelium (28) and regulate the formation, stabilization, remodeling, and function of the vasculature (29). In poorly differentiated prostate cancers from TRAMP mice, pericytes were abnormally shaped, variable in size, loosely associated with vessels, and had coverage that was not uniform (30). The vascular pattern in control tumors was very different from the large vessels remaining after RT, which were less torturous and had closely bound pericytes (Supplementary Fig. S3). This implies that these remaining vessels might be less permeable and more efficient in supplying blood and nutrients to surrounding tumor cells.

Our data suggest that RT retards angiogenesis; however, tumors regrow very effectively. The decrease of the expression of endothelial cell marker genes, such as *CD31*, *endoglin*, *Tie*, and *Flt-4* after RT is in keeping with their radiation-induced death. Loss of *endoglin*, a transforming growth factor- β binding protein, causes poor vascular smooth muscle development and arrested endothelial remodeling (31). Decreased expression of *Flt-4*, which functions at sprouting first lymphatic vessels from embryonic veins, has been associated with pathologic angiogenesis (24,32) and contributes to the debranched phenomena in irradiated tumors. Whereas biological targeting of neovascularization is popular, with and without RT, our study clearly indicates that angiogenesis may not be a major factor determining tumor regrowth after RT.

Microvessels within hypoxic regions of control tumors were presumably functionally inefficient because many of them were not perfused by Hoechst dye. Such areas may be presumed to be acutely hypoxic, which is supported by the lack of carbonic anhydrase IX expression, a marker associated with cells exposed to diffusion-limited chronic hypoxia over a long time period (33). Remarkably, nearly no microvessels were found in the hypoxic regions of irradiated tumors at 2 to 3 weeks, suggesting chronic hypoxia caused by vascular deficiency predominated. This conclusion was supported by carbonic anhydrase IX staining. This seems to suggest that the acutely hypoxic microvasculature is relatively sensitive to irradiation. This may be as a result of the induction of a p53-dependent proapoptotic phenotype by hypoxia (34,35) that might selectively radiosensitize endothelium in this area.

Reoxygenation during RT is an advantage for fractionated treatment, and we showed that RT decreased the percentage of hypoxia after 1 week but not after 1 day. The hypoxic fraction of SD-irradiated tumors gradually increased to control levels as tumors regrew, but this was not true for MF RT, suggesting that there is reoxygenation during and/or after RT and that MF RT prolongs this reoxygenation. More interesting was that there was an apparent shift from transient to chronic hypoxia at 2 to 3 weeks even after MF RT. This suggests a changing target for therapeutic strategies aimed at hypoxic cells even at later phases of a MF RT regimen.

One perhaps surprising finding from our study was that irradiated tumors had a lower percentage of necrosis than control tumors in spite of vascular loss, with the lowest values in MF-treated tumors. Moreover, the necrosis was more dispersed and located in the center of a region of chronic hypoxia after RT. Control tumors, on the other hand, usually had a large area

of central necrosis. The fact that necrosis was linked to the development of small, diffuse avascularized areas of chronic hypoxia again indicates the dynamic nature of the changes in the tumor microenvironment after RT.

TAMs are a major cellular component of human (36) and murine cancers (37,38). They can accumulate in hypoxic regions (10,19), and their number in these regions correlates with poor prognosis in several human cancers (14,15,39,40). Remarkably, CD68+ TAMs, which were randomly distributed in unirradiated tumors and not associated with hypoxic regions, began to accumulate in hypoxia regions after RT, coincident with the development of the avascularized chronic hypoxic regions.

Chronic hypoxia is known to produce macrophage chemoattractants, such as endothelial monocyte-activating polypeptide II, endothelin 2, and VEGF (10), to trigger TAM migration, which may form a depot for cytokine release that may promote an avalanche of endothelial cell death and impotent attempts at angiogenesis. We have shown previously that TAMs from irradiated TRAMP-C1 tumors expressed higher level of inducible nitric oxide synthase (21) at 2 to 3 weeks, the time of accumulation of TAMs in hypoxic regions. Nitric oxide generated from TAMs of irradiated tumors can increase hypoxia-inducible factor-1 α expression (41), which regulates >60 genes that are involved in angiogenesis, metabolism, cell death, and metastasis (42). These TAMs also express higher levels of arginase I and cyclooxygenase-2, which may contribute to enhanced tumor growth and proliferation after RT (21). TAMs within chronically hypoxic areas that develop would be prime targets for modulating tumor responses after RT.

In summary, we have shown in at least this murine prostate cancer model that RT can cause a shift from acute to chronic hypoxia by eliminating microvasculature in acutely hypoxic areas. The chronic hypoxia that develops becomes associated with TAMs that may form a depot for cytokines that dictate tumor regrowth even at the same time as vascular collapse leading to necrosis. This changing microenvironmental landscape after RT, if verified in human tumors, will need to be taken into account when targeting vasculature and/or hypoxia with therapeutic intent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The changes in MVD in TRAMP-C1 tumors after sham (control), SD, and MF RT. MVD was defined by the *CD31*⁺ area as a percent of the total tumor in the field of view. MVD of control tumors at 1d before RT was used as reference level for comparisons. The corresponding tumor diameter of control tumors at days 1, 3, 7, and 14 was 4.3, 7, 10.6, and 13.2 mm, respectively. Bar, SE of 5 fields (non-necrotic areas) of 3 to 6 tumors for each time point. *, $P < 0.05$ by unpaired t test.

Fig. 2.

The morphologic changes of TRAMP-C1 tumor vasculature after sham (control), SD, and MF RT. Endothelial cells were stained by anti-*CD31* antibody (*shown in white here*). Control tumors had relatively well-organized branching vessels (A-D; days 1, 3, 7, and 14). This well-organized structure was disrupted by either SD (E-H; days 1, 7, 14, and 21) or MF RT (I-L; days 1, 5, 12, and 19). The remaining vessels in the irradiated tumors at 3 wk were dilated with little or no branching; this is even more obvious in MF-irradiated tumors (L, arrows). Bar, 100 µm.

Fig. 3.

A representative RNase protection assay showing expression of angiogenesis-associated genes in TRAMP-C1 tumors after RT. The probe set is a mAngio-1 Multi-ProbeTemplate set from the BD RiboQuant system. L32 and glyceraldehyde-3-phosphate dehydrogenase housekeeping genes were used as loading controls. *A*, control tumors; *B*, SD-irradiated tumors; *C*, MF-irradiated tumors. The relative change of a representative angiogenic factor, tie, was calculated and plotted in *D*. The relative changes of other genes can be found in Supplementary Fig. S2. Bar, SE of 3 mice in the same plate. *, $P < 0.05$ by unpaired t test.

Fig. 4.

A, percent necrosis in TRAMP-C1 tumors after sham (control), SD, and MF RT. The largest cross section of the tumor by H&E staining was used to calculate necrosis percentage. Bar, SE of 5 to 10 tumors at each time point. *, $P < 0.05$ by unpaired t test. B, necrosis. Necrosis, delineated by the white outline, in control tumors was as large continuous zones (a-c; 3, 7, and 14 d). SD-irradiated (d-f; 7, 14, and 21 d) and MF-irradiated (g-i; 5, 12, and 19 d) tumors had small and randomly distributed areas of necrosis whereas large SD-irradiated tumors (e; 21 d) had a pattern in between control and MF-irradiated tumors.

Fig. 5.

A, the distribution of hypoxia, vasculature, and vascular perfusion in sham-irradiated (control; day14), SD-irradiated (day 21), and MF-irradiated (day 19) tumors. The hypoxic regions in control tumors were vascularized (a; con) but were not perfused by Hoechst 33342. There was almost no vasculature in the hypoxia regions of irradiated tumors (b, SD; c, MF). Green, pimonizadole stain for hypoxia; red, *CD31* stain for endothelial cells; blue, Hoechst 33342 for vascular perfusion. Bar, 100 µm. B, the change in hypoxia fraction after SD or MF RT is shown as a histogram. Tumor samples after MF RT were taken 2 d earlier than control and SD-RT tumors to account for the MF schedule. Bar, SE of 3 to 6 tumors for each time point. *, $P < 0.05$ by unpaired t test.

Fig. 6. The distribution of hypoxia and CD68+ TAMs in control (A), SD-irradiated (B), and MF-irradiated (C) tumors at different times after the initiation of RT. Green, anti-pimonizadole stain for hypoxia regions; red, anti-CD68 antibody for TAMs. Merged images, the colocalization of hypoxia and TAMs. The density of CD68+ TAMs in the hypoxic region is plotted in a histogram (D). Bar, SE of 5 fields of 5 tumors. *, $P < 0.05$ by unpaired t test. Bar, 80 μm .