

## Radiation and a Metalloporphyrin Radioprotectant in a Mouse Prostate Tumor Model

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**Abstract.** *Background: Antioxidants have the potential to protect normal tissues against radiation-induced damage, but must not protect tumor cells during radiotherapy. The major objectives were to determine whether a metalloporphyrin antioxidant affects prostate tumor response to radiation and identify possible mechanisms of interaction. Materials and Methods: C57BL/6 mice with RM-9 tumor were treated with manganese (III) meso-tetrakis(1,3-diethylimidazolium-2-yl)porphyrin (MnTDE-2-ImP) and 10 gray (Gy) radiation. Tumor volume was quantified and a subset/group was evaluated for hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), bone marrow-derived cell populations and cytokines. Results: The addition of MnTDE-2-ImP transiently increased tumor response compared to radiation alone. The group receiving drug plus radiation had reduced intratumoral HIF-1 $\alpha$  and decreased capacity to secrete TNF- $\alpha$ , whereas production of IL-4 was increased. There were no toxicities associated with combination treatment. Conclusion: The results demonstrate that MnTDE-2-ImP did not protect the RM-9 prostate tumor against radiation; instead, radiation effectiveness was modestly increased. Possible mechanisms include reduction of radiation-induced HIF-1 $\alpha$  and an altered cytokine profile.*

Although the cure rate for many tumors would be increased by radiation dose escalation, balancing the potential for cure against the risk for normal tissue injury is a complex endeavor (1). With even the best treatment protocols, some patients develop serious late radiation effects. Local control

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of prostate cancer is significantly improved when the radiation dose is increased above 72 Gray (Gy) (2). However, data suggest that severe complication rates double when conventional techniques are used to treat at doses >70 Gy (3) and patients with >25% of the rectum irradiated to 70 Gy have increased risk for grade 2 or higher toxicities (4). Serious consequences include proctitis, rectal bleeding, increased urinary frequency, urethral stricture and gastrointestinal complications.

Ionizing radiation induces formation of reactive oxygen species (ROS; e.g. O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, -OH) and other free radicals. The resulting DNA damage, lipid peroxidation, protein modification and reduced blood flow can all contribute to cell death. Although the underlying mechanisms responsible for late radiation effects remain unclear, studies implicate inflammatory cells called to the site of damage, ROS produced by the cells and cytokines that facilitate tissue repair (5, 6). It has been proposed that a persistent cytokine cascade determines the events leading to late radiation effects in normal tissues (7). Radiation-induced hypoxia may also perpetuate normal tissue toxicity by triggering continuous production of inflammatory and fibrogenic cytokines (8).

Because of normal tissue toxicity during radiotherapy and pathological conditions that include overproduction of unstable oxygen species (e.g. inflammatory disorders, pulmonary disease, cardiovascular disease), efforts are ongoing to develop new radioprotective drugs. Application of superoxide dismutases (SODs), key enzymes in defense against oxidative stress, has been limited due to short half-lives, lack of cellular uptake and hypersensitivity (6, 9, 10). The amino thiols, such as amifostine, provide some degree of radioprotection, but can lead to hypotension, nausea, vomiting and allergic reactions (11). Metal-containing SOD mimetics have now emerged as being especially promising (12). Of these, the metalloporphyrins possess at least four antioxidant properties, including scavenging of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>,

ONOO<sup>-</sup> and lipid peroxides (13). Thus, these drugs provide non-selective, broad-spectrum antioxidant activity.

MnTDE-2-ImP [manganese (III) meso-tetrakis(1,3-diethylimidazolium-2-yl)porphyrin] is a novel compound that attenuates expression of inflammatory genes (14), provides neuroprotection in brain ischemia (15) and reduces smoke-induced lung inflammation (16). Other studies have demonstrated that metalloporphyrin antioxidants can protect against radiation-associated destruction of monocyte/macrophage lineage cells (17), decrease severity of lung injury (18) and reduce plasma levels of cytokines associated with late radiation effects (19). In whole-body irradiated mice, substantial protection against oxidative damage and increased survival have been reported when MnTMPyP [manganese (III) tetrakis(*N*-methyl-2-pyridylporphyrin)] was administered before exposure (20). These results indicate that metalloporphyrin antioxidants may be useful during radiotherapy.

However, in order for a normal tissue radioprotectant to be useful during therapy, it must not protect the tumor. Moeller and colleagues demonstrated that reoxygenation after irradiation leads to increased free radicals that stabilize hypoxia-inducible factor-1 (HIF-1) and prevent its degradation (21). Administration of Mn(III) tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP<sup>5+</sup>) after radiation reduced free radical levels, induced regression of tumor vasculature and delayed tumor growth compared to either drug or radiation alone. The vascular regression was associated with a reduction in vascular endothelial growth factor (VEGF). Furthermore, this compound did not protect the tumor cells *in vitro* (22). These results suggest that Mn porphyrin compounds may increase tumor radioresponsiveness and also protect normal tissue. However, recent studies have shown that the timing of HIF-1 inhibition relative to when radiotherapy is administered may be crucial (23).

The major goal of the present study was to evaluate the effects of MnTDE-2-ImP in combination with radiation on tumor growth using the RM-9 mouse prostate tumor model. The RM-9 cells were originally developed with the specific intent to mimic as closely as possible the phenotypic and genetic characteristics of human prostate cancer (24). Body mass and animal behavior were closely monitored, since combination therapies can sometimes result in unexpected side-effects. Analyses of HIF-1 $\alpha$ , bone marrow-derived cell populations (leukocytes, erythrocytes, thrombocytes) and five different cytokines were performed to identify possible interactive mechanisms of the two treatment modalities.

## Materials and Methods

**Animals.** Male C57BL/6 mice (n=87) were purchased from Charles River Breeding Laboratories Inc. (Hollister, CA, USA) at 8-9 weeks of age and acclimatized for ~1 week. They were group-

housed and maintained in a room controlled for temperature, humidity, and a 12:12 h light:dark cycle. Food and water were provided *ad libitum*. The mice were routinely weighed and observed for signs of toxicity. Rapid CO<sub>2</sub> euthanasia was performed in compliance with the latest recommendations of the NIH Guide for the Care and Use of Laboratory Animals. The study was approved by the Loma Linda University Institutional Animal Care and Use Committee.

**Experimental design.** At study initiation, the animals were assigned to 5 groups: 1) No tumor control; 2) No treatment; 3) MnTDE-2-ImP; 4) Radiation; and 5) MnTDE-2-ImP + Radiation. The mice in all tumor-bearing groups (2 – 5) were further split into two subgroups: a) for euthanasia and *in vitro* analyses on day 12 after tumor cell implantation, together with the No Tumor group (n=45) and b) for tumor growth follow-up (n=42). Mice in the latter subset were euthanized individually when tumor volume reached the maximum allowed or if a 20% loss in body mass occurred. There were no significant differences between two replicate experiments, and hence the results were appropriately pooled.

**Tumor cell line, induction and volume.** RM-9 is a prostate cancer cell line originally derived from a *ras* + *myc* transformed/wild-type *p53* primary prostate tumor in C57BL/6 mice (24). This cell line has pathological and phenotypic characteristics similar to human prostate cancer. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD, USA) supplemented with 10% bovine calf serum (BCS; Hyclone Laboratories, Logan, UT, USA), 10 mM HEPES buffer, penicillin (100 IU/ml) and streptomycin (10 mg/ml) at 37°C in 5% CO<sub>2</sub>. For *in vivo* implantation, cells were harvested with 0.025% trypsin, washed, counted using trypan blue exclusion and adjusted to 5x10<sup>6</sup> cells/ml with phosphate-buffered saline (PBS). The cells were injected subcutaneously (*s.c.*) in the right hind flank (day 0). Tumor volume was calculated: (H x W x L)/2, where H=height, W=width, and L=length of the tumor. Animals followed for tumor growth were euthanized when the tumor volume reached ~2,000 mm<sup>3</sup>.

**MnTDE-2-ImP treatment.** The MnTDE-2-ImP was administered intraperitoneally (*i.p.*) at 6 mg/kg body mass to the appropriate groups beginning 1 day after tumor cell implantation and continued daily for a total of either 12 or 16 days. The dose and administration protocol were based on results from toxicology and normal tissue response studies, as reviewed by Crapo and colleagues (25). Animals that did not receive MnTDE-2-ImP were injected with PBS.

**Tumor irradiation.** Radiation was delivered on day 8 after tumor cell injection (tumor volume = ~650 mm<sup>3</sup>) using a <sup>60</sup>Co source and an Eldorado Model 'G'  $\gamma$ -irradiation machine (Atomic Energy of Canada Ltd, Commercial Products Division, Ottawa, Canada). A maximum of 8 tumors were irradiated simultaneously using a vertical beam projected to a 20 cm x 20 cm field size. Immediately prior to irradiation, the mice (including sham-irradiated animals) were anesthetized by *i.p.* injection of ketamine (80 mg/kg) and xylazine (5.2 mg/kg) in 0.1-0.2 ml/mouse. A 0.5 cm bolus ("superflab") was laid on top of each tumor to achieve electronic equilibrium. Tumors received a single fraction of 10 Gy at a dose rate of ~0.8 Gy/min; metal alloy blocks shielded the body.

**Quantification of HIF-1 $\alpha$ .** Tumors were excised and snap-frozen in liquid nitrogen on day 12. One-cubic-millimeter tissue pieces were isolated per specimen, pulverized and subjected to nuclear extraction (Nuclear Extract Kit, Active Motif, Inc., Carlsbad, CA, USA). The total protein content of each nuclear extract was quantified using a Bradford-based assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Ten micrograms of total protein were loaded in triplicate for each sample into wells of a HIF-1 ELISA kit (Active Motif); the results of the triplicate assays were combined into a single value/specimen for statistical purposes. Data are presented as light absorbance (O.D.) recorded after the final colorimetric reaction.

**Relative spleen mass.** Mice were weighed at the time of euthanasia (day 12) and spleens were excised and weighed. Spleen mass relative to body mass (RSM) was calculated as follows: RSM=organ mass (mg)/body mass (g).

**Hematological and spleen leukocyte analyses.** Whole blood was collected by cardiac puncture in syringes containing K<sub>2</sub>-EDTA; plasma was obtained by centrifugation at 4,000 xg for 5 min. An ABC Vet Hematology Analyzer (HESKA, Waukesha, WI) was used to quantify white blood cells (WBC), lymphocytes, monocytes, granulocytes, red blood cells (RBC), and platelets (PLT). The analyzer also provided hemoglobin (HGB) concentration, hematocrit (HCT, percentage of whole blood consisting of RBCs), mean corpuscular volume (MCV, mean volume per RBC), mean corpuscular hemoglobin (MCH, mean mass of HGB per RBC), mean corpuscular HGB concentration (MCHC, concentration of HGB per RBC), RBC distribution width (RDW, width of the RBC histogram produced by cell number x cell size), and the mean platelet volume (MPV, size of average platelet). Spleens were collected, weighed, prepared into single-celled suspensions, and erythrocytes were lysed (26). All spleens were uniformly diluted 1:5 for leukocyte analyses.

**Analysis of lymphocyte populations.** Flow cytometry was used to identify specific lymphocyte phenotypes in the blood and spleen. Fluorescence-labelled monoclonal antibodies (Pharmingen, San Diego, CA, USA), were directed against CD4<sup>+</sup> (T helper, Th), CD8<sup>+</sup> (T cytotoxic, Tc), CD19<sup>+</sup> (B), and NK1.1<sup>+</sup> natural killer (NK) cells. This immunophenotyping was carried out on 10,000 acquired events using a standard direct-staining technique and a four-color FACSCalibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA, USA). Analysis was performed using CellQuest Software version 3.1 (Becton Dickinson).

**Cell activation and cytokine quantification.** Spleen leukocytes were suspended in complete RPMI 1640 medium at 2x10<sup>6</sup> cells/ml and 200  $\mu$ l aliquots were dispensed into wells of 96-well microtiter plates with immobilized monoclonal antibodies against mouse CD3 (BioCoat™ anti-CD3 T Cell Activation Plates, BD Pharmingen, San Diego, CA). Supernatants were collected after 48 h of incubation at 37°C and interleukin-2 (IL-2), IL-4, IL-5, interferon- $\gamma$  (IFN- $\gamma$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were quantified using the Cytometric Bead Array Assay (CBA Assay, Becton Dickinson). Amplified fluorescence was detected with a flow cytometer (FACSCalibur™) to measure soluble analytes. Cytokine concentrations in test samples were interpolated from the appropriate standard curve.

**Statistical analysis.** Results were subjected to statistical analyses consisting of one-way analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) pairwise multiple comparison test. In addition, two-way ANOVA was used to analyze tumor volume, with group and time being the independent variables. Statistical significance was assumed when  $p$  was  $<0.05$ ;  $p < 0.1$  indicated a trend.

## Results

**Body mass and relative spleen mass.** There were no significant differences in body mass during most of the study. However, on day 12 after RM-9 cell injection, animals in all tumor-bearing groups weighed less than controls with no tumor ( $p < 0.001$ ). Mean values in grams were: 25.8 $\pm$ 0.3 (No tumor), 21.3 $\pm$ 0.3 (Tumor + No treatment), 22.2 $\pm$ 0.2 (Tumor + MnTDE-2-ImP), 21.1 $\pm$ 0.3 (Tumor + Radiation), and 21.0 $\pm$ 0.3 (Tumor + MnTDE-2-ImP + Radiation). Spleen mass in relation to body mass (RSM) was 28-40% greater for the tumor-bearing groups compared to normal control mice, but statistical analysis showed only a trend. The means were 31.1 $\pm$ 1.4 (No tumor), 42.0 $\pm$ 2.6 (Tumor + No treatment), 43.6 $\pm$ 3.4 (Tumor + MnTDE-2-ImP), 43.3 $\pm$ 4.6 (Tumor + Radiation) and 39.8 $\pm$ 2.1 (MnTDE-2-ImP + Radiation).

**Tumor volume.** Figure 1 shows tumor volume with time after RM-9 cell injection. Mice in the Radiation and MnTDE-2-ImP + Radiation groups had the slowest tumor progression and differed significantly from the groups receiving either no treatment or MnTDE-2-ImP alone at several time points. Post-hoc analysis indicated that the combination of drug plus radiation increased treatment efficacy at several time points compared to radiation alone. Table I presents the most pertinent  $p$ -values based on Tukey's test and results of two-way ANOVA.

**HIF-1 $\alpha$  expression in tumors.** There was a main effect of group on intratumoral HIF-1 $\alpha$  (Figure 2). Tumor irradiation alone resulted in higher levels of HIF-1 $\alpha$  compared to all other groups. Most importantly, addition of MnTDE-2-ImP to radiation significantly decreased HIF-1 $\alpha$  expression compared to radiation alone; values obtained after combination treatment were equivalent to those for non-treated tumors.

**Hematological analysis.** Figure 3 shows the numbers of WBC and the major leukocyte populations in the blood. With the exception of the group receiving only radiation, leukocytosis was present in all groups with tumor compared to the non-tumor bearing mice. The high WBC count in animals receiving no treatment was further increased by administration of MnTDE-2-ImP. Lymphocyte counts were reduced by radiation, regardless of drug, whereas monocytes were highest in the group receiving MnTDE-2-ImP. All

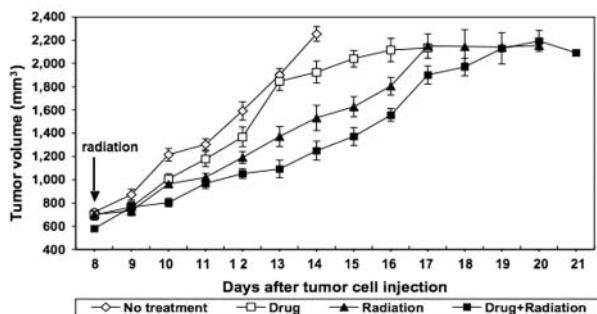


Figure 1. Tumor volume with time after RM-9 tumor cell implantation. Daily injections of MnTDE-2-ImP were initiated on day 1. At 12 days, ~1/2 of each tumor-bearing group was euthanized for in vitro analyses; the remaining mice/group were followed for tumor growth. Each point represents the mean ± SEM. Two-way ANOVA for days 8 to 14:  $p < 0.001$  for main effect of group and time;  $p < 0.001$  for group x time interaction. The most pertinent  $p$  values obtained with post-hoc Tukey's test are presented in Table I.

Table I. Selected  $p$ -values for tumor volume with time (days) after RM-9 tumor cell injection.

Days	Drug + Radiation versus:			No treatment versus:	
	No treatment	Drug	Radiation	Drug	Radiation
8	<0.05	-	<0.05	-	-
9	-	-	-	-	-
10	<0.001	<0.005	<0.05	<0.01	<0.001
11	<0.001	<0.05	-	-	<0.005
12	<0.001	<0.005	-	-	<0.001
13	<0.001	<0.001	<0.05	-	<0.001
14	<0.001	<0.001	-	-	<0.001
15	NA	<0.001	(0.066)	NA	NA
16	NA	<0.005	<0.05	NA	NA

Treatment with MnTDE-2-ImP was initiated 1 day after tumor cell injection; radiation (10 Gy) was delivered on day 8.  $P$ -values were obtained using Tukey's test. NA: not applicable. Two-way ANOVA:  $p < 0.001$  for main effect of time and for group x time interaction.

mice with tumor had some degree of granulocytosis, although statistical significance was not always obtained. The most striking increase occurred in animals treated only with MnTDE-2-ImP.

The RBC counts, hemoglobin, and hematocrit were similar among groups. The range of means was: RBCs ( $10^3$  cells/ $mm^3$ ),  $8.5 \pm 0.3$  (No tumor) to  $9.2 \pm 0.3$  (Tumor + No treatment); hemoglobin (g/dl),  $13.5 \pm 0.5$  (No tumor) to  $15.3 \pm 0.6$  (Tumor + No treatment); and hematocrit (%),  $40.8 \pm 1.0$  (Tumor + MnTDE-2-ImP + Radiation) to  $43.6 \pm 1.6$  (Tumor + No treatment). There were also no significant differences in MCH and RDW (data not shown). However, the volume per RBC (MCV; fl) was significantly decreased in the untreated tumor-bearing ( $47.4 \pm 0.40$ ), MnTDE-2-ImP ( $47.3 \pm 0.4$ ), and MnTDE-2-ImP + Radiation ( $46.8 \pm 0.4$ ) groups when compared to mice with no tumor ( $49.0 \pm 0.2$ ) ( $p < 0.05$ ). In addition, although the MCHC (g/dl) was higher in all tumor-bearing mice in relation to animals with no tumor ( $32.2 \pm 0.1$ ), significant elevation occurred only in the Tumor + MnTDE-2-ImP + Radiation group ( $36.9 \pm 0.9$ ) ( $p < 0.05$ ). Platelet counts ( $\times 10^3$  cells/ $mm^3$ ) were similar among groups, although a trend was noted for increased numbers in the Tumor + Radiation group ( $856 \pm 21$ ) compared to animals without tumor ( $723 \pm 44$ ). The volume per platelet (fl) was higher in the Tumor + No treatment ( $10.7 \pm 0.4$ ) and Tumor + MnTDE-2-ImP ( $10.8 \pm 0.4$ ) groups than in the No tumor group ( $8.7 \pm 0.2$ ) ( $p < 0.005$ ).

**WBC and major leukocyte populations in spleen.** In the spleen (Figure 4), the highest WBC counts were in the group treated only with MnTDE-2-ImP. Although all mice with tumor had high granulocyte counts, the enhancement

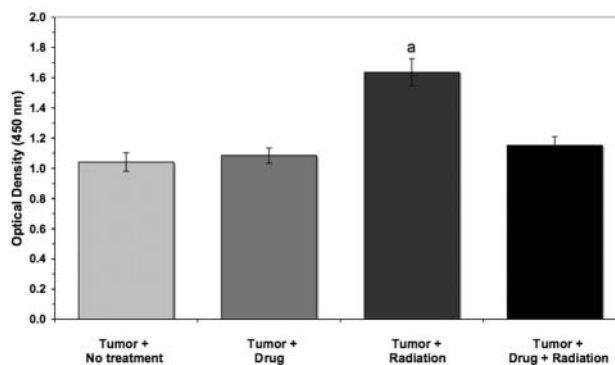


Figure 2. HIF-1 $\alpha$  expression in tumors. Each bar represents the mean ± SEM. a:  $p < 0.05$  vs. all other groups.

was most pronounced in the group receiving only MnTDE-2-ImP. Lymphocytes were reduced in the MnTDE-2-ImP + Radiation group versus all others except the one treated with radiation alone. All groups with tumor had relatively high numbers of monocyte/macrophages compared to mice without tumor; significant elevation was noted for the Tumor + No treatment, Tumor + MnTDE-2-ImP, and Tumor + Radiation groups.

**Leukocyte subpopulations in blood and spleen.** The numbers of T-, B-, and NK-cells are presented in Figure 5. In the blood, the Tumor + MnTDE-2-ImP group had the highest T-cell counts. B-cell counts were reduced in all tumor-bearing groups, especially in the two receiving radiation. All groups with tumor had relatively high NK-cell numbers, but significance was obtained only for the Tumor + No



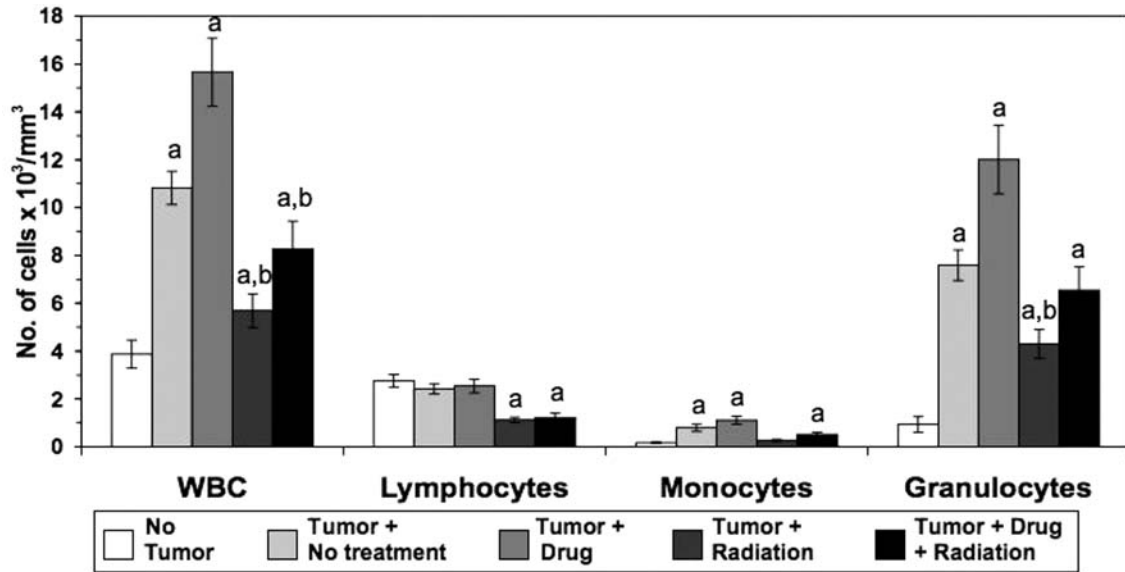


Figure 3. Number of white blood cells (WBC) and major leukocyte populations in blood. The cells were quantified using an automated hematology analyzer. Each bar represents the mean  $\pm$  SEM. a:  $p < 0.001$  vs. No tumor; b:  $p < 0.05$  vs. Tumor + No treatment.

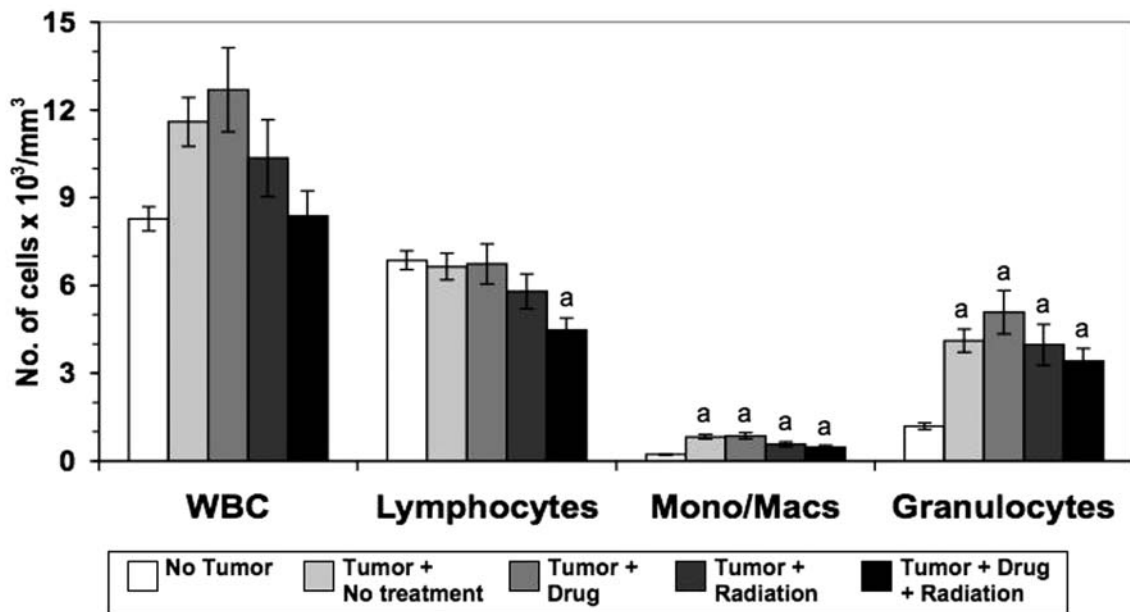


Figure 4. Number of white blood cells (WBC) and major leukocyte populations in spleen. The cells were quantified using an automated hematology analyzer after lysis of erythrocytes. Each bar represents the mean  $\pm$  SEM. a:  $p < 0.05$  vs. No tumor.

treatment and Tumor + Drug groups compared to No Tumor and Tumor + Radiation groups. In the spleen, B-cell counts were low in the Tumor + MnTDE-2-ImP + Radiation group, but there were no other significant differences. Figure 6 shows the counts obtained for the Th and Tc subsets in the blood and spleen. Th cells were similar among groups when comparisons were made within

either the blood or spleen. However, Tc cell counts were low in the blood in the two groups receiving radiation compared to the Tumor + MnTDE-2-ImP group; in the spleen, a similar but less pronounced pattern was seen.

*Cytokine production.* There were significant differences in splenocyte capacity to secrete four of the five quantified

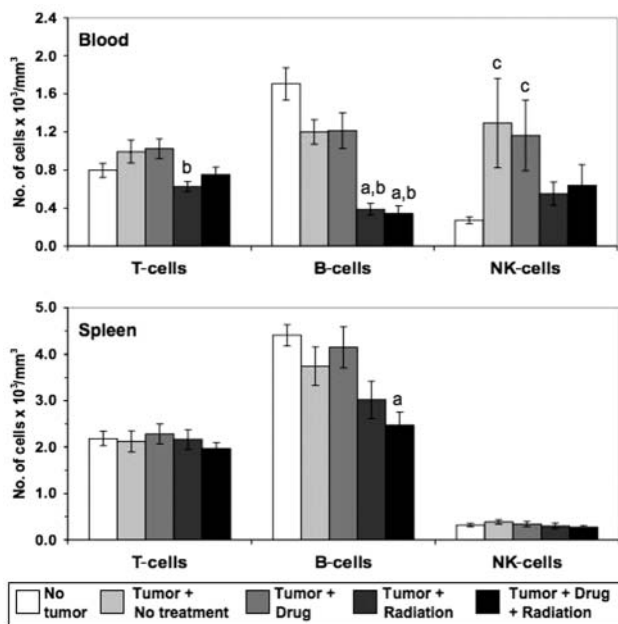


Figure 5. Major lymphocyte populations in blood and spleen. The T-, B-, and NK-cells were distinguished by the expression of CD3, CD19, and NK1.1, respectively, using fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean±SEM. a:  $p < 0.05$  vs. No Tumor; b:  $p < 0.05$  vs. Tumor + No treatment; c:  $p < 0.05$  vs. No Tumor and Tumor + Radiation.

cytokines (Figure 7). IL-2 was markedly increased in all tumor-bearing groups, with the lowest increase occurring in the tumor group treated with radiation alone. IL-4 was higher in the Tumor + MnTDE-2-ImP + Radiation group than in animals with no tumor and in those with tumor but no treatment. IFN- $\gamma$  was decreased in all tumor-bearing groups, but was not substantially influenced by the treatments. TNF- $\alpha$  was slightly decreased in the Tumor + MnTDE-2-ImP + Radiation group in relation to non-treated tumor-bearing animals. There were no significant differences in IL-5 (data not shown).

### Discussion

The data consistently show that MnTDE-2-ImP did not protect RM-9 prostate tumors against radiation effects. In fact, the results support the conclusion that addition of the drug to radiotherapy may increase the overall anti-tumor effect, albeit to a modest degree. In our previous studies, MnTDE-2-ImP had differential effects on the radiation response of RM-9 prostate tumor and normal small airway epithelial cells cultured *in vitro* (27). Based on <sup>3</sup>H-thymidine incorporation into DNA, the drug protected the normal, but not tumor, cells. Other investigators have reported that survival of mice with Lewis lung tumor was

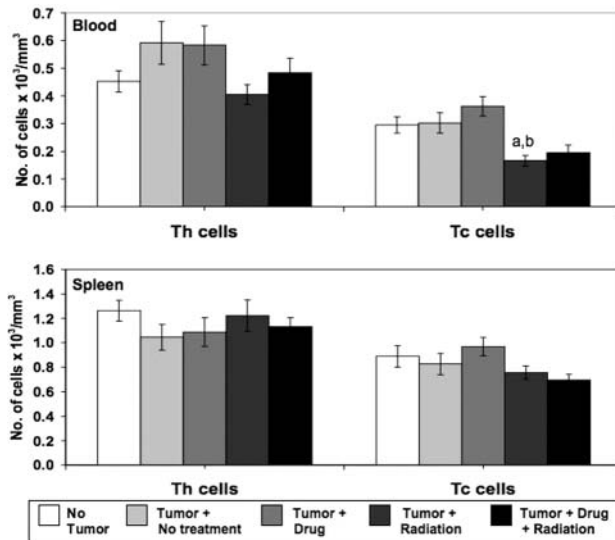


Figure 6. T helper (Th) and T cytotoxic (Tc) cells in blood and spleen. The cell subsets were distinguished by the expression of CD3/CD4 and CD3/CD8, respectively, using fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean±SEM. a:  $p < 0.05$  vs. No tumor; b:  $p < 0.05$  vs. Tumor + No treatment.

improved after treatment with an MnSOD-plasmid/liposome preparation and radiation compared to irradiated controls (28). Overexpression of MnSOD in breast carcinoma, glioma, and melanoma cell lines has resulted in loss of the neoplastic phenotype (29-31). More recently, Vujaskovic and colleagues demonstrated that MnTE-2-PyP, a drug closely related to MnTDE-2-ImP, protects against radiation-induced pulmonary injury (18) and also enhances radiation efficacy (32). Other studies have shown that mammary adenocarcinoma growth is delayed by MnTDE-2-ImP in the absence of radiation, thereby indicating that these types of drugs may independently counteract tumor progression (33).

A possible explanation for RM-9 tumor growth delay in mice receiving both MnTDE-2-ImP and radiation in the present study is that the drug down-regulated radiation-induced enhancement of intratumoral HIF-1 $\alpha$ . Solid tumors, including those of the prostate, have regions of hypoxia (34), a condition that can be exacerbated by radiation. Indeed, irradiated RM-9 tumors had significantly higher levels of HIF-1 $\alpha$  compared to all other groups. HIF-1 $\alpha$  is a transcription factor that is up-regulated when oxygen level is low, leading to expression of proteins involved in neoangiogenesis, cell survival, glucose metabolism, invasion, and metastasis (33, 35). The importance of the factor in facilitating tumor aggressiveness is emphasized by efforts to identify HIF-1 $\alpha$  inhibitors that may serve as anticancer therapeutics (36). Somewhat unexpectedly, Moeller *et al.* have shown that HIF-1 $\alpha$  activity does not occur immediately

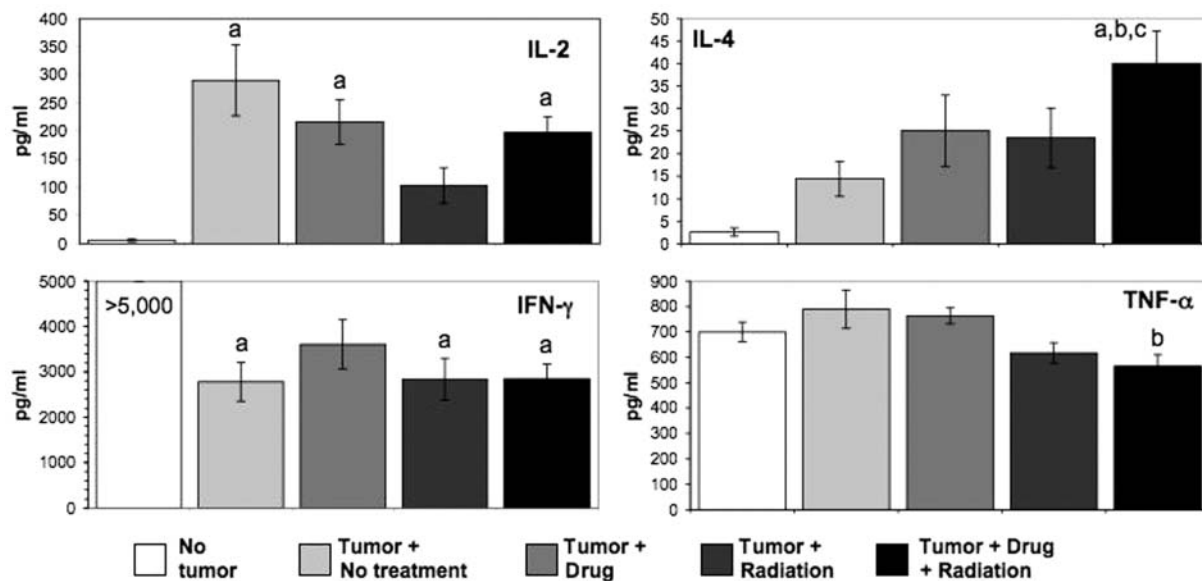


Figure 7. Concentration of cytokines secreted by activated splenocytes. Activation was accomplished using anti-CD3 antibody; the cytokines in supernatants were quantified using a cytometric bead array assay and flow cytometry. Each bar represents the mean  $\pm$  SEM. a:  $p < 0.05$  vs. No tumor; b:  $p < 0.05$  vs. Tumor + No treatment; c:  $p < 0.05$  vs. Tumor + Radiation.

in response to ROS generated at the time of irradiation. Instead, mRNAs transcribed by HIF-1 $\alpha$  are sequestered in stress granules within hypoxic cells; translation into proteins is thus prevented until ROS are produced during reoxygenation (21). However, it appears that full expression of only some genes may be prevented. Furthermore, these investigators have demonstrated that HIF-1 $\alpha$  can enhance tumor cell apoptosis, proliferation, and metabolic rate *in vitro*, processes that may increase tumor radiosensitivity (23). *In vivo*, however, the anti-angiogenesis effect of blocked HIF-1 $\alpha$  apparently overrides any tumor radiosensitizing properties that the factor may have.

Although radiation is immunosuppressive, radiation-induced damage can provide a "danger" signal, resulting in immune activation that in some cases may contribute to tumor control (37). Furthermore, studies have demonstrated that prostate cancer cells are immunogenic; several vaccines have already entered clinical trials (38). The trend toward splenomegaly observed in all RM-9 tumor-bearing groups is consistent with immune activation. Tumor presence also induced granulocytosis (*i.e.* primarily neutrophils), but the increase was especially noticeable in the group treated with MnTDE-2-ImP. Granulocytes are known to possess anti-tumor properties (39). Thus, it appears that these cells, which are part of innate immunity, may have contributed to the observed delay in RM-9 tumor growth. However, more research is obviously needed to confirm this possibility. Our analysis of specific lymphocyte populations showed that their numbers tended to reflect tumor presence and susceptibility

to radiation (B>T>NK and Tc>Th). MnTDE-2-ImP alone appeared to have no effect.

Increased tumor aggressiveness has been correlated with a wide range of cytokines derived from tumor cells and leukocytes (40, 41). Mn-SOD mimetics block HIF-1 $\alpha$ , NF- $\kappa$ B and AP-1 signaling pathways, and hence also the release of tumor survival cytokines under conditions of oxidative stress (22, 42, 43). Moeller *et al.* have reported that MnTE-2-PyP<sup>5+</sup> prevented tumor cell production of soluble factors that protect endothelial cells against radiation damage and that combination of the mimetic with radiation caused synergistic devascularization of mouse mammary tumors (33). In the present study, IL-4, a Th2 cell-derived cytokine, was elevated in the tumor-bearing group receiving MnTDE-2-ImP plus radiation. This cytokine is a potent anti-angiogenesis factor that acts both locally and systemically (44). Another striking feature was the association between low IFN- $\gamma$  secretion and tumor presence. The reduction was significant in all tumor-bearing groups except the one treated only with MnTDE-2-ImP, suggesting that the drug ameliorated the depressive effect of the tumor. IFN- $\gamma$ , a Th1 cytokine, is a potent activator of both innate and adaptive immunity (45) and has anti-angiogenic (46) and anti-fibrotic (47) properties. Its down-regulation may provide an escape mechanism for the tumor. The level of IL-2, a Th1 cytokine that functions in an autocrine/paracrine manner, was generally increased by tumor presence. Radiation alone decreased the enhancement, possibly due to apoptosis and/or induction of hyporesponsiveness (48).

## Conclusion

In summary, administration of MnTDE-2-ImP led to changes that suggest enhanced radiation efficacy in the RM-9 prostate tumor model that mimics human prostate cancer. The primary effects appear to be in down-regulation of radiation-induced HIF-1 $\alpha$  expression and up-regulation of IL-4. Both of these effects would act to inhibit tumor angiogenesis. A compound that would protect normal tissues while also facilitating radiation-induced tumor control would be an attractive adjunct to radiotherapy, especially for cancers located in highly radiosensitive parts of the body.

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