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Superoxide dismutase mimetic reduces hypoxia-induced $O_2^{\cdot -}$, TGF-β, and VEGF production by macrophages

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Abstract
Normal tissue injury poses a major limitation to the success of radiation therapy (RT) in the treatment of solid tumors. We propose that radiation-induced lung injury is a result of chronic oxidative stress propagated by hypoxia-induced macrophage activation and cytokine production. Therefore, the objective of our study was two-fold. First, *in vivo* studies were conducted to support our hypothesis suggesting radiation injury is characterized by chronic hypoxia associated with increased macrophage infiltration/activation and pro-fibrogenic/angiogenic cytokine production. Second, we investigated the proposed mechanism of radiation injury *in vitro*. We demonstrate that hypoxia (0.5% O_2) elicits macrophages to produce higher levels of $O_2^{\cdot -}$, TGF-β, and VEGF than normoxia. Our hypothesis that $O_2^{\cdot -}$ is contributing to increased macrophage cytokine production was supported by a significant reduction in TGF-β and VEGF when redox signaling was minimized using a small molecular weight metalloporphyrin antioxidant, MnTE-2-PyP^{5+}.

Keywords: *Radiation fibrosis, TGF-β, VEGF, ROS, SOD-mimetic, macrophages*

Introduction

The effectiveness of radiation therapy (RT) in the treatment of thoracic tumors is limited by the radiation tolerance of surrounding normal lung tissue. Radiation pneumonitis (RP), an interstitial pulmonary inflammation, occurs in 5–30% of patients receiving thoracic irradiation [1–6]. There is considerable variation in individual risk for development of radiation induced normal tissue injury. Factors that have been correlated with the risk of pulmonary injury from RT include dose-volume factors and individual factors (age, gender, smoker vs. non-smoker, pulmonary function) [7,8]. Radiation-induced lung injury is characterized by a latent period that can last for months or years following radiation exposure and can result in life threatening and debilitating pulmonary toxicity. Advances in normal tissue radiobiology demonstrate that ionizing radiation triggers a cascade of genetic and molecular events that proceed during a latent period of pulmonary injury; however, the precise mechanisms underlying radiation-induced lung injury are still unclear.

In our model of radiation-induced lung injury, macrophage accumulation and activation was associated with hypoxia and was a prominent histological feature underlying the development of post-radiation lung injury [9,10]. Macrophages represent one of the primary cell types involved in radiation injury. In response to low oxygen tension, macrophages are recruited from the circulation, where they are activated to undergo respiratory burst releasing superoxide anion ($O_2^{\cdot -}$) and nitric oxide (·NO) [11]. Both reactive species are involved in production of

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other reactive oxygen and nitrogen species (such as peroxynitrite, carbonate radical, nitrogen dioxide, and hydroxyl radical), which contribute to the development of chronic oxidative stress. Oxidative stress can affect signaling events that in turn increase leukocyte migration and vascular permeability, upregulate TGF-β, promote collagen formation, induce VEGF and promote angiogenesis [12–14]. The central hypothesis of this study is that radiation-induced lung injury is a consequence of post-radiation continuous production of ROS mediated by hypoxia and generated by macrophages.

The hypothesis is based on our previous findings, which have shown extracellular superoxide dismutase (EC-SOD) overexpression in rodents significantly reduces oxidative stress and offers significant protection from radiation-induced lung injury [15]. Furthermore, supporting evidence for our hypothesis comes from previous studies conducted by our laboratory in which *in vivo* administration of MnTE-2-PyP^{5+} was found to alleviate oxidative stress as well as pathological changes associated with long term RT injury [16].

Herein, we propose that chronic oxidative stress, more specifically the production of $O_2^{\cdot-}$ by macrophages, is a result of tissue hypoxia. Furthermore, we hypothesize that changes in pro-fibrogenic and pro-angiogenic cytokine levels, specifically, TGF-β and VEGF, in irradiated healthy tissue are a result of increased $O_2^{\cdot-}$ signaling. Our proposed mechanism of radiation injury was investigated *in vitro* using a small molecular weight metalloporphyrin antioxidant MnTE-2-PyP^{5+} [17–19]. Our study hypothesized that MnTE-2-PyP^{5+} treatment would ameliorate oxidative stress and suppress cytokine production *in vitro*, that otherwise facilitates pulmonary pneumonitis and fibrosis *in vivo*.

Materials and methods

Animals

Experiments were performed using female Fisher-344 rats (150–200 g) with prior approval from the Duke University Institutional Animal Care and Use Committee. All animals were housed 2–4 per cage based on animal weight and maintained under identical standard laboratory conditions. Food and water were provided *ad libitum*.

Irradiation

Animals were anaesthetized with an intraperitoneal injection of ketamine (67 mg/kg) and xylazine (4.5 mg/kg) prior to sham irradiation ($n = 10$) or irradiation ($n = 10$). Animals received 28 Gy single dose radiation delivered to the right hemithorax with 6 MV photons. Unirradiated parts of the thorax and body were shielded using Cerrobend blocks.

Immunohistochemistry and quantification

Animals were followed for 14 weeks post radiation. At 14 weeks, animals from both the control group and the group receiving radiation were euthanized with pentobarbital sodium overdose (100 mg/kg). Lungs were fixed by tracheal instillation of 10% neutral buffered formalin, then embedded in paraffin. The paraffin-embedded tissues were sectioned (5 microns). Tissues were treated overnight at 4°C with primary antibody against carbonic anhydrase IX (CAIX) (1:100, Abcam, Cambridge, MA) for detection of hypoxia, 8-OHdG (1:1000, JaICA, Shizuoka, Japan) for determination of oxidative stress, the macrophage marker ED-1, and polyclonal antibodies for VEGF and TGF-β1 (1:200, Santa Cruz Biotechnology, CA). Slides were incubated with species-specific secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The sections were washed three times in phosphate-buffered saline for 5 min between incubations. The sections were then incubated with ABC-Elite (Vector Labs, Burlingame, CA), developed using DAB working solution (Laboratory Vision, Fremont, CA) and counterstained with Harris hematoxylin (Fisher Scientific, Pittsburgh, PA).

To quantify the total number of macrophages present in control vs. irradiated lung tissue 14 weeks post radiation, total macrophages per slide were counted by light microscopy at 20 × image magnification, and macrophage per square centimeter of lung tissue was calculated.

Bronchoalveolar lavage

Twelve female Fisher 344 rats weighing between 170 and 200 g were anesthetized with 100 mg/kg sodium pentobarbital dissolved in 1 ml phosphate buffered saline (PBS, Gibco) injected intraperitoneally. Lungs were lavaged with 1 ml warm PBS via an 18G catheter inserted through a small incision in the trachea eight times. Cell suspensions were centrifuged at 5000*g* for 10 min at 4°C to obtain a cell pellet. Cell viablility was analysed by trypan blue dye exclusion. Viable cells were plated at 2.5×10^5 in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS and incubated at 37°C in a 5% CO_2 humidified atmosphere overnight with 1 μg/ml LPS (Sigma-Aldrich, St Louis, MO).

Superoxide dismutase mimetic

MnTE-2-PyP^{5+}, a potent scavenger of reactive oxygen and nitrogen species, particularly $O_2^{\cdot-}$ [17,18] and $ONOO^-$ [19] was prepared as previously described [17].

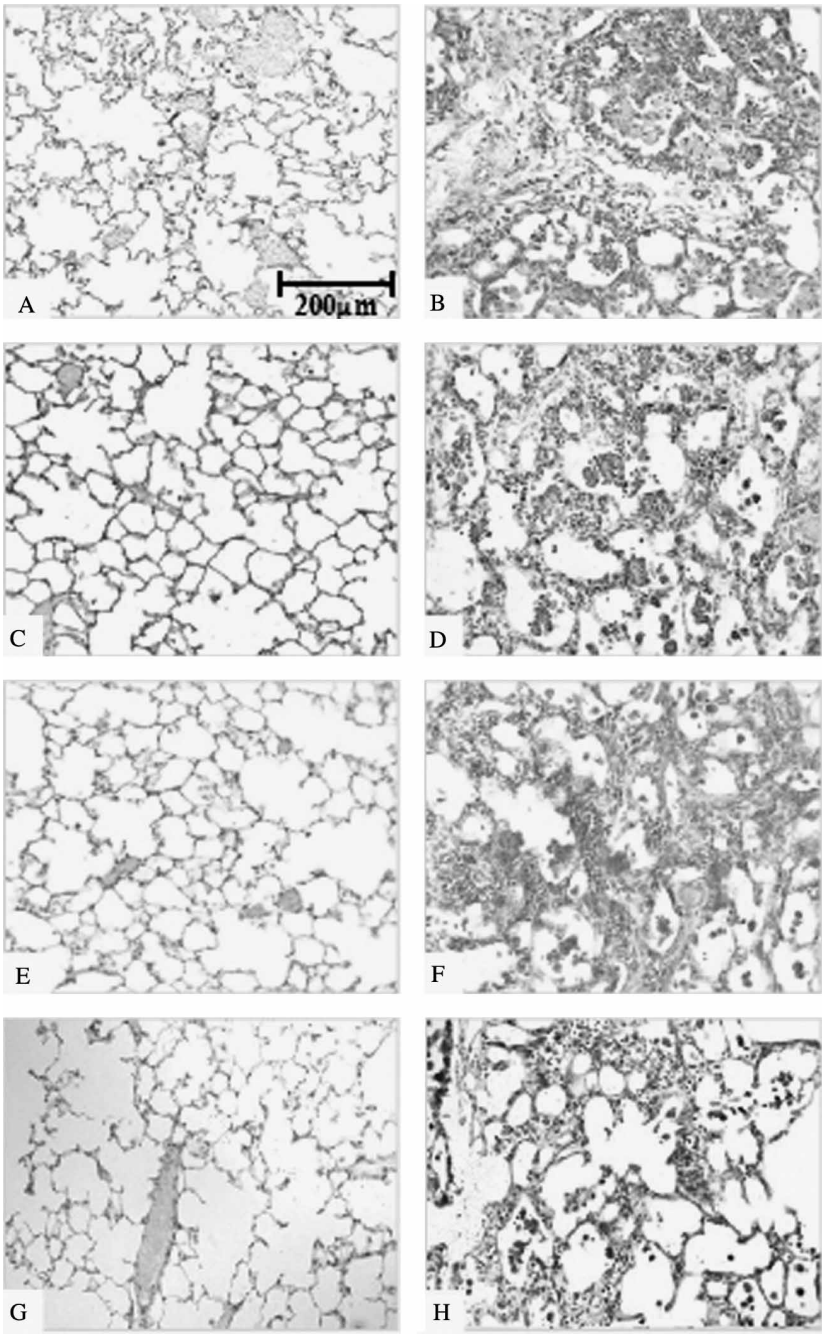

Figure 1. Histologic comparison between sham-irradiated and irradiated rat lungs 14 weeks post radiation. Each image is 20× magnification. CAIX staining for hypoxia is significantly stronger in the irradiated group (B) as compared to the control (A). Lung tissues were immunostained for 8-OHdG, a marker for oxidative stress and DNA damage, VEGF, and TGF-β. Increase in oxidative stress was detected in irradiated rat lungs (D) as compared to control (C). VEGF expression in control (E) and irradiated rat lungs (F) and TGF-β expression in control (G) and irradiated rat lungs (H) demonstrate increased cytokine activity in irradiated animals as compared to control.

Hypoxia

As a control, alveolar macrophages were incubated in a circling water bath pre-heated to 37°C for 2 h with or without 80 μM MnTE-2-PyP^{5+} under normoxic conditions (21% O_2). To determine $O_2^{\cdot-}$ production by hypoxic alveolar macrophages and the effect of MnTE-2-PyP^{5+} cells were incubated in a hypoxia chamber at 0.5% O_2 for 2 h at 37°C with or without 80 μM MnTE-2-PyP^{5+}. At the end of each treatment, cell culture medium was removed and the reduction of cytochrome *c* by $O_2^{\cdot-}$ was recorded. Cell culture medium was stored at −20°C for further analysis. All experiments were performed in duplicate.

Superoxide quantification

Superoxide release by macrophages was determined using the ferricytochrome *c* method by McCord and Fridovich [20]. Cells were incubated in 2 ml phenol

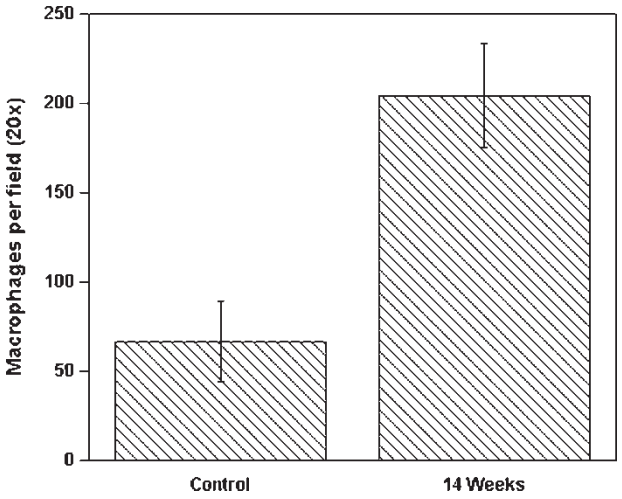

Figure 2. Macrophage per field in control and irradiated rat lungs 14 weeks post radiation. Lung tissues were immunostained for ED-1, an activated macrophage marker. Images were taken at 20 × magnification and the total number of macrophages per field was counted. The data represent the average of five animals per group. Irradiated rat lungs demonstrate a significantly higher amount of macrophages than the control ($P < 0.02$).

red free HBSS with 31.4 μl/ml of a 1 mM stock solution of equine ferricytochrome *c* (cyt *c* #7752 Sigma-Aldrich, St Louis, MO). The cyt *c* reduction by $O_2^{\cdot -}$ induces an increase in the absorbance at 550 nm which is measured from 100 μl aliquots before and after the treatment using a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The change in cyt *c* absorbance upon reduction by $O_2^{\cdot -}$ at 550 nm ($\varepsilon_{550} = 21{,}000\,M^{-1}\,cm^{-1}$) was used to calculate the levels of $O_2^{\cdot -}$ [21]. All experiments were run in duplicate.

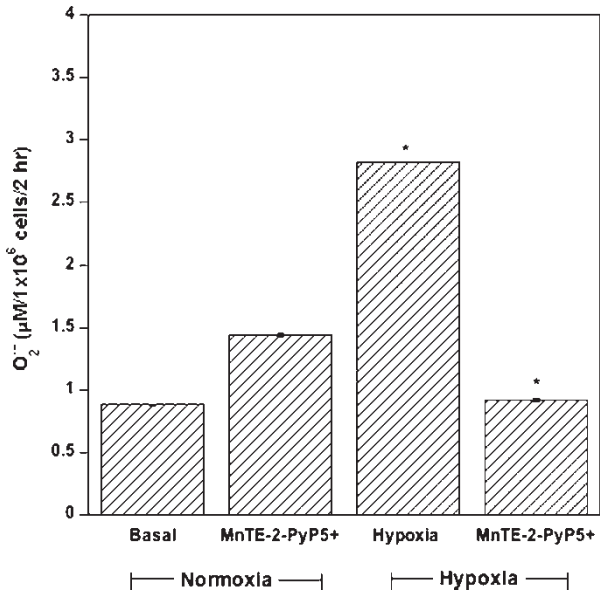

Figure 3. Cytochrome *c* analysis of superoxide production by alveolar macrophages. Cells were incubated overnight in DMEM -10% FBS with 1 μg/ml LPS. The following day, media was aspirated and replaced with cytochrome *c* assay mixture. Macrophages were exposed to 0.5% O_2 (hypoxia) or 21% O_2 (normoxia) for 2 h with or without MnTE-2-PyP^{5+} (80 μM). Experiments were run in duplicate and readings were recorded in triplicate. Results are represented as mean ± SEM. $^{\star}P < 0.02$.

Active TGF-β production

Active TGF-β was assayed in collected cell supernatant by a commercially available sandwich ELISA (Biosource, Camarillo, CA). The assay was performed according to the manufacturer's instructions. Data are expressed in pg/ml. The experiments were done in duplicate.

VEGF production

To measure VEGF production by hypoxic macrophages, cells were incubated for 8 h under chronic hypoxia (0.5% O_2) or under normoxia (21% O_2) as a control. For determination of VEGF levels in response to decreased $O_2^{\cdot -}$, cells were treated with or without 80 μM MnTE-2-PyP^{5+}. Extracellular VEGF was determined in collected cell supernatant by sandwich ELISA (Biosource, Camarillo, CA) following the manufacturer's instructions. Data are expressed in pg/ml. The experiments were done in duplicate.

Statistical analysis

Data are shown as mean ± SEM. Statistical comparisons were performed using an unpaired Student's *t*-test. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Immunohistochemistry and quantification

Histological analysis of paraffin embedded lung tissue samples from irradiated and sham- irradiated rats 14 weeks post radiation, demonstrated increased severity of pulmonary damage in the irradiated rats. CAIX staining indicated a significant increase in hypoxia in irradiated rats as compared to non-irradiated animals (Figure 1A, B). In addition, increased macrophage infiltration (detected by ED-1 staining, Figure 2) was associated with strong oxidative stress (8-OHdG, Figure 1C, D). There was strong expression of TGF-β and VEGF (Figure 1E–H) in irradiated animals that was not present in non-irradiated animals. Therefore, *in vivo*, hypoxia is associated with increased macrophage accumulation, oxidative stress, and pro-fibrogenic/pro-angiogenic cytokine activity following RT.

Superoxide production

To establish whether low oxygen tension could activate the phagocytic burst in alveolar macrophages,

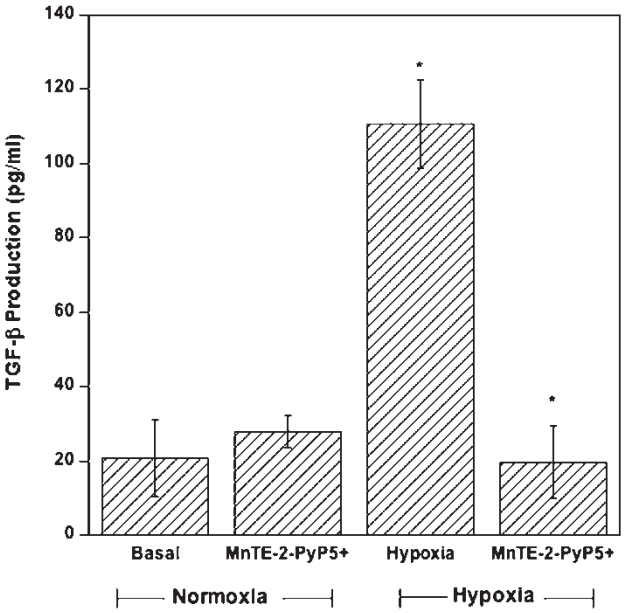

Figure 4. Active TGF-β levels produced by alveolar macrophages after two-hour exposure to normoxia (21% O_2) or hypoxia (0.5% O_2) with or without 80 μM MnTE-2-PyP^{5+}. Experiments were run in duplicate. Results are represented as mean ± SEM. $\star P < 0.05$.

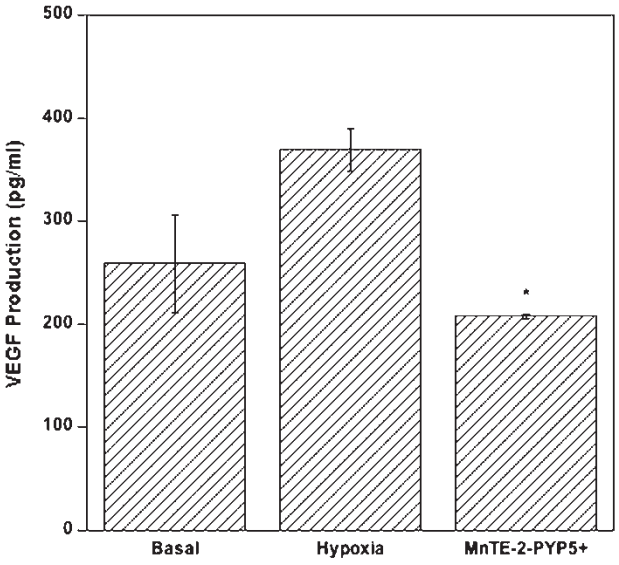

Figure 5. VEGF expression in alveolar macrophages after 8 h incubation under normoxia (21% O_2) or hypoxia (0.5% O_2) with or without 80 μM MnTE-2-PyP^{5+}. Experiments were run in duplicate. Results are represented as mean ± SEM. $\star P < 0.02$.

cells were exposed to normoxia (20% O_2) or hypoxia (0.5% O_2) for 2 h. Figure 3 demonstrates that under normoxia, alveolar macrophages produced low submicromolar levels of $O_2^{\cdot-}$ (0.88 μM/10^6 cells), which was significantly increased to 2.82 μM$O_2^{\cdot-}$/10^6 cells by incubation under hypoxic conditions for 2 h ($P = 0.01$). Treatment with 80 μM MnTE-2-PyP^{5+} returned $O_2^{\cdot-}$ production to normal levels of 0.92 μM $O_2^{\cdot-}$/10^6 cells ($P = 0.03$).

Active TGF-β levels

Here, we sought to determine whether macrophages stimulated by hypoxia also increased their expression of TGF-β. Figure 4 shows that after 2 h under hypoxia, alveolar macrophages increased their production of TGF-β from 20.718 to 110.684 pg/ml which was effectively reduced by MnTE-2-PyP^{5+} to 19.583 pg/ml ($P = 0.03$).

VEGF production

We incubated alveolar macrophages under hypoxia to determine whether VEGF expression was upregulated by low oxygen tension. Figure 5 demonstrates that hypoxia stimulated a 42% increase in VEGF production over baseline levels. As was the case with TGF-β, VEGF was reduced to normal levels when MnTE-2-PyP^{5+} was applied for the duration of exposure ($P = 0.0002$).

Discussion

The present study extends our previous findings that the temporal onset of radiation-induced lung injury is positively correlated with macrophage infiltration, activation, and increased oxidative injury around focal areas of hypoxia suggesting macrophages have an important role in post-radiation lung injury [1,2,22,23]. A potential role for hypoxia in the development of RT-induced injury was first suggested in a canine model of RT-induced peripheral neuropathy [24]. Additional evidence supporting post radiation normal tissue hypoxia was described in a rat model of radiation-induced spinal cord injury [25]. The authors showed a dose dependent temporal and spatial association of hypoxia, VEGF upregulation, and radiation-induced damage to blood spinal cord barrier and concluded that hypoxia perpetuates endothelial permeability damage in the central nervous system after irradiation. Similarly, we have found hypoxia to appear early prior to the overt onset of any histologic or symptomatic evidence of injury, which was followed by an increase in macrophage accumulation to the irradiated tissue [1]. Based on this evidence, we propose hypoxia plays a central role in generating a non-wound healing response that perpetuates radiation-induced lung injury through continuous generation of macrophage-produced reactive oxygen species (ROS) and profibrogenic/proangiogenic cytokines.

In this study, we sought to understand the mechanisms of ROS and cytokine activation/release by macrophages in irradiated, non-tumor bearing lung tissue. We were able to detect severe hypoxia in Fischer 344 rat lungs 14 weeks following 28 Gy irradiation delivered to the right hemithorax (Figure 1) which was associated with a significant increase in macrophage accumulation (Figure 2, $P = 0.005$) and elevated levels of oxidative stress (Figure 1). In addition,

increases in TGF-β and VEGF were also observed at the same time point (Figure 1). Both TGF-β and VEGF play essential roles in the processes that facilitate post radiation lung injury, including angiogenesis, collagen formation, endothelial cell death, and vascular permeability [26–28]. In addition to histological analysis of irradiated lung tissue, we investigated whether hypoxia induces macrophage ROS production *in vitro*. Our data show that hypoxia (0.5% O_2) stimulated the oxidative burst in macrophages, increasing the extracellular superoxide concentration by 3.2-fold (Figure 3). Along with increased $O_2^{\cdot-}$, we found that TGF-β and VEGF production by macrophages was upregulated *in vitro* under simulated hypoxic conditions. Our results demonstrate that macrophages incubated under hypoxic conditions increase TGF-β activation by 5.4-fold over a period of 2 h. This was associated with a 1.4-fold increase in VEGF production. Therefore, our results have shown that the macrophage respiratory burst, more specifically $O_2^{\cdot-}$ is a regulator of TGF-β and VEGF production *in vitro,* which coincide with our observations *in vivo*. These data demonstrate a strong relationship between hypoxia and macrophage associated ROS/cytokine production.

ROS-induced activation of TGF-β has been demonstrated *in vivo* several hours after ionizing RT, implicating TGF-β as a sensor of environmental oxidative stress [29–31]. Radiation injury is characterized by a chronic tissue remodeling process that results in vascular congestion, capillary permeability, edema, and excessive fibroblast proliferation within the alveolar septa, all of which can be induced by deregulated TGF-β and VEGF in response to radiation injury and low oxygen tension [25,32–34]. Knockout studies with TGF-β or components of its signal transduction pathway have shown higher normal tissue tolerance with no significant histological or functional damage following radiation exposure [31,35,36]. Past studies conducted by our lab have shown increased levels of TGF-β in its active form to be associated with heavy macrophage infiltrate into the alveolar space and increased alveolar wall thickness simultaneous to the onset of functional damage [1,16,23]. ROS have been implicated in cell signaling pathways that regulate transcription factor activation and downstream changes in gene expression. It has recently been shown that the potent SOD mimetic, MnTE-2-PyP^{5+}, affects transcription factor activation by eliminating ROS signaling molecules [17–19,37,38]; therefore, experiments using MnTE-2-PyP^{5+}, were designed to target macrophage cytokine production through $O_2^{\cdot-}$ scavenging. When macrophages were co-cultured under hypoxia with SOD mimetic, MnTE-2-PyP^{5+}, $O_2^{\cdot-}$ production was suppressed to baseline levels (Figure 3). Along with decreased $O_2^{\cdot-}$ production, TGF-β and VEGF levels were significantly reduced (Figures 4 and 5). Thus, increased elimination of superoxide coincided with a significant reduction in TGF-β (5.6-fold reduction) and VEGF (1.8-fold reduction) such that normal levels of both growth factors were sustained under hypoxia ($P < 0.02\%$). These results suggest that $O_2^{\cdot-}$ induces macrophage production of TGF-β and VEGF, which can be suppressed by the SOD like action of MnTE-2-PyP^{5+}.

We have previously reported that *in vivo*, acute hypoxia develops early following RT and is accompanied by macrophage infiltration and increased levels of VEGF and TGF-β expression [1]. Herein, we showed that *in vitro*, hypoxia stimulates macrophages to produce proangiogenic (VEGF) and profibrogenic (TGF-β) cytokines, key mediators in the development of radiation-induced pulmonary pneumonitis and fibrosis, in an $O_2^{\cdot-}$ dependent manner. These results are conclusive with our previous *in vivo* studies where MnTE-2-PyP^{5+} ameliorated radiation-induced cytokine cascade and oxidative injury that precedes the onset of occult pulmonary injury [16].

Acknowledgements

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