

# Darbepoietin Alfa Potentiates the Efficacy of Radiation Therapy in Mice with Corrected or Uncorrected Anemia

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## Abstract

Darbepoietin alfa (DA) is a long-acting analogue of erythropoietin that has reduced receptor affinity and enhanced biological activity. Experiments were done to test the hypothesis that correction of anemia in tumor-bearing mice by DA would increase tumor oxygenation and potentiate radiation-induced tumor cell killing. A SCC VII tumor model was used to study tumor responses to fractionated radiation therapy in mice with anemia induced by total body irradiation. Administration of DA reduced the extent and duration of anemia and associated tumor hypoxia, protected the bone marrow cells and prevented the body weight loss from the effect of irradiation, and facilitated the recovery in a time-dependent manner, with the administration of DA prior to total body irradiation having the greatest protective effect. When combined with fractionated radiation therapy, DA increased the tumor growth delay time from 2.7 days for irradiation alone to 7.3 to 10.6 days for combination of DA and irradiation. The effect of DA on tumor responses to fractionated radiation therapy was observed when DA was given 18 to 4 days before starting radiation therapy, but DA was also equally effective as a radiosensitizer when given only 2 hours before fractionated irradiation therapy. Weekly dosing of DA was as efficacious for the enhancement of radiation responses of tumors as biweekly dosing. Similar results were obtained in the RIF-1 fibrosarcoma tumor model. These studies show that DA can effectively correct anemia in tumor-bearing mice and sensitize tumor cells to fractionated radiation therapy. Importantly, DA was also able to sensitize tumors to radiation in mice with uncorrected anemia and hypoxia, suggesting that the effect of DA on radiosensitivity was independent of these factors and a different mechanism of action may be responsible for this effect. (Cancer Res 2005; 65(1): 284-90)

## Introduction

Anemia is common in cancer patients secondary to their disease and/or treatment. In a retrospective review, the overall prevalence of anemia (hemoglobin <12 g/dL) at the time of presentation for radiation therapy was ~41%, whereas at the completion of radiation therapy it was 54% (1). Anemia in radiation therapy patients is of potential clinical significance because of the association of anemia and pretreatment tumor  $pO_2$  with decreased local tumor control and lower rates of disease-free and overall

survival in patients with head and neck, cervical, and other cancers (2–9) treated with radiation therapy. This relationship has been attributed to the correlation between anemia and tumor hypoxia (10–12) and the association of hypoxia with decreased radiosensitivity (13). Studies suggest that correction of anemia may enhance radiosensitivity of tumors in both preclinical (14–16) and clinical (14, 17, 18) studies, although this is still somewhat controversial (19–21). Nevertheless, it has been estimated that increasing the hemoglobin by 20% would produce a theoretical decrease in hypoxic tissue volume of ~30% (22).

Erythropoietin has been used preclinically to restore anemia-induced reduction in tumor radiosensitivity (14–16), and the use of recombinant human erythropoietin (rHuEPO) to correct anemia in patients receiving radiation therapy is under investigation with mixed results (21, 23–33). However, nothing is known about the effect of darbepoietin alfa (DA), a long-acting hyperglycosylated analogue of rHuEPO containing five N-linked carbohydrate chains (34), on tumor sensitivity in the presence of anemia. DA has a higher molecular weight, sialic acid content, and negative charge than rHuEPO, which results in an increased serum circulating half-life, lower affinity for the erythropoietin receptor (EPO-R), and higher biological activity than rHuEPO. Notably, it produces a faster rate of hemoglobin rise and a higher stable plateau hematocrit than rHuEPO (34, 35). We hypothesized that DA would increase the relative radiosensitivity of tumors in anemic mice secondary to increased tumor oxygenation and decreased tumor hypoxia. Results from experiments described here show that DA potentiates the radiation responses of tumors in mice with corrected anemia but also surprisingly show a significant radiosensitization in the absence of the correction of preexisting anemia when DA was given within 2 hours of radiation therapy.

## Materials and Methods

**Tumor Models.** The murine squamous cell carcinoma SCC VII (36) and fibrosarcoma RIF-1 (37) tumor models were used for the experiments described below. Briefly, C3H mice (male, ages 8–10 weeks; body weight, 22–25 g) were purchased from Charles River Lab (Wilmington, MA) and injected with  $5 \times 10^5$  SCC VII or RIF-1 cells in 0.05 mL Hank's solution s.c. in the back ~10 mm proximal to the base of the tail. Tumors reached ~100 to 150 mm<sup>3</sup> in volume 11 days (SCC VII tumors) or 14 days (RIF-1 tumors) after tumor implantation. Mice with tumors ranging from 100 to 150 mm<sup>3</sup> in size were randomized into groups prior to fractionated radiation therapy. On the day fractionated radiation therapy was initiated, there was no statistically significant difference in tumor size between the DA-treated and control groups. The length, width, and height of the tumors were measured with calipers immediately before treatment and thrice a week thereafter until the tumor volume reached at least four times (4×) the original pretreatment volume. Tumor volume (mm<sup>3</sup>) was calculated according to the formula: tumor volume =  $\pi/6 \times \text{length} \times \text{width} \times \text{height}$ . The data are expressed as relative tumor volume normalized to the pretreatment volume measured on the day when the

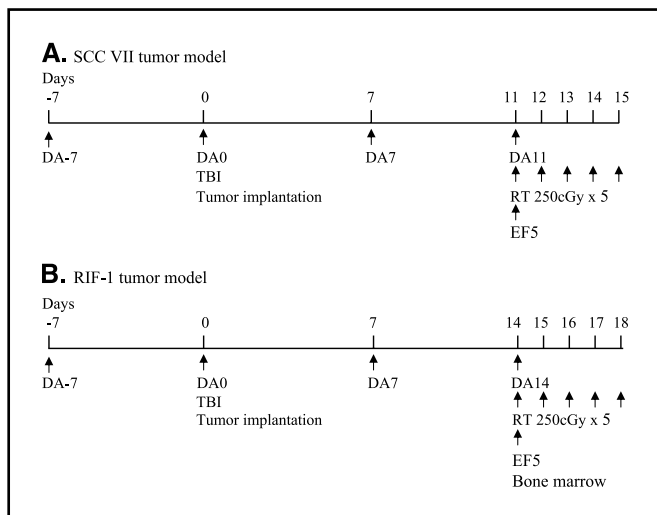
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fractionated local radiation therapy was started. The tumor volume quadrupling time (in days) was determined by a best-fit regression analysis. The tumor growth delay time is the difference between the tumor volume quadrupling time of treated tumors compared with that of untreated control tumors. Both the tumor volume quadrupling time and tumor growth delay time was calculated for each individual animal and then averaged for each group. Body weight was measured twice a week. Mice were maintained under specific pathogen-free conditions in the Stanford Research Animal Facility. All animal experiments were approved by and complied with the regulations of the Stanford University Animal Care Panel.

**Experimental Design.** A single dose of 500 cGy total body irradiation (TBI) was used to induce a stable anemia in the mice. TBI was given on day 0 and tumor cells were implanted 4 hours following TBI. Fractionated local tumor radiation therapy was initiated 11 or 14 days after TBI/tumor implantation for SCC VII and RIF-1 tumors, respectively. Tumors were treated daily with 250 cGy/d for five consecutive days. TBI and local tumor irradiation was given using a Philips RT-250 200 kVp X-ray unit (12.5 mA; Half Value Layer, 1.0-mm Cu) at a dose rate of 140 cGy/min. DA (Aranesp, Amgen, Inc., Thousand Oaks, CA) was diluted in sterile normal saline (0.9% sodium chloride) and given i.p. at a dose of 30  $\mu$ g/kg body weight every 2 weeks (38). There were four different regimens for administration of DA [i.e., (1) DA-7: DA administration starting 7 days before TBI on day 0; (2) DA0: starting on the same day of TBI (2 hours before TBI); (3) DA7: 7 days after TBI; and (4) DA11: 11 days after TBI]. In RIF-1 tumor model, DA was also given at 14 days after TBI (DA14, instead of DA11) because it took 14 days for these tumors to reach a volume of  $\sim$ 100 to 150 mm<sup>3</sup>. For all the regimens, administration of DA was repeated every 2 weeks (biweekly) until the completion of each experiment. Mice in control groups were injected i.p. with the same volume (10  $\mu$ L/g body weight) of normal saline. The study design is shown in Fig. 1.

In one experiment, weekly administration of DA was studied in the SCC VII tumor model to determine if weekly dosing would be more efficacious than biweekly dosing. The experimental design was otherwise identical to that described above.



**Figure 1.** Schemes showing the timing of experiment schedules for (A) SCC VII tumor model and (B) RIF-1 tumor model. Mice were irradiated with 500 cGy TBI on day 0 and implanted with SCC VII or RIF-1 tumor cells 4 hours following TBI. Fractionated local tumor radiotherapy of 250 cGy daily for five consecutive days was given from days 11 to 15 or from days 14 to 18 for SCC VII and RIF-1 tumor models, respectively. DA (30  $\mu$ g/kg) was administered i.p. starting 7 days before (DA-7), or on the same day of (DA0), or 7 days (DA7), or 11 days (DA11) after TBI/tumor implantation and repeated every 2 weeks thereafter until the completion of each experiment. In RIF-1 tumor model, the last regimen of DA administration was started 14 days after TBI (DA14, instead of DA11).

In some experiments, 150  $\mu$ L of blood was obtained from the retro-orbital vein of representative mice for complete blood counts (CBC). The same mouse was bled once every 2 weeks. In other experiments, mice were sacrificed at specific time points and tumors were removed for EF-5 staining as described below. In some cases, femurs were removed for bone marrow colony assays. In all experiments, comparisons were made to normal nonanemic mice and similarly TBI-irradiated anemic mice with tumors that had not received DA.

A Student's *t* test was used for the comparison of results from the different treatment groups.

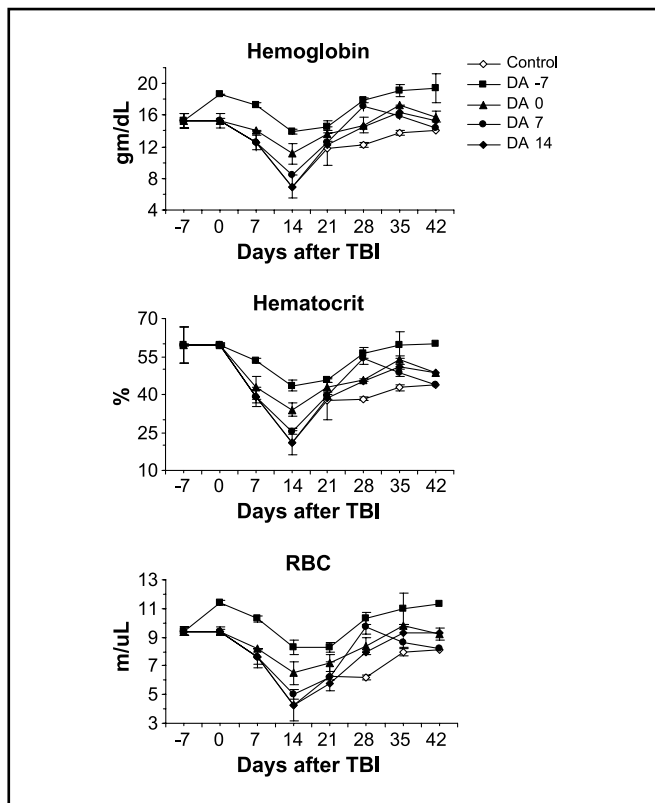
**Bone Marrow Colony Assays.** Mice were irradiated with 500 cGy TBI and RIF-1 tumor cells were implanted 4 hours later. DA was given every 2 weeks. Mice were sacrificed 14 days after TBI. Femurs were harvested and bone marrows were flushed into McCoy's medium with 2% serum (Life Technologies, Grand Island, NY). Bone marrow cells were counted and plated in a progenitor assay system (Stem Cell Technologies, Vancouver, BC, Canada) to detect erythroid progenitor cells (BFU-E) and granulocyte macrophage colony-forming cells (GM-CFC). BFU-E colonies were grown in stem cell methylcellulose cultures (Stem Cell Technologies) with 100 ng/mL rrSCF, 100 ng/mL rmIL-3 and rhIL-6, and 10 units/mL rHuEPO. GM-CFC colonies were cultured in Iscove's modified Dulbecco's medium with 0.33% agar and 100 ng/mL rmIL-3 and 2.5 ng/mL rrSCF. Cultures were incubated for 7 to 8 days in 5% CO<sub>2</sub> and 5% O<sub>2</sub> at 37°C and colonies >50 cells were counted.

**Assessment of Tumor Hypoxia.** Tumor hypoxia was assessed in freshly frozen tumors using a hypoxia marker EF5 as previously described (39). Briefly, mice were irradiated with 500 cGy TBI. SCC VII or RIF-1 tumor cells were implanted 4 hours after TBI. DA was given every 2 weeks. Eleven days (SCC VII tumors) or 14 days (RIF-1 tumors) after TBI, EF5 (University of Pennsylvania, Philadelphia, PA) was injected i.p. at a volume of 0.2 mL of 10 mmol/L stock solution. Tumors were collected 2 hours after EF5 injection. Tumors were then sectioned, and slides were stained with anti-EF5 antibody ELK3-51 conjugated with Cy3 for detecting the specific and nonspecific EF5 binding. Digital photomicrographs were taken at 100 $\times$  magnification (10  $\times$  10) with a fluorescence microscope (Nikon E800, Tokyo, Japan). The Cy3 signal was analyzed with Adobe Photoshop program. The EF5 binding due to hypoxia was determined by correction for nonspecific and endogenous binding.

**Measurement of Erythropoietin Receptor Expression on SCC VII and RIF-1 Cells.** Erythropoietin receptor expression on tumor cells was assayed as previously described (40). Briefly, cells were washed with PBS, pelleted, and lysed using buffer containing guanidine thiocyanate and  $\beta$ -mercaptoethanol as described by the manufacturer (Stratagene, La Jolla, CA, Absolutely RNA RT-PCR Miniprep Kit). Total RNA was isolated and then treated with DNase (Invitrogen, Carlsbad, CA). cDNA was synthesized (Invitrogen SuperScript II RT-PCR System) using oligo-dT as a primer. A portion of the RNA was set aside to which reverse transcriptase was not added, and this served as a measure of genomic DNA contamination. The cDNA-RNA hybrids were then treated with RNase H. Quantitative RT-PCR was done with 50 ng cDNA/rxn, 2X Taqman Universal Mix (Perkin-Elmer, Boston, MA), gene-specific primers and fluorogenic probe (593-654 bp for mouse EPO-R), using the ABI PRISM 7900HT Sequence Detection System. Gene expression was reported as copy number and then normalized to mouse cyclophilin. An EPO-R-negative control cell line 32D-clean and EPO-R-responsive cell line 32D + mEpoR were included as the internal controls (41).

## Results

**Effect of DA on Anemia in TBI Irradiated Mice.** The effect of DA on the TBI-induced anemia was initially studied by treating mice with DA every 2 weeks beginning 7 days before (DA-7), or on the same day of (DA0), or 7 (DA7) or 14 (DA14) days after 500 cGy TBI. Blood samples were collected weekly for CBCs. Hemoglobin,



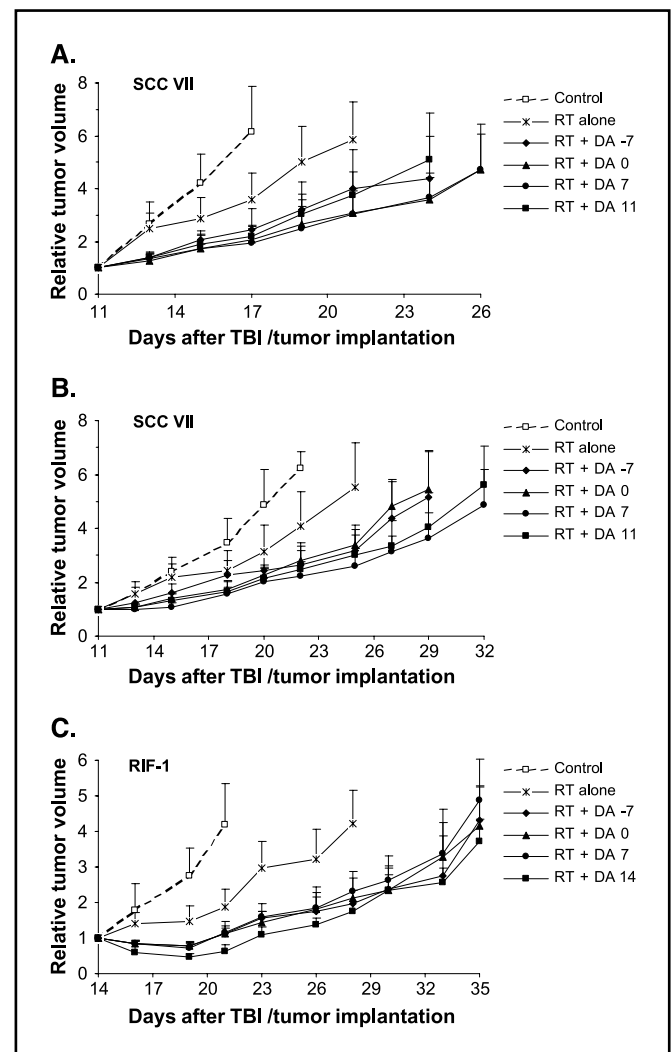
**Figure 2.** Effects of DA on the recovery of (A) hemoglobin, (B) hematocrit, and (C) RBC counts from TBI-induced anemic mice. Mice were irradiated with 500 cGy TBI on day 0. DA was given every 2 weeks starting on days -7, 0, 7, and 14 following TBI. A control group irradiated with TBI only without DA treatment was included for comparison. There were four mice per time point per group. Points, mean; bars,  $\pm$  SD.

hematocrit, RBC, WBC, neutrophil, lymphocyte, and platelet counts were assessed as a function of time following TBI. A control group (treated with TBI only) is included for comparison. As shown in Fig. 2, administration of DA 7 days before TBI (DA-7) prevented the reduction of hemoglobin, hematocrit, and RBC. Administration of DA on the same day as TBI (DA0) minimized, but did not preclude, the TBI-induced nadir in hemoglobin, hematocrit, and RBC. In all other groups, there was a  $>50\%$  reduction from baseline in hemoglobin, hematocrit, and RBC by 14 days after TBI. All of the DA regimens studied accelerated recovery from TBI-induced anemia. DA had little effect on the other cell lineages studied (data not shown).

**Effect of DA on Tumor Growth in Anemic Mice.** Because erythropoietin can interact directly with endothelial cells and elicit an angiogenic response (42, 43), it was important to study the effect of DA on tumor growth *in vivo*. Mice were treated with TBI, and SCC VII tumor cells were implanted s.c. 4 hours after TBI. DA was given at 30 g/kg every 2 weeks. The control group was treated at the same time points with the same volume of normal saline. Administration of DA did not significantly alter the growth of the SCC VII tumors in these anemic mice, with  $4\times$  tumor growth time of  $6.9 \pm 1.5$  days for DA-treated mice versus  $5.9 \pm 1.4$  days for the control mice ( $P = 0.25$ ).

**Effect of DA on Tumor Responses to Local Fractionated Radiation Therapy in Anemic Mice.** Mice were irradiated with 500 cGy TBI, and SCC VII tumors were implanted 4 hours after TBI. Eleven days after TBI/ tumor implantation, mice were irra-

diated with the fractionated local tumor radiation. DA was given every 2 weeks. Control groups consisted of mice irradiated with TBI only (Control) and TBI-irradiated mice treated with local tumor irradiation as above but no DA (radiotherapy alone). The results of this experiment are shown in Fig. 3A and Table 1. Radiation alone had a modest effect on the tumor growth rate with a tumor growth delay time of 2.7 days, but all four DA regimens resulted in significantly increased tumor growth delay (7.3-10.6 days) and tumor volume quadrupling times (11.1-14.5 days) compared with irradiation alone ( $P < 0.01$ ; Table 1). The four DA treatment groups did not differ significantly from one another ( $P = 0.1-0.9$ ), although there was a trend towards the DA0 and DA7 regimens being more effective for the enhancement of radiation effects on the tumors.



**Figure 3.** Effects of DA on radiation response of (A-B) SCC VII carcinoma and (C) RIF-1 fibrosarcoma tumors in anemic mice. For SCC VII tumor model, mice were irradiated with 500 cGy TBI and implanted with SCC VII tumor cells 11 days before fractionated radiation. Fractionated radiotherapy was given locally to tumors from days 11 to 15. DA was administered (A) every 2 weeks or (B) every week. For RIF-1 tumor model, mice were irradiated with 500 cGy TBI and implanted with RIF-1 tumor cells 14 days before fractionated radiation. Fractionated radiotherapy was given locally to tumors from days 14 to 18. DA was administered every 2 weeks. There were 6 to 10 mice per group. Points, mean relative tumor volume as a function of days after TBI/tumor implantation; bars,  $\pm$  SD.

**Table 1.** Comparison of tumor growth time in anemic mice treated with fractionated radiation therapy and DA

	SCC VII tumor (biweekly DA)			SCC VII tumor (weekly DA)			RIF-I tumor (biweekly DA)		
	No. mice	TVQT (d)	TGD (d)	No. mice	TVQT (d)	TGD (d)	No. mice	TVQT (d)	TGD (d)
Control	10	3.9 ± 1.3		10	7.9 ± 1.8		10	6.9 ± 1.3	
Radiation alone	10	6.5 ± 1.9	2.7 ± 1.9	10	11.5 ± 3.1	3.5 ± 3.1	10	14.4 ± 3.0	7.5 ± 3.0
Radiation + DA-7	7	11.4 ± 3.5	7.5 ± 3.5	10	16.3 ± 3.2	8.3 ± 3.2	8	21.0 ± 1.1	14.1 ± 1.1
Radiation + DA0	6	14.5 ± 3.4	10.6 ± 3.4	10	15.3 ± 2.5	7.4 ± 2.5	8	20.8 ± 2.4	13.9 ± 2.4
Radiation + DA7	8	13.5 ± 3.0	9.7 ± 3.0	10	19.2 ± 2.9	11.3 ± 2.9	8	20.2 ± 1.8	13.3 ± 1.8
Radiation + DA11 (DA14 for RIF-1)	10	11.1 ± 3.6	7.3 ± 3.6	10	18.0 ± 2.7	10.1 ± 2.7	8	21.9 ± 1.2	15.0 ± 1.2

NOTE: Tumor growth curves are showed in Fig. 3A-C.

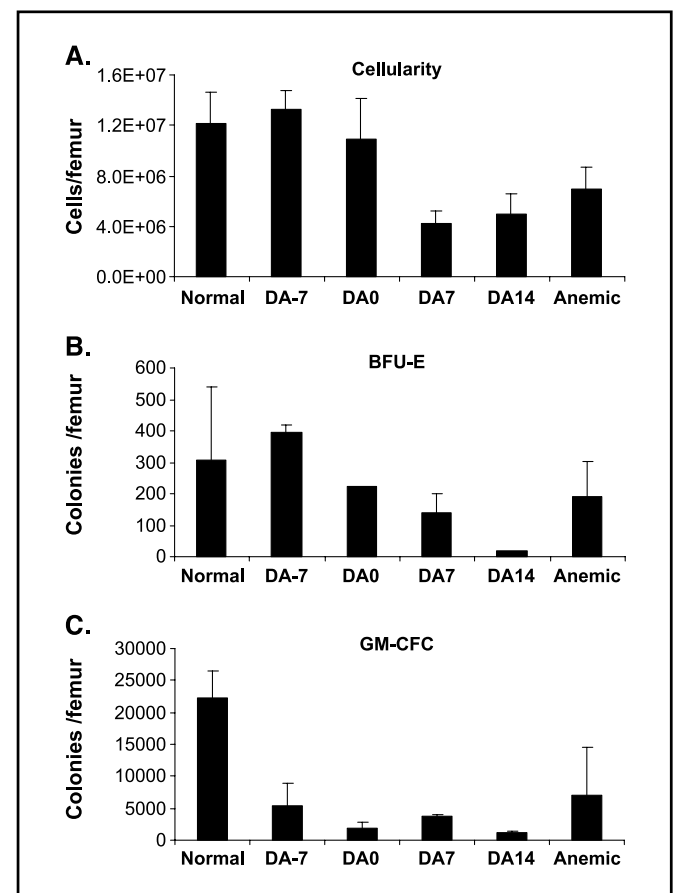
Abbreviations: TVQT, tumor volume quadrupling time; TGD, tumor growth & delay.

The effect of weekly DA was also studied in the SCC VII tumor model to determine if weekly dosing would be more efficacious than dosing every 2 weeks. The experimental design was otherwise identical to that described above. The results are shown in Fig. 3B and Table 1. Radiation alone delayed tumor growth by 3.5 ± 3.1 days. Weekly administration of DA in combination with fractionated radiation increased the tumor growth delay time from 3.5 days to 7.4–11.3 days ( $P < 0.01$  compared with radiation alone) and inhibited tumor growth by 55% to 64% on day 22 compared with 34% for irradiation alone. However, the four administration regimens of DA produced similar results with no statistically significant differences in tumor growth delay time ( $P > 0.05$ ). Also, there were no statistically significant differences in tumor growth delay time between the groups treated with weekly or biweekly DA in the SCC VII tumor model ( $P = 0.1-0.6$ ).

In order to confirm that the radiosensitization effect of DA was not unique to the SCC VII tumor model, the effect of biweekly DA on the response of established RIF-1 fibrosarcoma tumors in anemic mice was studied. As before, mice were irradiated with 500 cGy TBI and implanted with RIF-1 tumor 14 days before initiation of the local tumor fractionated radiation. DA was started either 7 days before, or 0, 7, or 14 days after TBI and repeated every 2 weeks thereafter. Data are shown in Fig. 3C and Table 1. Fractionated radiation therapy alone inhibited tumor growth by 7.5 ± 3.0 days ( $P < 0.001$  compared with untreated control). Administration of DA in combination with fractionated radiation significantly inhibited RIF-1 tumor growth and produced tumor growth delay times of 13.3 to 15.0 days ( $P < 0.001$  compared with radiotherapy alone). Again, there were no statistically significant differences in tumor growth delay time among all the groups treated with DA ( $P = 0.1-0.8$ ).

**Effect of DA on TBI-induced Body Weight Loss.** In the SCC VII tumor model with biweekly dosing of DA, the body weight of all mice fell 10% following 500 cGy TBI. Mice that had received DA 7 days before TBI (DA-7) had recovered to baseline body weight by the first day of fractionated irradiation treatment. This group maintained body weight throughout treatment. Mice treated with DA on the same day of TBI (DA0) seemed to have improved body weight during and after fractionated irradiation, but there was no statistical difference from controls ( $P > 0.05$ ). Also, there were no statistical differences between the other DA

treatment groups and controls ( $P > 0.05$ ). In the weekly dosing study of the SCC VII tumor model, the body weight of mice fell ~5% to 10% (with most animals experiencing ~5% weight loss).



**Figure 4.** Effects of DA on (A) cellularity, (B) BFU-E, and (C) GM-CFC of bone marrow progenitor cells from anemic mice. Mice were irradiated with 500 cGy TBI and implanted with RIF-1 tumor 14 days before collection of bone marrow. DA was administered every 2 weeks. Mice with RIF-1 tumors irradiated with TBI only without DA treatment were included as TBI anemic control (*Anemic*). Normal mice bearing RIF-1 tumors were also included as a normal nonanemic control (*Normal*). There were two mice (four femurs) per group. Columns, mean no. cells per femur or colonies per femur; bars, ± SD.

The weight of all animals gradually recovered, and there were no statistically significant differences between the weights of any of the study groups at any of the time points studied. Skin reactions at the site of local tumor irradiation were similar for irradiated mice treated with or without DA.

**Effect of DA on Bone Marrow Progenitor Cells from Anemic Mice.** As described above in the RIF-1 tumor study, mice were irradiated with 500 cGy TBI and implanted with RIF-1 tumor cells. DA was given every 2 weeks. Normal nonanemic mice with implanted RIF-1 tumors, which did not receive TBI or DA, were included as a normal control. Femoral bone marrow cells were collected from all groups 14 days after TBI/tumor implantation.

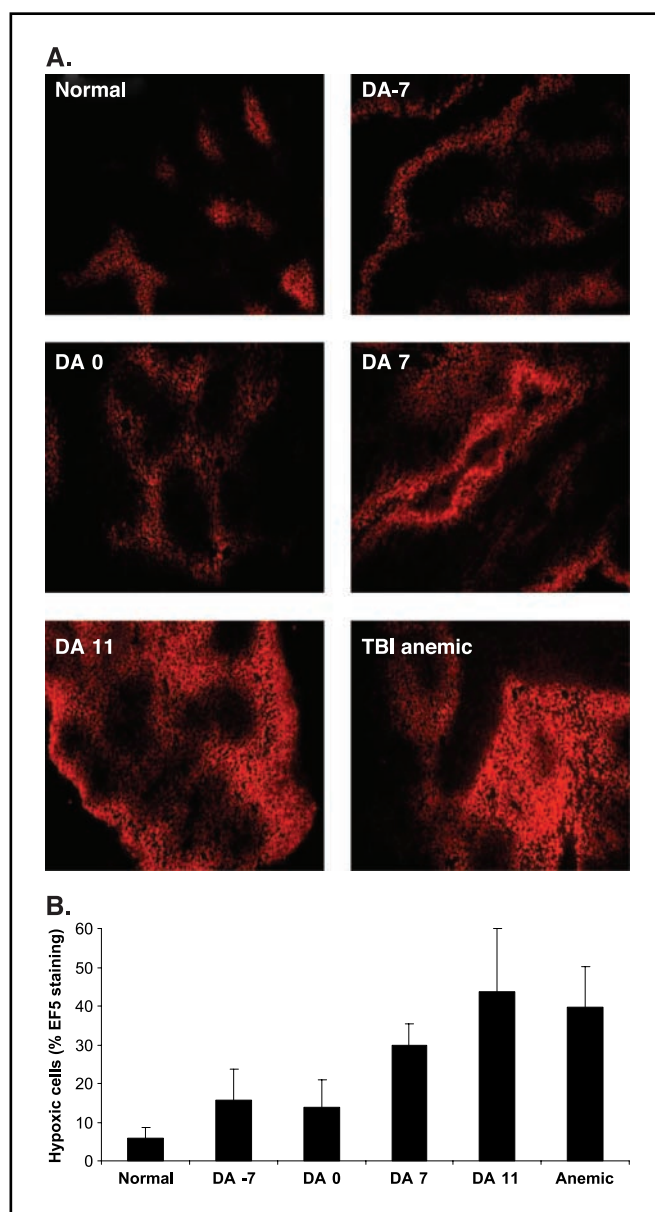
Bone marrow cellularity data are shown in Fig. 4A. The cellularity in the marrow from normal nonanemic mice (normal) and TBI anemic control mice without DA treatment (anemic) was  $1.21 \pm 0.25 \times 10^7/\text{femur}$  and  $0.69 \pm 0.18 \times 10^7/\text{femur}$  ( $P < 0.05$ ), respectively. Mice with TBI-induced anemia that received DA treatment starting on days  $-7$  and  $0$  had a bone marrow cellularity of  $1.33 \pm 0.14 \times 10^7$  and  $1.09 \pm 0.33 \times 10^7/\text{femur}$ , respectively. That was very similar ( $P > 0.05$ ) to that from normal nonanemic mice. In mice that received DA treatment starting on days  $7$  and  $14$ , the bone marrow cellularity was  $0.42 \pm 0.11 \times 10^7$  and  $0.5 \pm 0.16 \times 10^7/\text{femur}$  ( $P > 0.05$  compared with anemic control), respectively. Therefore, bone marrow cellularity was normal when DA treatment was given before (DA-7) or on (DA0) TBI day. This could either be because DA prevented the radiation-induced death of cells or stimulated the recovery of bone marrow cells.

Data from the BFU-E assays are presented in Fig. 4B. Normal nonanemic mice had  $305 \pm 233$  BFU-E per femur. TBI anemic control mice had  $192 \pm 110$  BFU-E per femur. BFU-E in anemic mice treated with DA on days  $7$  and  $0$  was  $390$  to  $440$  colonies/femur, which was  $2$ - to  $2.5$ -fold greater than TBI anemic controls. Mice that received DA on day  $7$  had similar BFU-E growth ( $141 \pm 58/\text{femur}$ ) to TBI anemic mice. Mice that received DA on the same day of harvest (day  $14$ ) had fewer BFU-E ( $40/\text{femur}$ ) than TBI anemic mice.

Data from GM-CFC assays are presented in Fig. 4C. Normal nonanemic mice produced the greatest number of GM-CFC ( $22,250 \pm 4,300/\text{femur}$ ). Mice that were irradiated with TBI and treated with DA beginning on days  $-7$ ,  $0$ , and  $7$  had numbers of GM-CFC ( $\sim 5,500$ - $2,000$  colonies/femur) that were very similar to TBI anemic controls ( $7,100 \pm 7,350/\text{femur}$ ). The group (DA14) that received DA on the same day of harvest had fewer GM-CFC ( $1,150 \pm 350/\text{femur}$ ) than TBI anemic control mice that did not receive DA. However, there were no statistically significant differences in the number of GM-CFC colonies among all the groups treated with DA and TBI anemic control without DA ( $P > 0.05$ ).

Taken together, these data suggest that the protective and/or stimulatory effect of DA is specific and is evident in terms of bone marrow cellularity and BFU-E but not for GM-CFC.

**Assessment of Tumor Hypoxia.** Tumors were harvested from additional mice from all groups in the SCC VII tumor model with biweekly DA after EF5 injection on day  $11$ . Normal mice implanted with tumors not treated with TBI and DA were included as a normal control for nonanemic host mice. As can be seen in Fig. 5A, tumors from normal nonanemic mice (normal) had the lowest level of EF5 staining, and tumors from TBI-induced anemic mice with no DA treatment (TBI anemic) had the highest EF5 staining (i.e., the lowest level of oxygenation). Early



**Figure 5.** Effect of DA on tumor hypoxia of SCC VII tumors in anemic mice. Mice were treated as described in Materials and Methods. Tumor samples were collected 11 days after TBI/tumor implantation and stained with an anti-EF5 antibody ELK3-51 conjugated with Cy3. A, digital photomicrographs were taken at  $100\times$  magnification ( $10 \times 10$ ) with a Nikon fluorescence microscope. B, Cy3 red signal was analyzed with Adobe Photoshop program. Columns, mean of four sections per tumor; bars,  $\pm$  SD.

administration of DA markedly decreased the percentage of hypoxic cells in the tumor, with treatment either 7 days before TBI (DA-7) or on the day of TBI (DA0) being most effective for the minimization of tumor hypoxia, although these tumors still had a higher level of EF5 staining than normal control tumors. Tumors from mice treated with DA on days  $7$  and  $11$  had EF5 staining similar to that of TBI anemic control. Fig. 5B shows quantification of EF5 staining. EF5 stained  $5.9 \pm 2.8\%$  of cells from tumors of normal nonanemic mice and  $39.9 \pm 4.1\%$  of cells from tumors of TBI-induced anemic mice (anemic). Tumors treated with DA on days  $-7$ ,  $0$ ,  $7$ , and  $11$  had  $15.6 \pm 8.1\%$ ,  $14.0 \pm 8.1\%$ ,  $29.8 \pm 5.5\%$ , and  $43.7 \pm 16.2\%$  of the EF5 staining,

respectively. Tumors from the weekly dosing experiment with DA in the SCC VII model and the biweekly dosing experiment in the RIF-1 model were also stained with EF5 and showed similar results to those described above for biweekly dosing in the SCC VII model (data not shown).

#### Expression of Erythropoietin Receptors on Tumor Cells.

The level of expression of the EPO-R on the tumor cells was measured as described in Materials and Methods. Both SCC VII and RIF-1 tumor cells had a low level of EPO-R mRNA ( $24 \pm 11$  and  $92 \pm 7$  copies, respectively) compared with the EPO-R negative 32D-clean cells ( $8367 \pm 467$ ), whereas an erythropoietin-responsive cell line 32D + mEpoR ( $60716 \pm 1949$  copies) and mouse bone marrow ( $17661 \pm 191$  copies) had a high level of EPO-R mRNA expression.

## Discussion

The results presented here show that DA can effectively correct TBI-induced anemia in tumor-bearing mice and increase the relative radiosensitivity of established SCC VII and RIF-1 tumors to fractionated radiotherapy. Surprisingly, the results also show radiosensitization in the absence of the correction of anemia, with no significant difference between the magnitude of sensitization in mice first treated with DA only 2 hours prior to the first fraction of local tumor radiation therapy (DA regimens: DA11 for SCC VII and DA14 for RIF-1) as compared with DA administration 18, 11, or 4 days prior to initiation of fractionated radiation therapy. This is an entirely novel finding and was observed in both the SCC VII and RIF-1 tumor models. Furthermore, there was no significant difference in efficacy between biweekly and weekly dosing with DA. DA protected the hemoglobin, RBC, and bone marrow cells from the effects of TBI and stimulated the recovery in a time-dependent manner, with the administration of DA prior to TBI being most effective. Early administration of DA (DA-7 and DA0) also prevented the TBI-induced body weight loss and facilitated the recovery of body weight loss secondary to the radiotherapy. Studies with the hypoxia marker EF5 showed that early administration of DA in anemic mice improved tumor oxygenation and reduced tumor hypoxia in both SCC VII and RIF-1 tumors.

Whereas radiosensitization secondary to the correction of preexisting anemia was expected and has been reported by other groups using erythropoietin (14–16), the observations of radiosensitization in the presence of uncorrected anemia and unaltered hypoxia in the DA treated anemic mice are novel and provocative. These results cannot be explained by the observed effects on tumor oxygenation or bone marrow cells because these effects required DA administration days prior to local tumor irradiation. As reported, erythropoietin possesses pleiotropic biological activities through interaction with different receptors in addition to its established erythropoietic effects (reviewed in refs. 44–46). Although many tumor cells express EPO-R, the fact that both SCC VII and RIF-1 tumor cells do not express EPO-R makes it unlikely that DA has an EPO-R-mediated cytotoxic effect on these tumor cells. Instead, it suggests that DA may be acting via other receptors to which it may bind on tumor cells to affect apoptotic- and/or DNA damage-related signal transduction pathway, or via effects on T-cell-mediated antitumor immune responses (47), or via effects on the tumor microenvironment or other tissues (e.g., endothelial and other pluripotent cells; refs. 42, 43) that mediate this effect on tumor cells. Therefore, both

direct and indirect biological effects of DA may play an important role in the radiosensitization effect of tumor cells. It is also possible that DA may have multiple effects that may depend in part on the presence or absence of preexisting anemia. Clearly, elucidation of the mechanism of action of DA-mediated tumor radiosensitization is critical and is an active area of investigation.

Although our data suggest that the radiosensitization effect of DA is independent of correction of anemia, one cannot entirely rule out a contribution of oxygenation to enhanced radiosensitivity. For example, the rate of correction of anemia may influence tumor response to irradiation. In a pilot study with head and neck cancer patients undergoing radiation therapy and treatment with rHuEPO, locoregional tumor control was improved in patients achieving a rapid increase in hemoglobin (23). This may be relevant in some situation because DA produces a faster increase in hemoglobin and a higher stable plateau level of hematocrit than rHuEPO (34, 35). Perhaps DA will be more efficacious than rHuEPO as a radiosensitizer in anemic patients. Another important consideration for the potential use of DA in the setting of radiation therapy is whether or not the patients have had an acute change in hemoglobin (e.g., treatment related) or have had chronic relatively stable anemia. This is important because acute changes in hemoglobin have been reported to alter intratumoral hypoxia and reduce radiosensitivity (2, 48), whereas adaptive hematopoietic responses over time in the presence of chronic anemia have less clear effects and could even potentially mitigate the effects of chronic anemia on the tumor microenvironment (2, 49). Historically, data from numerous studies have suggested the presence of a direct relationship between anemia and hypoxia, with correction of anemia increasing the radiosensitivity of tumors (14–17, 50).

Studies of the use of DA in combination with radiation therapy are in the early stages and much remains to be learned. The results presented here are provocative, but further study is warranted to elucidate the mechanism of action of DA on tumors and normal tissues under anemic and nonanemic conditions and to understand differences in the biological activity of DA as compared with erythropoietin. Nevertheless, the magnitude of DA mediated radiosensitization is significant and this effect is reproducible in different tumor models. Given the lack of correlation between the correction of anemia and radiosensitization, anemia alone would not be an indication for use of DA in this setting for purposes of enhancing the efficacy of radiation therapy. Rather, the data presented here and our preliminary data showing radiosensitization of tumors by DA in nonanemic mice suggest that DA may have potential utility as a radiosensitizer in both the presence and absence of anemia, via mechanisms independent of anemia/hypoxia, that have yet to be elucidated.

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