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Effect of a Metalloporphyrin Antioxidant (MnTE-2-PyP) on the Response of a Mouse Prostate Cancer Model to Radiation

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Abstract. *Background: Metalloporphyrin antioxidants can protect tissues against radiation-induced damage. However, for effective use in radiotherapy as normal tissue radioprotectants, they must not protect the cancer. The major objectives were to evaluate the effects of Mn (III) tetrakis (N-ethylpyridinium-2-yl) porphyrin (MnTE-2-PyP) on tumor response to radiation and to explore mechanisms responsible for the observed effects. Materials and Methods: C57BL/6 mice were subcutaneously (s.c.) injected with RM-9 prostate tumor cells on day 0 and grouped according to treatment with MnTE-2-PyP (s.c. 6 mg/kg/day beginning on day 1 for 16 maximum days), 10 Gray (Gy) single fraction radiation on day 7, a combination of both or neither. Subsets per group and non-tumor bearing controls were evaluated for leukocyte populations, red blood cell (RBC) and platelet characteristics and cytokines on day 12; the remaining mice were followed for tumor growth. Results: Although radiation alone significantly slowed tumor growth and the addition of MnTE-2-PyP resulted in slightly slower tumor progression, the difference between radiation and radiation plus drug was not statistically significant. However, the treatment with drug alone significantly elevated T (helper, Th and cyotoxic, Tc) and natural killer (NK) cells in the spleen, B-cells in the blood and spleen, and the capacity to produce interleukin-2. The addition of the drug to radiation did not ameliorate the depression seen in all the major leukocyte types, but did protect against radiation-induced decreases in RBC counts, hemoglobin and hematocrit. Vascular endothelial growth factor (VEGF) increased in the plasma from both the irradiated groups and a trend for increased transforming growth factor-β1 (TGF-β1) was noted with radiation alone. Conclusion: MnTE-2-PyP did not protect RM-9 prostate tumors against radiation damage and was not toxic under the conditions used. The drug-induced enhancement of certain immune parameters suggests that MnTE-2-PyP may be beneficial not only as a normal tissue radioprotectant, but also as a facilitator of antitumor immunity.*

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Radiation therapy is a highly relevant modality in the treatment of prostate cancer. However, sensitivity of the surrounding tissue to radiation inevitably leads to normal tissue damage with varying degrees of severity. An optimal radiation dose requires maximum tumor destruction with minimal damage to normal tissues. Higher doses of radiation have been reported to cause a variety of side-effects, ranging from short-term acute to long-term and chronic (1). The nature and duration of the side-effects depend on the radiosensitivity of the tissue, total radiation dose, fractionation scheme, and the overall health of the patient.

Radiation induces tissue toxicity directly by damaging DNA and indirectly *via* the production of reactive oxygen species (ROS). The elevated ROS interact with cellular components vital to various signaling cascades, apoptosis/cell cycle regulation and vascular proliferation/angiogenesis. In this context, the fate of the cells ultimately depends on the interaction of ROS with these cellular processes. Cytokines secreted by leukocytes are among the many molecules expressed in response to ROS. Since they play important roles in cellular signaling, the pattern of cytokine expression due to elevated ROS could be especially crucial in the response of cells to radiation (2). Tumor growth modulation by cytokines can have both direct and indirect effects on angiogenesis, as well as on host anti-tumor immunity mediated by T lymphocytes and natural killer (NK) cells. Transforming growth factor-beta1 (TGF-β1) and vascular endothelial growth factor (VEGF) are two major cytokines that respond to

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oxidative stress and regulate angiogenesis (3-7). In contrast, the ability to produce interleukin-2 (IL-2), a major cytokine secreted by activated T helper 1 (Th1) lymphocytes, important in facilitating immune attack against tumors, can be compromised by exposure to radiation (8).

TGF-β1 is an effective and ubiquitous modulator of cell growth and its biphasic role in carcinogenesis is significant. In early stage carcinogenesis, it acts as a tumor suppressor, inhibiting cell growth; in later stages, it functions as a tumor promoter, enhancing cell growth, invasion, and metastasis, in part by decreasing the host-tumor immune response (7, 9, 10). TGF-β1 has been shown to be a key modulator of angiogenesis by regulating endothelial cell proliferation, migration, extracellular matrix (ECM) metabolism, and the expression of adhesion molecules (5, 7). It is important to also note that TGF-β1 is a highly immunosuppressive cytokine that is secreted by the Th3 subset, as well as by many malignant cell types (11).

VEGF plays a central role in both normal and tumor angiogenesis (12). It enhances proliferation, migration and invasion of endothelial cells into tumors, resulting in new blood vessel formation and thus also increases the risk of tumor progression. Low oxygen tension causes up-regulation of VEGF. As tumors develop and become more malignant, they become more hypoxic because of the inability of the existing vasculature to supply sufficient oxygen to all parts of the tumor mass; radiation compounds these hypoxic conditions by damaging already established vessels and causing elevated ROS levels. These conditions lead to altered gene expression in favor of increased cell survival, angiogenesis and metastasis (13). Elevated levels of VEGF have been associated with increased tumor metastasis and poor prognosis for patients with cancer of the prostate (14, 15), as well as other body sites.

IL-2 has long been known to be essential for T-cell growth and differentiation and acts as a signaling molecule by binding to an IL-2 receptor complex on the surface of lymphocytes, thereby inducing effector cell functions that include the destruction of tumor cells. The administration of IL-2 resulted in eradication of advanced carcinomas in some patients, but serious toxicities and unpredictability in therapeutic effectiveness have limited its usefulness (16). Recent studies have demonstrated that IL-2 is indispensable not only for T-cell activation, but also for the maintenance of tolerance to self antigens (17).

Antioxidants are responsible for detoxifying ROS and are the central cellular defense mechanism against oxidative damage. The groups of antioxidants include superoxide dismutases (SODs), catalase (CAT) and glutathione (GPx). SODs play a critical role in the eradication of ROS, being responsible for the dismutation of superoxide, $O_2^{\bullet-}$, to H_2O_2, which can then be reduced to H_2O and O_2 by CAT. It has been shown that elevated levels of SODs have a protective effect against oxidative stress (18). The clinical use of endogenous SODs has unfortunately been limited due to their short circulating half-lives, hypersensitivity induction, large molecular weight (M_r ~30 kDa), poor cell delivery and cost of production. As a result, SOD mimetics have been developed in efforts to overcome these limitations. The basic properties required are stability and non-toxicity, in addition, they should have low molecular mass and most importantly scavenge effectively for ROS (18, 19).

Small molecular weight metalloporphyrin SODs can function as SOD mimetics. In addition to scavenging $O_2^{\bullet-}$, they also scavenge H_2O_2, lipid peroxyl radicals and reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), and the carbonate radical ($CO_3^{\bullet-}$) (20, 21). It has been reported that SOD mimetics are effective free radical scavengers in models of stroke (22), diabetes (23) and sickle cell anemia (24). They have been shown to protect normal tissues against radiation-induced damage presumably by preventing or at least minimizing oxidative stress (19). In addition, there is mounting evidence that SOD mimetics may enhance tumor radioresponsiveness (25). Our previous studies with the RM-9 mouse prostate model have also shown that the use of SOD mimetics may facilitate the anti-tumor effect of radiation (26).

Mn (III) tetrakis (*N*-ethylpyridinium-2-yl) porphyrin (MnTE-2-PyP) has been shown to protect normal tissues from radiation-induced injury (19) and has been highly effective in a wide variety of other models of oxidative injury (19, 27-29). This compound contains a Mn^{2+} at the center of a porphyrin ring with imidazole side chains (Figure 1). Alternate oxidation-reduction reactions of the Mn moiety are responsible for the dismutation of $O_2^{\bullet-}$ into oxygen and hydrogen peroxide. This mechanism of O_2 scavenging is similar to the naturally occurring SOD.

In our previous studies with MnTDE-2-ImP, a SOD mimetic structurally similar to MnTE-2-PyP, administration to RM-9 prostate tumor-bearing mice that also received radiation treatment significantly slowed tumor growth and reduced the expression of hypoxia-inducible factor-1α (HIF-1α) in comparison to animals that received radiation alone (26). HIF-1α, an oxygen-regulated transcription factor, is known to regulate the genes for the hypoxia responsive element (HRE), including those for VEGF and TGF-β1. Examining VEGF and TGF-β1 levels might give insight into mechanisms responsible for the activity of SOD mimetics in combination with radiation. Thus, the major objectives of this study were to determine if MnTE-2-PyP could be used in combination with radiation in the RM-9 mouse prostate tumor model and to further investigate possible mechanisms of interaction. The emphasis was on leukocyte populations that respond to tissue damage, secrete cytokines and have antitumor properties.

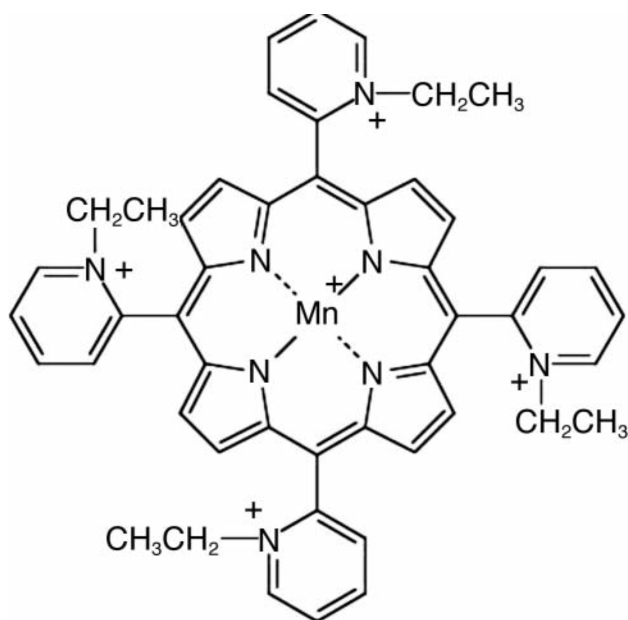

Figure 1. *Structure of MnTE-2-PyP. A porphyrin complex with a Mn+ center and four positively charged imidazole side groups.*

Materials and Methods

Animals. Eight- to 9-week-old male C57BL/6 mice (n=87) were purchased from Charles River Breeding Laboratories Inc. (Hollister, CA, USA) and acclimatized for about 1 week prior to initiation of the study. They were housed under standard temperature, humidity and 12-h light/dark cycling conditions. Food and water were provided *ad libitum*. The mice were weighed and observed routinely for signs of toxicity. Rapid CO_2 euthanasia was performed under the approved Institutional Animal Care and Use Committee (IACUC) protocol and in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Treatment groups. Animals were randomly assigned to 5 groups based on the treatment received. Four groups were tumor-bearing and assigned as follows: i) no treatment, ii) drug, iii) radiation, and iv) drug + radiation. A fifth group with no tumor was designated to serve as the baseline control for the *in vitro* assays that were also performed on a subset from each of the tumor-bearing groups. The mice used for the *in vitro* analyses, both with and without tumor (n=8-10/group), were euthanized 12 days after tumor cell implantation, all the remaining animals had tumors (n=10-12/group) and were euthanized individually when the tumor volume reached the maximum allowed (~2,000 mm^3) or at termination of the study on day 21.

Tumor cell line. RM-9 is a prostate cancer cell line that was originally derived from a ras+myc transformed/wild-type *TP53* primary prostate tumor induced in the Zipras/myc-9-infected mouse prostate reconstitution (MPR) model using C57BL/6 mice. The RM-9 cells were provided by Dr. Timothy C. Thompson at the Baylor College of Medicine in Houston, TX, USA. This cell line has been extensively evaluated for pathological and phenotypic characteristics and was selected because of its close similarity to human prostate cancer (30). The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD, USA) completed 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 a humidified atmosphere containing 5% CO_2.

Tumor induction and measurement. Prior to *in vivo* implantation, the cells were harvested with 0.025% trypsin, washed in PBS and counted using the trypan blue exclusion method. After adjustment to 5×10^6 cells/ml, 0.1ml (5×10^5) were injected subcutaneously (*s.c.*) in the right hind flank of the respective mice on designated day 0. The height (H), width (W) and length (L) of the tumors were measured using vernier calipers and the volume was calculated using a formula that approximates a hemi-ellipsoid: (H x W x L)/2.

MnTE-2-PyP treatment. MnTE-2-PyP was synthesized by Ricerca Biosciences, LLC, Concord, OH, USA, following procedures previously described (31). Drug treatment of the relevant groups commenced on day 1 after RM-9 cell implantation and was administered *s.c.* at a dose of 6 mg/kg body mass. Treatment continued daily for a total of 12-16 days. The groups that did not receive drug were injected with equivalent volumes of phosphate buffered saline (PBS) to maintain equivalent handling. The time/dose schedule for administration of the drug was based on our previous studies (26).

Tumor irradiation. Radiation was delivered on day 7, the time at which the tumor was palpable. The mice were anesthetized by intraperitoneal (*i.p.*) injection of ketamine (80 mg/kg) and xylazine (5.2 mg/kg); all the animals (including controls with no tumor) received anesthetic, regardless of irradiation, in order to equalize stress factors. Metal alloy blocks were placed to shield the rest of the body so that only the tumor and underlying leg tissue was irradiated. A 0.5 cm bolus was laid on top of each tumor to achieve electronic equilibrium. The tumors received a single fraction of 10 gray (Gy) at a dose rate of ~0.8 Gy/min using a ^{60}Co source and an Eldorado Model 'G' γ-irradiation machine (Atomic Energy of Canada Ltd, Commercial Products Division, Ottawa, Canada).

Blood and tissue collection. Whole blood was collected by cardiac puncture with syringes containing K_2-EDTA. The plasma was obtained by micro-centrifugation at 4,000 g for 5 min and stored at –80˚C. The spleens were also collected, weighed and homogenized into single cell suspensions in complete RPMI-1640 medium as previously reported (26). The spleen mass relative to body mass (RSM) was calculated: RSM = organ mass (mg)/body mass (g).

Hematological and flow cytometry analyses. An automated hematology analyzer (HESKA, Waukesha, WI, USA) was used to quantify the white blood cells (WBC), major leukocyte populations, platelets, hemoglobin (HGB) concentration and red blood cells (RBC), mean corpuscular volume (MCV, average RBC volume), mean corpuscular hemoglobin (MCH, average mass of HGB per RBC), mean corpuscular HGB concentration (MCHC, average HGB concentration per RBC), RBC distribution width (RDW, width of RBC based on cell number x cell size)and mean platelet volume (MPV, average platelet size) (32). To quantify specific lymphocyte populations, the WBC were labeled with CD45 for leukocyte identification, followed by fluorescence-labeled monoclonal

antibodies (Pharmingen, San Diego, CA, USA) against CD3, CD4, CD8, CD19 and NK1.1 markers for identification of total T, T helper (Th), T cytotoxic (Tc), B and natural killer (NK) cells, respectively. The samples were evaluated with a four-color FACSCalibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA, USA) and 5,000-10,000 events were analyzed with CellQuest Software version 3.1 (Becton Dickinson).

Spleen leukocyte analyses. An aliquot of each splenocyte suspension was run through an automated hematology analyzer, (HESKA) to quantify the total and major leukocyte populations as described previously (32). The erythrocytes in the splenocyte suspensions were then lysed and the leukocytes analyzed for T, Th, Tc, B and NK cells by flow cytometry as described above for blood lymphocytes.

TGF-β1 and VEGF quantification in plasma. The active plus latent forms of TGF-β1 and VEGF were quantified using commercial enzyme-linked immunoassay (ELISA) kits (Quantikine; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The optical density was read at 450 nm with a correction wavelength of 570 nm using a microplate reader (Infinite™ 200 series, Tecan, San Jose, CA, USA). Concentrations (pg/ml) were calculated using the appropriate standards provided by the manufacturer.

IL-2 quantification in spleen supernatants. Briefly, single-celled suspensions of splenic leukocytes at 2×10^6/ml were activated with immobilized anti-CD3 (BioCoat™ anti-CD3 T-cell activation plates; BD Pharmingen, San Diego, CA, USA). After a 48-h incubation, the supernatants were collected and IL-2 was quantified by the Luminex-based bead array method using the LINCOplex detection system (Linco Research, Inc., St. Charles, MO, USA) following the manufacturer's instructions.

Statistical analysis. The data from the *in vitro* analyses were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's HSD (honestly significant difference) pairwise multiple comparison test, if indicated. Two-way ANOVA was used to evaluate tumor progression, with group and time as the independent variables. Data are reported as mean±standard error of the mean (SEM). The Kaplan-Meier method was used to detect any association between treatment received and survival, where the end-point examined was the time interval beginning on the day of RM-9 cell implantation and ending when the tumor reached a volume of ~2,000 mm^3. A $p<0.05$ indicated significance, whereas $p<0.1$ was selected to indicate a trend.

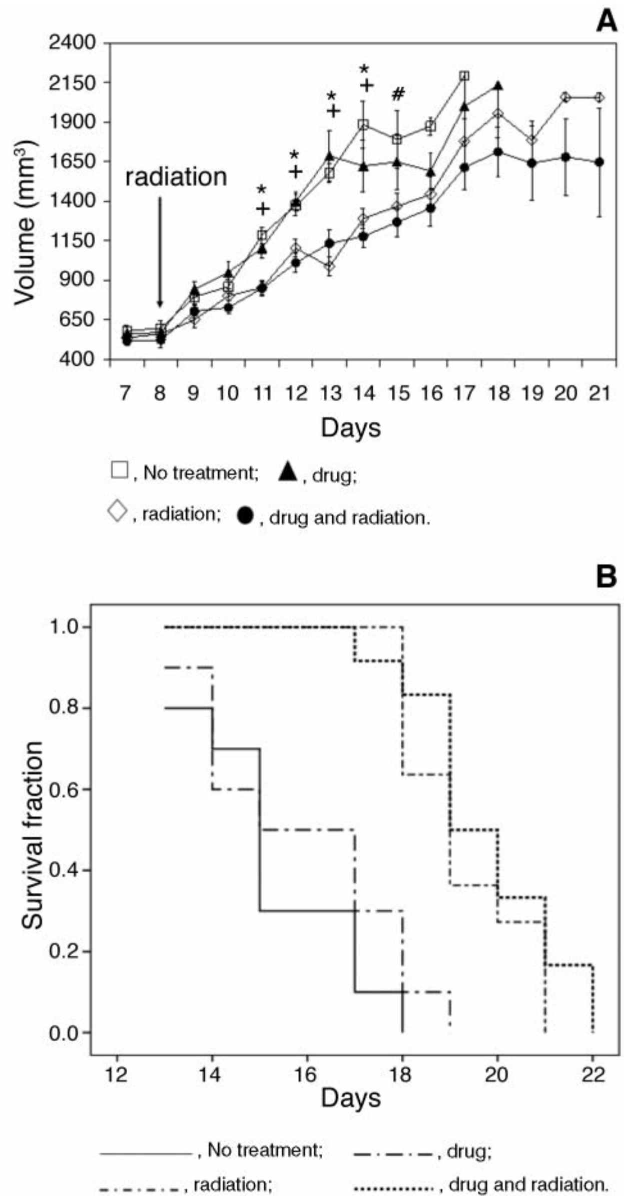

Figure 2. *Tumor volume and animal survival. The data are shown for 10-12 mice/group. Panel A: Each point represents mean±SEM. *p<0.05, Radiation vs. no treatment and drug; +p<0.05, drug + radiation vs. no treatment and drug; #p<0.05, drug + radiation vs. no treatment. Panel B: Kaplan-Meier survival curves of RM-9 prostate tumor-bearing mice.*

Results

Body mass and relative spleen mass (RSM). All the mice, either with or without tumor, survived for >12 days after inoculation with RM-9 cells. The mice in all the tumor-bearing groups weighed more than the non-tumor-bearing controls on days 7-8, but the difference was transient. At the time of euthanasia for *in vitro* analyses (day 12), body mass ranged from 23.2±0.5 g (drug + radiation) to 25.6±0.4 g (no tumor). However, at this same time-point, the groups receiving either drug alone or radiation alone had increased spleen mass relative to body mass compared to the control mice with no tumor ($p<0.05$). Mean RSM values were 34.1±2.7 (no tumor), 42.2±1.2 (no treatment), 46.0±1.2 (drug), 45.1±4.0 (radiation) and 37.6±2.5 (drug + radiation).

Tumor volume and survival analyses. As shown in Figure 2A, the mice in the radiation and drug + radiation groups had the slowest tumor progression. Tumor volumes in the drug + radiation group were not significantly different *versus* the

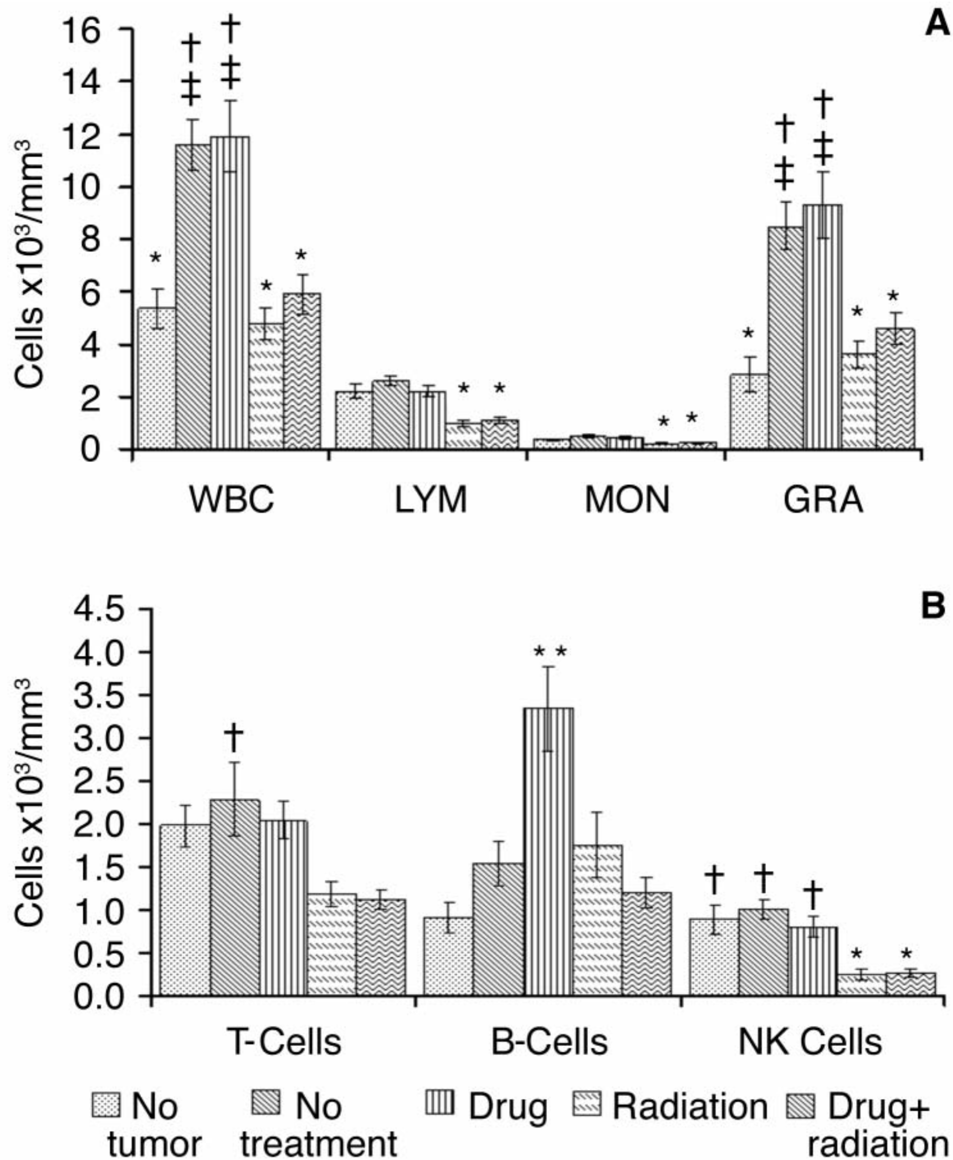

Figure 3. *WBC and major leukocyte populations in the blood. WBC and major leukocyte types obtained with an automated hematology analyzer (panel A). Specific lymphocyte populations obtained using fluorescence-labeled monoclonal antibodies and flow cytometry (panel B). Each bar represents mean±SEM for n=8-10 mice/group. ‡p<0.05 vs. No tumor; *p<0.05 vs. non-irradiated tumor-bearing groups; †p<0.05 vs. irradiated groups; **p< 0.05 vs. all other groups; WBC: white blood cells, LYM: lymphocytes, MON: monocytes, GRA: granulocytes.*

radiation group. A slightly lower tumor volume was observed in the group treated with drug alone compared to the non-treated controls. Although, there was no significant difference in survival time between the radiation and the drug + radiation groups, Figure 2B shows that the fraction of surviving mice in the drug + radiation group was higher across the entire length of the study. By day 18 there were 0% survivors in the non-treated group, whereas in the drug, radiation, and drug + radiation groups there were 10% , 58% and 85% survivors, respectively.

WBC, major leukocyte populations, and lymphocyte subpopulations in blood. Leukocytosis due to a dramatic increase in granulocytes was observed in the non-treated and drug only (tumor-bearing) groups when compared with the non-tumor bearing controls and the irradiated groups (p<0.05) (Figure 3A). On the other hand, the two groups that received radiation did not exhibit elevation in any leukocyte population. Similar patterns were observed in all three cell types (lymphocytes, monocytesand granulocytes), regardless of drug treatment.

Table I. *T lymphocyte subpopulations in blood.*

Cells and ratio	Group				
		Tumor			
	No tumor	No treatment	Drug	Radiation	Drug + radiation
$CD4^+$ Th ($10^6/mm^3$)	1.11±0.15	1.21±0.14	1.25±0.13	0.72±0.10[a]	0.60±0.09[b]
$CD8^+$ Tc ($10^6/mm^3$)	0.87±0.10	0.87±0.11	0.79±0.09	0.47±0.06[c]	0.44±0.06[d]
CD4:CD8	1.23±0.12	1.43±0.09	1.59±0.06	1.79±0.08[c]	1.70±0.08[e]

Values represent mean±SEM for n=8-10 mice/group. Data were obtained using fluorescent monoclonal antibodies and flow cytometry. [a]$p<0.05$ *vs*. No treatment and drug; [b]$p<0.05$ *vs*. no tumor, no treatment and drug; [c]$p<0.05$ *vs*. no tumor and no treatment; [d]$p<0.05$ *vs*. all non-irradiated groups; [e]$p<0.05$ *vs*. no tumor.

Figure 3B shows the effect of treatment on the lymphocyte populations in the blood. Although a decrease in T-cells was noted in both the irradiated groups, significance was observed only *versus* the non-treated controls ($p<0.05$). B-cells were elevated in the drug only group, and this increase was significant when compared to all other groups ($p<0.01$). Radiation caused a significant decrease in NK cells when compared to all other groups ($p<0.01$).

Further analysis of the T-cell subpopulations in the blood showed a significant radiation effect on both the $CD4^+$ Th and $CD8^+$ Tc cells (Table I). Radiation alone reduced the Th population in comparison to non-treated mice and those given drug alone, whereas Tc cells were lower than in the no tumor, no treatment groups ($p<0.05$). Drug plus radiation treatment significantly reduced both the Th and Tc cell counts *versus* the no tumor group; some differences were also noted compared to one or more of the tumor-bearing groups ($p<0.05$). The CD4:CD8 ratio was elevated in the group receiving only radiation compared to the no tumor and no treatment groups, whereas the mice treated with both drug and radiation had an increased ratio compared only to the no tumor group ($p<0.05$).

RBC and platelets in blood. As shown in Table II, the presence of tumor, treatment with radiation and/or drug caused relatively few perturbations in RBC characteristics. Radiation alone reduced the RBC count ($p<0.05$) and there was a trend for low hemoglobin and hematocrit compared to one or more of the other groups. RBC distribution width (RDW) in the irradiated group receiving no drug increased compared to drug alone ($p<0.05$). There were no significant differences among groups in platelet counts or volume.

Table II. *Summary of erythrocyte and platelet characteristics.*

Test	Group				
		Tumor			
	No tumor	No treatment	Drug	Radiation	Drug + radiation
RBC ($10^6/mm^3$)	8.75±0.19	9.08±0.17	8.67±0.16	8.22±0.28[a]	8.61±0.22
HGB (g/dl)	13.0±0.32	13.7±0.24	13.0±0.25	12.4±0.46[b]	12.9±0.31
HCT (%)	39.8±0.99	41.6±0.79	39.7±0.75	37.7±1.36[b]	39.3±1.03
MCH (pg)	14.9±0.07	15.1±0.07	15.0±0.13	15.1±0.07	15.0±0.10
MCHC (g/dl)	32.6±0.11	32.8±0.14	32.8±0.21	32.9±0.08	33.0±0.16
MCV (μm^3)	45.5±0.19	45.8±0.20	45.8±0.13	45.7±0.15	45.6±0.16
RDW (%)	14.9±0.15	14.9±0.21	14.5±0.14	15.4±0.19[c]	14.7±0.17
PLT ($10^5/mm^3$)	843.4±29.98	929.9±23.87	901.1±41.34	892.1±46.39	893.7±25.62
MPV (μm^3)	10.1±0.20	9.9±0.21	9.7±0.29	9.6±0.14	9.7±0.20

Values were obtained with an automated hematology analyzer and represent mean ±SEM for 8-10 mice/group. RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, RBC distribution width; PLT, platelet count; MPV, mean platelet volume. [a]$p<0.05$ *vs*. No treatment; [b]$p<0.1$ *vs*. no treatment; [c]$p<0.001$ *vs*. drug and $p<0.1$ *vs*. drug + radiation.

Table III. *T lymphocyte subpopulations in spleen.*

Cells and ratio	Groups				
		Tumor			
	No tumor	No treatment	Drug	Radiation	Drug + radiation
$CD4^+$ Th ($10^6/mm^3$)	2.75±0.51	3.92±0.26	4.97±0.45[a]	2.09±0.53[b]	1.17±0.36[b]
$CD8^+$ Tc ($10^6/mm^3$)	1.53±0.28	2.48±0.16	2.99±0.33[a]	1.09±0.28[b]	0.72±0.20[b]
CD4:CD8	1.82±0.05	1.59±0.06	1.74±0.06	2.05±0.12[c]	1.96±0.13[d]

Values represent mean±SEM for n=8-10 mice/group. Data were obtained using fluorescent monoclonal antibodies and flow cytometry. [a]$p<0.05$ *vs*. no tumor, radiation, and drug + radiation; [b]$p<0.05$ *vs*. no treatment and drug; [c]$p<0.05$ *vs*. no treatment; [d]$p<0.1$ *vs*. no treatment.

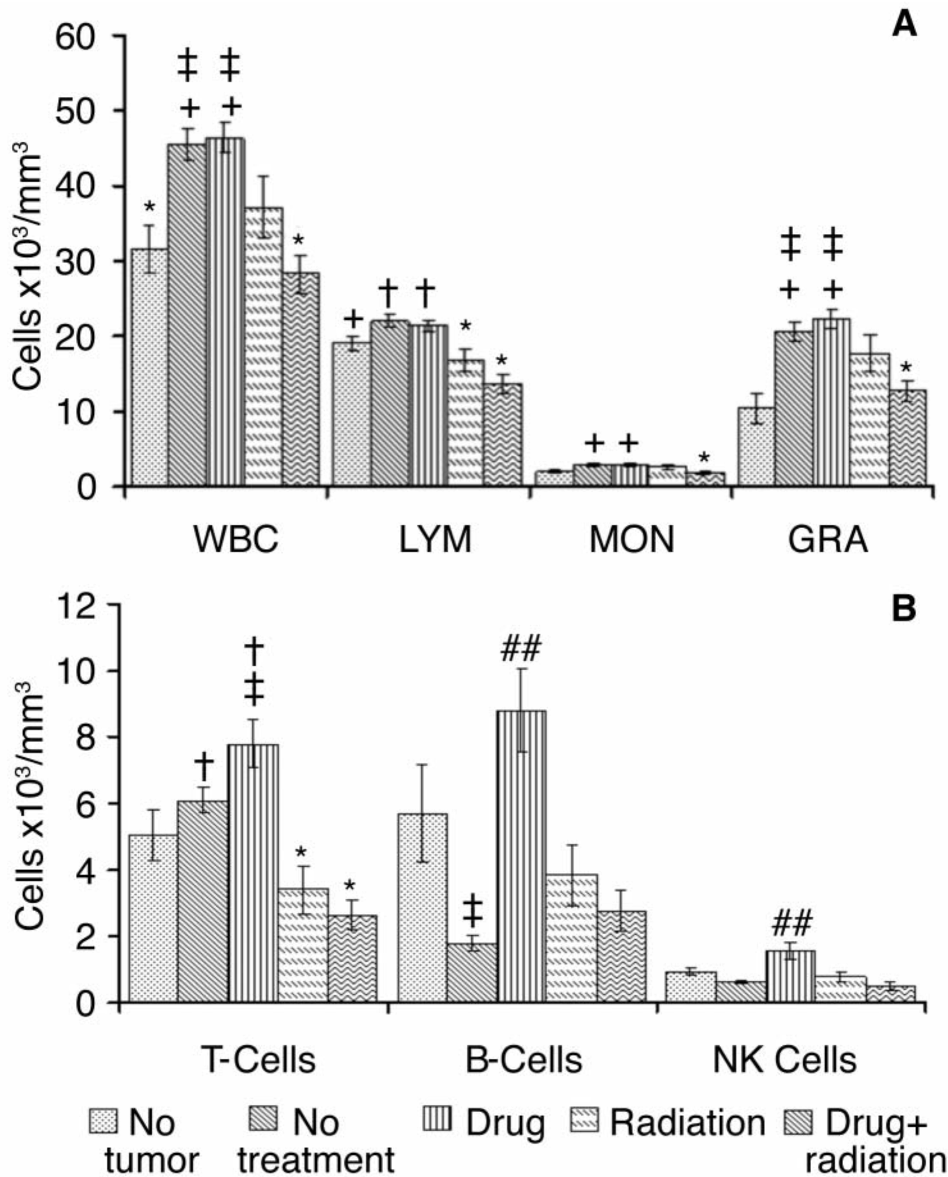

Figure 4. *WBC and major leukocyte populations in the spleen. WBC and major leukocyte types obtained with an automated hematology analyzer (panel A). Specific lymphocyte populations obtained using fluorescence-labeled monoclonal antibodies and flow cytometry (panel B). Each bar represents mean±SEM for n=8-10 mice/group. ‡p<0.05 vs. No tumor; *p<0.05 vs. no treatment and drug; +p<0.05 vs. drug + radiation; †p<0.05 vs. radiation and drug + radiation; ##p<0.05 vs. all other tumor-bearing groups; WBC: white blood cells, LYM: lymphocytes, MON: monocytes, GRA: granulocytes.*

WBC, major leukocyte populations, and lymphocyte subpopulations in spleen. The leukocyte counts in the spleen showed a significant increase associated with tumor presence (Figure 4A). However, radiation in combination with the drug induced a significant decrease in the WBC when compared to non-irradiated tumor-bearing groups (p<0.05). This same pattern was noted for the granulocytes and to a lesser extent also for lymphocytes. Monocyte/macrophage counts were similar among all groups except the one receiving drug plus radiaton treatment, which had significantly reduced counts *versus* the non-irradiated groups with tumor (p<0.05).

Specific lymphocyte populations in the spleen are shown in Figure 4B. In the tumor-bearing mice, drug alone significantly increased T-cell numbers compared to the no tumor and both irradiated groups (p<0.05). Significant drug-induced enhancement of B-cells and NK cells was also noted. In addition, both irradiated groups had lower T-cell counts *versus*

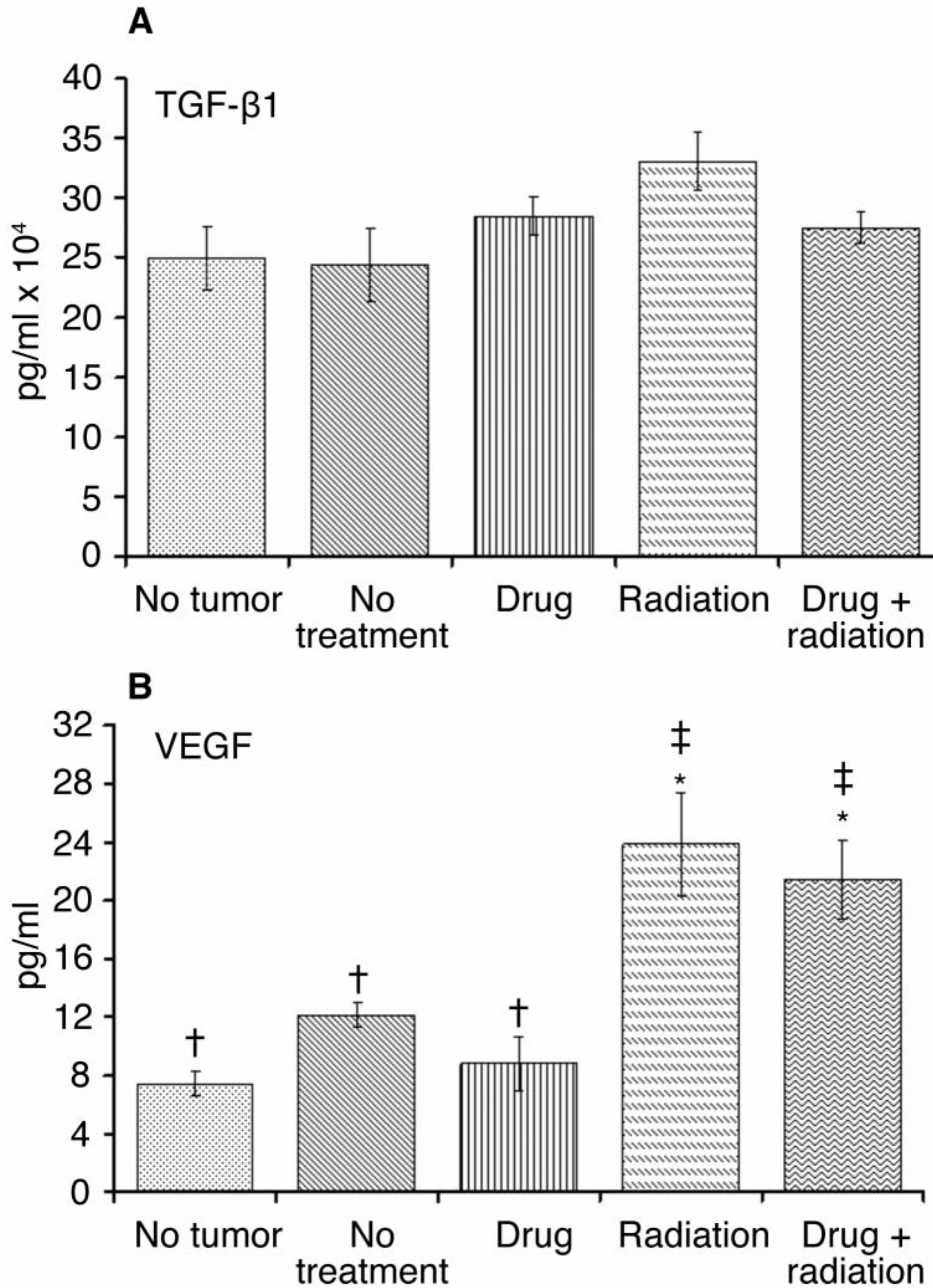

Figure 5. *TGF-β1 and VEGF concentrations in plasma. The data were obtained using ELISA procedures. Each bar represents mean±SEM for n=8-10 mice/group. ‡p<0.05 vs. No tumor; *p<0.05 vs. non-irradiated tumor-bearing groups; †p<0.05 vs. irradiated groups. For radiation versus no tumor and no treatment groups, the p-value was <0.1.*

the two non-irradiated groups with tumor and B-cell numbers were depressed in the mice with non-treated tumors ($p<0.05$).

Lymphocyte subpopulation data are presented in Table III. $CD4^+$ Th and $CD8^+$ Tc cell counts were highest in the group receiving drug alone ($p<0.05$ *versus* no tumor and both irradiated groups). Radiation, either with or without drug, resulted in decreased numbers of these cells compared to the two non-irradiated groups with tumor ($p<0.05$). A significant increase in the CD4:CD8 ratio was noted for the group receiving only radiation compared to the non-treated group ($p<0.05$).

TGF-β1 and VEGF in plasma. No significant difference was observed in TGF-β1 (latent plus active forms) regardless of tumor presence or treatment (Figure 5). However, a trend

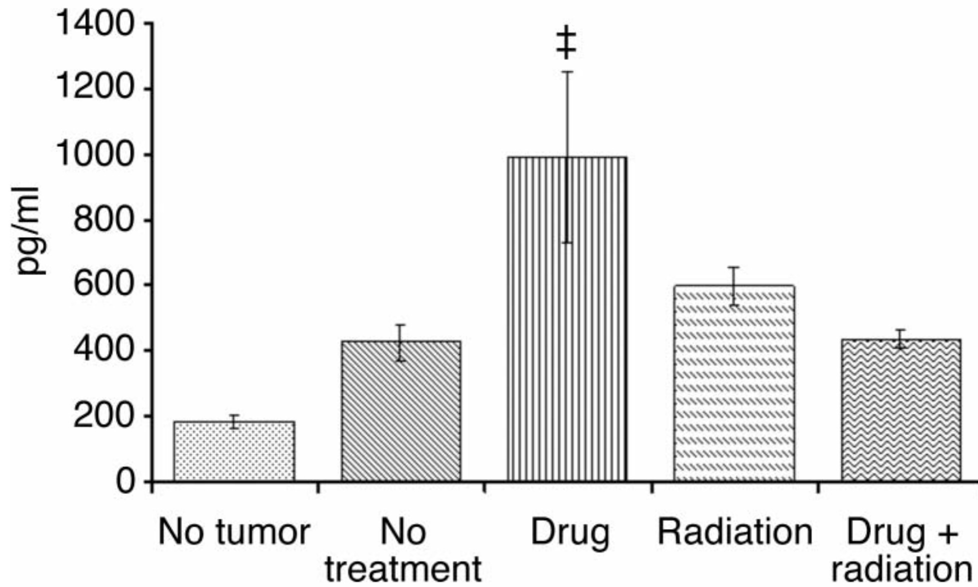

Figure 6. *IL-2 concentration in activated spleen cell supernatants. The data were obtained using an ELISA procedure after activation with anti-CD3 monoclonal antibody. Each bar represents mean±SEM for n=8-10 mice/group. ‡$p<0.05$ vs. No tumor and drug + radiation.*

($p<0.1$) for an increase was observed in the group receiving only radiation in comparison to the no tumor and no treatment groups. A significant interaction for TGF-β1 was observed with two-way ANOVA between radiation and drug treatment ($p<0.05$). In contrast, the groups that received radiation expressed significantly elevated levels of VEGF in comparison with the no tumor and non-irradiated groups ($p<0.05$) (Figure 5). Treatment with drug, either alone or in combination with radiation, did not show a significant effect on VEGF expression.

IL-2 production by splenocytes. The level of IL-2 production in response to anti-CD3 activation was consistently higher in the groups with tumor compared to the non-tumor-bearing control (Figure 6). A significantly higher level was obtained in the group receiving drug alone compared to the control group with no tumor and the tumor-bearing group receiving both the drug and radiation ($p<0.05$).

Discussion

The data presented here demonstrated that the tumor volumes were significantly reduced by radiation regardless of drug treatment. Although there was no statistically significant difference between the radiation and drug + radiation groups, the rate of tumor growth was slowest in the group receiving both treatments (*i.e.* more mice survived with tumor volumes ≤2,000 mm^3 by the end of the study). These data showed that MnTE-2-PyP did not protect the tumor against radiation-induced damage in this pre-clinical model of prostate cancer.

The immunodepressive effect of radiation was exhibited by low levels of the three major leukocyte populations (lymphocytes, monocytes and granulocytes) in the blood. Similar findings were also noted in the spleen, but with a less pronounced outcome. This radiation effect was comparable regardless of drug treatment, signifying that MnTE-2-PyP did not exacerbate the radiation-induced decrease in any of these white blood cell types. Furthermore, the depressive effect of radiation on RBC counts, hemoglobin and hematocrit and increase in RBC distribution width did not occur when the drug was used together with radiation. This latter finding suggested that the risk of anemia associated with radiation therapy might be minimized by administration of MnTE-2-PyP. A recent review concluded that low hemoglobin and/or a high level of tumor hypoxia correlated with a poor prognosis for patients with prostate and other types of cancers (33). The few and transient differences in body mass observed among our experimental groups, as well as the maintenance of near-normal activity by the treated mice, support the conclusion that health was not seriously compromised by the treatment regimens. These are all important observations, since different forms of therapy, especially when used concurrently, can sometimes cause unexpected and severe toxicities.

Although MnTE-2-PyP did not significantly affect radiation-associated changes in the distribution of specific leukocyte populations at the time of assessment, there were some striking elevations in certain cell types when the drug was used alone. The highest levels of total $CD3^+$ T, $CD4^+$ Th and $CD8^+$ Tc lymphocytes were found in the spleens from the group receiving only MnTE-2-PyP, while a normal CD4:CD8 ratio was

maintained. These cells have long been known to be of great importance in tumor destruction by direct cell-to-cell contact and regulation of antitumor immune responses by virtue of the cytokines they secrete. The exceptionally high level of IL-2, a Th1 cell-derived cytokine, secreted by activated splenocytes in the group treated with MnTE-2-PyP suggested that these cells are able to respond well. IL-2 is essential for optimal T-cell activation and subsequent expression of their cytotoxic and helper activities. Indeed, convincing evidence that cancer of the prostate and other body sites can be immunogenic have led to clinical trials with vaccines, some of which include IL-2 (34, 35). However, tumors evade and/or suppress the immune system in a variety of ways such as down-regulation of tumor antigens, impairment of antigen presentation and secretion of immunosuppressive cytokines (36). Reports have indicated that treatment with antioxidants can diminish the immune evasion/suppression mediated by tumor cells (37) and cause them to lose their "altered phenotype" (38). Therefore, it seems possible that by scavenging for ROS, MnTE-2-PyP may modify the pathways by which tumors evade the immune system.

The drug-induced increase in the splenic NK cell population was a response that should not be overlooked. These cells play an important role in innate immunity and have the ability to recognize and eradicate tumor cells, as well as virus-infected and other aberrant cells, by detecting phenotypic changes on the target (39, 40). NK cell activation is dependent on a balance between inhibitory and activation signals and the ability to discriminate between normal and tumor cells based on differences in major histocompatibility complex (MHC) class I expression. The MHC-specific inhibitory receptors on the surface of NK cells recognize loss of expression on tumor cell surfaces, leading to a failure of receptors to deliver inhibitory signals (39). Additionally, a number of ligands present on tumors are recognized by receptors on NK cells that trigger activation signals (39, 41) that, together with failure to deliver inhibitory signals, shift the balance towards NK-mediated tumor cell death.

Interestingly, significant elevation of B-cells was observed in both the blood and spleens from the mice treated only with MnTE-2-PyP compared to all other groups. B-cells are a critical component of the immune system in that they respond to antigens with the production of antibodies and secretion of cytokines (42, 43). Although optimal B-cell activation is often thought to require T-cell help, elevation in these cells can also occur *via* a T-cell-independent pathway (44-46). CD40, a member of the TNF-receptor superfamily expressed on the surface of Th cells (47), has also been recently found on B-cells and is associated with T-cell-independent B-cell activation (43). Ligation with CD40L on dendritic and other antigen-presenting cells (APC) activated B-cells and induced their proliferation, survival and differentiation (46). In addition, APC produce B-cell-activating factor (BAFF), a ligand in the TNF family that promotes the generation and survival of mature B-cells that can induce activation independently of T-cells (45). It may be that MnTE-2-PyP up-regulates one or more of the above processes.

Furthermore, ROS have been reported to play a variety of roles in B-cell activation, proliferation, survival and maintenance (43). ROS elevation can lead to oxidative stress that hinders cell proliferation and death (48, 49). Liu *et al.* have reported the suppressive effect of ROS on CD40-induced B-cell activation (50). In contrast, tumors require appreciably higher levels of ROS to support their survival. In tumor metabolism, ROS can promote carcinogenesis, metastasis, survival and progression (51, 52). Thus, while tumor cells would be negatively affected by MnTE-2-PyP due to their dependence on elevated oxidative stress (53), B-cells could, on the other hand, be enhanced by reduction of ROS (52).

As expected, the highest level of plasma TGF-β1 was found in the group receiving radiation alone and a trend for enhancement was noted compared to the no tumor and no treatment groups. This factor is up-regulated by radiation and is associated with late radiation effects such as fibrosis, increased tumor resistance to radiation and the suppression of antitumor immunity (53, 54). Although statistical significance was not obtained, TGF-β1 concentration was closer to normal when MnTE-2-PyP was combined with radiation. This latter finding, if confirmed in future studies, would be consistent with the drug-induced enhancements noted here in IL-2 production and certain immune cell populations. Lack of statistical significance in our study may be related to the up/down fluctuations that are known to occur in TGF-β1 with time after radiation.

Although an effect of MnTE-2-PyP on the circulating VEGF concentration was not seen in the mice receiving radiation treatment, a highly significant increase was observed in both irradiated groups. The lack of a significant decrease in VEGF that regulates angiogenesis, as we originally expected based on HIF-1α data in our previous study with a different metalloporphyrin antioxidant (26), might have been due to the fact that ROS are not the only molecules that can enhance its production. It is also possible that the absence of a drug effect on VEGF was related to differences in metalloporphyrin antioxidant efficacies in mediating a particular effect. Another possibility is that the plasma level of VEGF at the time of assessment may not necessarily have reflected the level of the factor within the tumor mass itself.

Although our focus was on treatment-related changes in the leukocyte populations and cytokines that can affect tumor growth, it should be pointed out that the presence of the RM-9 tumor, without any treatment, caused at least two significant changes. A dramatic increase in granulocytes was seen in both the blood and spleens from the non-treated tumor-bearing group, an enhancement that remained evident also in the group given only MnTE-2-PyP. This finding suggested that the RM-9 tumor cells may secrete a factor that stimulates granulopoiesis. In contrast, B-cell numbers in the spleens from these mice were significantly below normal, an effect that was

counteracted by MnTE-2-PyP as discussed above. The underlying mechanisms responsible for these tumor-induced aberrations remain to be elucidated.

Conclusion

The administration of MnTE-2-PyP together with radiation did not enhance RM-9 tumor growth, thus the drug does not protect tumors against radiation in this prostate tumor model. There were no observed adverse effects due to the addition of MnTE-2-PyP to the radiation treatment, instead, the drug normalized several RBC parameters that differed significantly from normal in the animals receiving radiation.

The drug did not have a statistically significant effect on tumor volume when used alone or together with radiation, nonetheless, the slowest tumor growth occurred in the group treated with both modalities. Furthermore, the group receiving MnTe-2-PyP had relatively high levels of T, Th, Tc, and NK cells in the spleen, high B-cell counts in both blood and spleen, and high capacity to produce IL-2. These findings support the possibility that the drug has potential to enhance antitumor immune responses.

MnTE-2-PyP may be useful not only as a normal tissue radioprotectant based on its ability to scavenge ROS, but may also serve to enhance mechanisms that are vital for effective immune responses against neoplastic cells and decrease the risk of anemia in patients undergoing radiotherapy. The data reported here are unique and should stimulate further research with metalloporphyrin antioxidants.

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References

1 Storey MR, Pollack A, Zagars G, Smith L, Antolak J and Rosen I: Complications from radiotherapy dose escalation in prostate cancer: preliminary results of a randomized trial. Int J Radiat Oncol Biol Phys *48*: 635-642, 2000.

2 Aebersold DM, Burri P, Beer KT, Laissue J, Djonov V, Greiner RH and Semenza GL: Expression of hypoxia-inducible factor-1{alpha}: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res *61*: 2911-2916, 2001.

3 Koli K, Myllarniemi M, Keski-Oja J and Kinnula VL: Transforming growth factor-beta activation in the lung: focus on fibrosis and reactive oxygen species. Antioxid Redox Signal *10*: 333-342, 2008.

4 Jobling MF, Mott JD, Finnegan MT, Jurukovski V, Erickson AC, Walian PJ, Taylor SE, Ledbetter S, Lawrence CM, Rifkin DB and Barcellos-Hoff MH: Isoform-specific activation of latent transforming growth factor beta (LTGF-beta) by reactive oxygen species. Radiat Res *166*: 839-848, 2006.

5 Li C, Guo B, Bernabeu C and Kumar S: Angiogenesis in breast cancer: the role of transforming growth factor beta and CD105. Microsc Res Tech *52*: 437-449, 2001.

6 Barcellos-Hoff MH and Dix TA: Redox-mediated activation of latent transforming growth factor-beta 1. Mol Endocrinol *10*: 1077-1083, 1996.

7 Abate-Shen C and Shen MM: Molecular genetics of prostate cancer. Genes Dev *14*: 2410-2434, 2000.

8 Kusunoki Y and Hayashi T: Long-lasting alterations of the immune system by ionizing radiation exposure: implications for disease development among atomic bomb survivors. Int J Radiat Biol *84*: 1-14, 2008.

9 Bierie B and Moses HL: TGF-beta and cancer. Cytokine Growth Factor Rev *17*: 29-40, 2006.

10 Rich J, Borton A and Wang X: Transforming growth factor-beta signaling in cancer. Microsc Res Tech *52*: 363-373, 2001.

11 Teicher BA: Transforming growth factor-{beta} and the immune response to malignant disease. Clin Cancer Res *13*: 6247-6251, 2007.

12 Walsh DA: Pathophysiological mechanisms of angiogenesis. Adv Clin Chem 44: 187-221, 2007.

13 Vaupel P and Mayer A: Hypoxia and anemia: effects on tumor biology and treatment resistance. Transfus Clin Biol *12*: 5-10, 2005.

14 Duque JLF, Loughlin KR, Adam RM, Kantoff P, Mazzucchi E and Freeman MR: Measurement of plasma levels of vascular endothelial growth factor in prostate cancer patients: relationship with clinical stage, Gleason score, prostate volume, and serum prostate-specific antigen. Clinics *61*: 401-408, 2006.

15 Green MM, Hiley CT, Shanks JH, Bottomley IC, West CM, Cowan RA and Stratford IJ: Expression of vascular endothelial growth factor (VEGF) in locally invasive prostate cancer is prognostic for radiotherapy outcome. Int J Radiat Oncol Biol Phys *67*: 84-90, 2007.

16 Jin P, Wang E, Provenzano M, Stroncek D and Marincola FM: Gene expression signatures of interleukin-2 *in vivo* and *in vitro* and their relation to anticancer therapy. Crit Rev Immunol *27*: 437-448, 2007.

17 Bachmann MF and Oxenius A: Interleukin 2: from immunostimulation to immunoregulation and back again. EMBO Rep *8*: 1142-1148, 2007.

18 Salvemini D, Riley DP and Cuzzocrea S: SOD mimetics are coming of age. Nat Rev Drug Discov *1*: 367-374, 2002.

19 Vujaskovic Z, Batinic-Haberle I, Rabbani ZN, Feng QF, Kang SK, Spasojevic I, Samulski TV, Fridovich I, Dewhirst MW and Anscher MS: A small molecular weight catalytic metalloporphyrin antioxidant with superoxide dismutase (SOD) mimetic properties protects lungs from radiation-induced injury. Free Radic Biol Med *33*: 857-863, 2002.

20 Ferrer-Sueta G, Vitturi D, Batinic-Haberle I, Fridovich I, Goldstein S, Czapski G and Radi R: Reactions of manganese porphyrins with peroxynitrite and carbonate radical anion. J Biol Chem *278*: 27432-27438, 2003.

21 Spasojevic I, Batinic-Haberle I and Fridovich I: Nitrosylation of manganese (II) tetrakis(*N*-ethylpyridinium-2-yl) porphyrin: a simple and sensitive spectrophotometric assay for nitric oxide. Nitric Oxide *4*: 526-533, 2000.

22 Shimizu K, Rajapakse N, Horiguchi T, Payne RM and Busija DW: Protective effect of a new nonpeptidyl mimetic of SOD, M40401, against focal cerebral ischemia in the rat. Brain Res *963*: 8-14, 2003.

23 Haskins K, Bradley B, Powers K, Fadok V, Flores S, Ling X, Pugazhenthi S, Reusch J and Kench J: Oxidative stress in type 1 diabetes. Ann NY Acad Sci *1005*: 43-54, 2003.

24 Kaul DK, Liu XD, Zhang X, Ma L, Hsia CJC and Nagel RL: Inhibition of sickle red cell adhesion and vasoocclusion in the microcirculation by antioxidants. Am J Physiol Heart Circ Physiol *291*: H167-175, 2006.

25 Moeller BJ, Batinic-Haberle I, Spasojevic I, Rabbani ZN, Anscher MS, Vujaskovic Z and Dewhirst MW: A manganese porphyrin superoxide dismutase mimetic enhances tumor radioresponsiveness. Int J Radiat Oncol Biol Phys *63*: 545-552, 2005.

26 Gridley DS, Makinde AY, Luo X, Rizvi A, Crapo JD, Dewhirst MW, Moeller BJ, Pearlstein RD and Slater JM: Radiation and a metalloporphyrin radioprotectant in a mouse prostate tumor model. Anticancer Res *27*: 3101-3109, 2007.

27 Chang L-YL, Subramaniam M, Yoder BA, Day BJ, Ellison MC, Sunday ME and Crapo JD: A catalytic antioxidant attenuates alveolar structural remodeling in bronchopulmonary dysplasia. Am J Respir Crit Care Med *167*: 57-64, 2003.

28 Mackensen GB, Patel M, Sheng H, Calvi CL, Batinic-Haberle I, Day BJ, Liang LP, Fridovich I, Crapo JD, Pearlstein RD and Warner DS: Neuroprotection from delayed postischemic administration of a metalloporphyrin catalytic antioxidant. J Neurosci *21*: 4582-4592, 2001.

29 Lubbers NL, Polakowski JS, Crapo JD, Wegner CD and Cox BF: Preischemic and postischemic administration of AEOL10113 reduces infarct size in a rat model of myocardial ischemia and reperfusion. J Cardiovasc Pharmacol *41*: 714-719, 2003.

30 Thompson TC, Timme TL, Park SH, Yang G and Ren C: Mouse prostate reconstitution model system: a series of *in vivo* and *in vitro* models for benign and malignant prostatic disease. Prostate *43*: 248-254, 2000.

31 Batinic-Haberle I, Spasojevic I, Hambright P, Benov L, Crumbliss AL and Fridovich I: Relationship among redox potentials, proton dissociation constants of pyrrolic nitrogens, and *in vivo* and *in vitro* superoxide dismutating activities of manganese(III) and iron(III) water-soluble porphyrins. Inorg Chem *38*: 4011-4022, 1999.

32 Miller GM, Andres ML and Gridley DS: NK cell depletion results in accelerated tumor growth and attenuates the antitumor effect of total body irradiation. Int J Oncol *23*: 1585-1592, 2003.

33 Varlotto J and Stevenson MA: Anemia, tumor hypoxemia, and the cancer patient. Int J Radiat Oncol Biol Phys *63*: 25-36, 2005.

34 Arlen PM and Gulley JL: Therapeutic vaccines for prostate cancer: a review of clinical data. Curr Opin Investig Drugs *6*: 592-596, 2005.

35 Brill TH, Kubler HR, von Randenborgh H, Fend F, Pohla H, Breul J, Hartung R, Paul R, Schendel DJ and Gansbacher B: Allogeneic retrovirally transduced, IL-2- and IFN-gamma-secreting cancer cell vaccine in patients with hormone refractory prostate cancer – a phase I clinical trial. J Gene Med *9*: 547-560, 2007.

36 Croci DO, Zacarias Fluck MF, Rico MJ, Matar P, Rabinovich GA and Scharovsky OG: Dynamic cross-talk between tumor and immune cells in orchestrating the immunosuppressive network at the tumor microenvironment. Cancer Immunol Immunother *56*: 1687-1700, 2007.

37 Malmberg KJ: Effective immunotherapy against cancer: a question of overcoming immune suppression and immune escape? Cancer Immunol Immunother *53*: 879-892, 2004.

38 Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M and Telser J: Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol *39*: 44-84, 2007.

39 Scott GB, Meade JL and Cook GP. Profiling killers: unravelling the pathways of human natural killer cell function. Brief Funct Genomic Proteomic *7*: 8-16, 2008.

40 Cerwenka A and Lanier LL: NKG2D ligands: unconventional MHC class I-like molecules exploited by viruses and cancer. Tissue Antigens *61*: 335-343, 2003.

41 Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, Nanni M, Biassoni R, Bottino C, Moretta A and Moretta L: Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. Eur J Immunol *31*: 1076-1086, 2001.

42 Zinkernagel RM: What is missing in immunology to understand immunity? Nat Immunol *1*: 181-185, 2000.

43 Lee JR: Reactive oxygen species play roles on B cell surface receptor CD40-mediated proximal and distal signaling events: effects of an antioxidant, *N*-acetyl-L-cysteine treatment. Mol Cell Biochem *252*: 1-7, 2003.

44 Craxton A, Magaletti D, Ryan EJ and Clark EA: Macrophage- and dendritic cell-dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. Blood *101*: 4464-4471, 2003.

45 Shulga-Morskaya S, Dobles M, Walsh ME, Ng LG, MacKay F, Rao SP, Kalled SL and Scott ML: B-cell-activating factor belonging to the TNF family acts through separate receptors to support B-cell survival and T-cell-independent antibody formation. J Immunol *173*: 2331-2341, 2004.

46 Bergamin F, Vincent IE, Summerfield A and McCullough KC: Essential role of antigen-presenting cell-derived BAFF for antibody responses. Eur J Immunol *37*: 3122-3130, 2007.

47 Rakhmilevich AL, Buhtoiarov IN, Malkovsky M and Sondel PM: CD40 ligation *in vivo* can induce T-cell independent antitumor effects even against immunogenic tumors. Cancer Immunol Immunother *57*: 1151-1160, 2008.

48 Moon EY, Lee JH, Oh SY, Ryu SK, Kim HM, Kwak HS and Yoon WK: Reactive oxygen species augment B-cell-activating factor expression. Free Radic Biol Med *40*: 2103-2111, 2006.

49 Tohyama Y, Takano T and Yamamura H: B-cell responses to oxidative stress. Curr Pharm Des *10*: 835-839, 2004.

50 Liu J, Yoshida Y and Yamashita U: Suppressive effect of reactive oxygen species on CD40-induced B-cell activation. FEBS Letters *581*: 5043-5049, 2007.

51 Storz P: Reactive oxygen species in tumor progression. Front Biosci *10*: 1881-1896, 2005.

52 Wu WS: The signaling mechanism of ROS in tumor progression. Cancer Metastasis Rev *25*: 695-705, 2006.

53 Andarawewa KL, Paupert J, Pal A and Barcellos-Hoff MH: New rationales for using TGFbeta inhibitors in radiotherapy. Int J Radiat Biol *83*: 803-811, 2007.

54 Bommireddy R and Doetschman T: TGFbeta1 and Treg cells: alliance for tolerance. Trends Mol Med *13*: 492-501, 2007.

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