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Minocycline Modulates Cytokine and Gene Expression Profiles in the Brain After Whole-body Exposure to Radiation

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Abstract. *An effective countermeasure against radiation damage to normal tissues is urgently needed. The major goal of the present study was to determine if minocycline could modify the immunomodulatory effects of radiation on the brain. C57BL/6 mice were treated with minocycline intraperitoneally for 5 days beginning immediately before total-body exposure to 0, 1, 2 and 3 Gray (Gy) ^{60}Co γ-rays. Brains were collected on days 4 and 32 post-irradiation for cytokine and gene analyses. Minocycline treatment significantly increased the levels of interleukin (IL)-10, IL-15 and vascular endothelial growth factor (VEGF) in the brain on day 4 in one or more irradiated groups compared to radiation-alone ($p<0.05$). IL-10 is anti-inflammatory, IL-15 can prevent apoptosis and VEGF is nuroprotective. On day 32, the drug decreased IL-1β in the 2- Gy group ($p<0.05$ vs. 2-Gy alone); this cytokine is implicated in immune-related central nervous system pathologies. Microarray analysis of brains on day 32 showed that while radiation increased expression of inflammatory genes such as Il1f10, Il17, Tnfrsf11b, Tnfsf12, Il12b and Il1f8, these were no longer up-regulated in the minocycline-treated groups. Similarly, the pro-apoptotic gene Bik and nitric oxide synthase producer (Nostrin) were no longer up-regulated in the drug-treated groups. Pathway analysis based on gene data suggested that catenin-β1 and tumor suppressor-related transcription regulation were significantly activated by radiation and/or minocycline (activation z-score >2.0). Overall, the data warrant further testing of minocycline as a potential neuroprotectant against radiation-induced damage.*

There continues to be great urgency to develop safe and effective agents that protect against the damaging effects of radiation on normal tissues in the context of radiological/nuclear events. As astronauts may be exposed to doses as high as 1-3 Gray (Gy) during a solar particle event (SPE) (1), the development of effective radioprotectants is also important to agencies such as the National Aeronautics and Space Administration (NASA). Due to the highly radiosensitive nature of the hematopoietic system and gastrointestinal tract, most studies to date have focused on pharmacological agents that mitigate the damage associated with acute radiation syndrome (ARS) (2). Although various cytokines and growth factors appear promising, minimal efficacy and side-effects remain problematic (3). Currently, although there are several agents that have Investigational New Drug (IND) status, granulocyte colony-stimulating factor (G-CSF) is the only drug approved by the Food and Drug Administration (FDA) of the United States as an Emergency Use IND. In contrast, research on agents with potential to protect the central nervous system (CNS) against radiation damage has been minimal and needs to be further explored.

Although the brain is considered to be relatively radioresistant, irradiation of neural tissues is often associated with localized inflammation (4, 5). The neuroinflammatory milieu generated by resident and infiltrating populations in response to radiation includes pro-inflammatory cytokines and other factors indicative of increased and prolonged oxidative stress. For example, the acute response to radiation in the brain involves an increase in inflammatory cytokines and mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), intercellular adhesion molecule-1 (ICAM-1) and cyclooxygenase-2 (COX-2), as well as activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and activator protein-1 (AP-1) (6-8). Prolonged oxidative stress, in turn, is responsible for the long-term pathogenesis of radiation-induced brain injury (9, 10). Indeed, irradiation of the brain has already been shown to increase apoptosis in the neural stem cell pools of the hippocampus, a structure critical to memory and other behaviors (11, 12). Furthermore, radiation-induced decrement in stem cell populations has been shown to impact behavior in animal models (13, 14).

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Numerous antioxidants are in various stages of development for protection against or mitigation of oxidative stress triggered by radiation exposure (15). These include superoxide dismutase (SOD) mimetics (16), resveratrol (17), melatonin (18) and others. Currently, however, only amifostine is approved by the FDA for use in a clinical setting. Although amifostine, a free radical scavenger, can be useful as a radioprotectant of healthy tissues, it has numerous side-effects, *e.g.* nausea/vomiting, acute hypersensitivity reaction, pruritis, urticaria, seizures and reduction in blood pressure (http://www.drugs.com/sfx/amifostine-side-effects.html). In some cases, the side-effects are serious and require discontinuation of the drug.

Minocycline is a semi-synthetic tetracycline derivative that is commonly prescribed for a variety of bacterial infections. However, the drug has a number of actions unrelated to its anti-microbial properties that may be useful for therapy. Although the mechanisms of action are still being worked out, minocycline can efficiently cross the blood-brain barrier (BBB) and exert neuroprotective effects in animal models of cerebral ischemia, traumatic brain injury and several other pathologies related to the CNS (19). The drug is being evaluated as a possible adjunct treatment for early-stage schizophrenia and other psychiatric disorders (20-22). Minocycline reportedly also has anti-apoptotic, anti-inflammatory and anti-tumorigenic effects that include reduction of glioma cell invasiveness (23). Perhaps most important to the present study is that minocycline has free radical scavenging properties (24) and potential to protect neurons against radiation-induced apoptotic death (25). In addition, minocycline can be administered orally and has a relatively long half-life, characteristics that are favorable in case of a nuclear disaster or during deep space travel where medical support is likely to be minimal.

The data presented here are part of a larger study that involved whole-body irradiation of mice, with the intent to assess the effects of minocycline on the hematopoietic system in the context of ARS followed by recovery (26, 27). However, brains were also harvested to evaluate the potential of minocycline as an anti-inflammatory radioprotectant of the CNS in an animal model under conditions that simulate exposures that may occur during a radiological/nuclear event on Earth, as well as irradiation during missions in space. Cytokine levels and genes related to cytokines and neurotoxicity were evaluated in brain tissue, both with and without minocycline treatment. The unique data generated in this study support further investigation of this drug under conditions simulating a radiation catastrophe.

Materials and Methods

Animals and study design. Female C57BL/6 mice (n=80; 8-9 weeks of age; Charles River Breeding Laboratories, Inc. Hollister, CA, USA) were acclimatized for 5-7 days in large plastic cages (n=10/cage) under standard vivarium conditions. Animals were assigned to 8 groups (10 mice/group): a) deionized water (dH_2O) + 0 Gy; b) dH_2O + 1 Gy; c) dH_2O +2 Gy; d) dH_2O + 3 Gy; e) minocycline + 0 Gy; f) minocycline + 1 Gy; g) minocycline + 2 Gy; and h) minocycline +3 Gy. Animals were rapidly euthanized on days 4 and 32 post-irradiation using 100% CO_2 in compliance with the recommendations of the National Institutes of Health and the Panel of Euthanasia of the American Veterinary Medical Association. Brains were collected following euthanasia, cut into right and left hemispheres, and immediately snap-frozen in liquid nitrogen. All procedures were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Drug treatment and irradiation of mice. Minocycline hydrochloride was purchased from Triax Pharmaceuticals, LLC, Cranford, NJ, USA. Animals in the respective treatment groups were injected intraperitonealy (*i.p.*) with the drug (45 mg/kg in 0.1 ml) or dH_2O immediately before irradiation. A Co-60 source (Eldorado machine, Atomic Energy of Canada Ltd, Commercial Products Division, Ottawa, Canada) was used to administer 1, 2 or 3 Gy whole-body radiation at 1.58 Gy/min to mice placed individually into rectangular plastic aerated boxes (30×30×60 mm^3). A second injection of minocycline (45 mg/kg) or dH_2O was administered to the appropriate groups immediately after irradiation. Three consecutive injections of minocycline (22.5 mg/kg) or dH_2O were then administered on the following three days post-irradiation. Sham-irradiated groups were given similar treatment, but without the radiation.

Analysis of cytokines. For cytokine analysis, left hemispheres obtained from mice on days 4 and 32 were thawed. Homogenates were prepared using phosphate buffered saline (PBS without calcium and magnesium) containing a cocktail of protease inhibitor tablets from Hoffman-La Roche, Inc. (Pleasanton, CA, USA) and 0.05% Tween-20. One ml of buffer solution was used for every 0.2 g tissue for homogenization, followed by centrifugation. The supernatants were stored at –80˚C until analysis. Supernatants from brain homogenates were thawed and analyzed for 22 different cytokines and chemokines using the Mouse Cytokine/Chemokine Milliplex MAP Kit (Millipore, MA, USA) and Luminex100 (Linco Research, Inc., St. Charles, MO, USA) as per the manufacturer's instructions. The cytokines/chemokines evaluated were as follows: IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN–γ), IFN-γ-induced protein 10 (IP-10), keratinocyte chemoattractant (KC), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), regulated and normal T-cell expressed and secreted (RANTES) and TNF-α. Vascular endothelial growth factor (VEGF) and matrix metallopeptidase-9 (MMP-9) were analyzed *via* enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) as per the vendor's protocol.

Microarray analysis of cytokine and neurotoxicity gene expression. Expression of cytokine- and neurotoxicity-related genes in brain samples collected on day 32 post-irradiation was determined using quantitative real-time polymerase chain reaction (qRT-PCR). The PAMM-21 Mouse Cytokine and PAMM-96 Mouse Neurotoxicity gene arrays were purchased from SABiosciences/Qiagen, Frederick, MD, USA; each array evaluated 84 genes. Standard procedures were used throughout and have been previously described in detail (28).

Biological pathway analysis. Although we only characterized a small subset of functionally-related genes, we performed the Upstream Regulator Analysis using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc., Redwood City, CA, USA; www.ingenuity.com). We combined data collected *via* the two qRT-PCR arrays described above for this analysis. IPA upstream analysis is based solely on changes in expression of genes known to be downstream from the transcription regulator based on the literature. Our goal here was to provide information regarding signaling pathways in the brain that may be involved under the conditions of our study.

Statistical analysis. Difference in gene expression was determined using the Student's *t*-test at the SABiosciences/Qiagen Technical Core. Other data were analyzed by two-way analysis of variance (ANOVA). Tukey's test was performed for pair-wise multiple comparisons when indicated. Means and standard errors of means (SEM) are presented. A *p*-value of <0.05 indicated significance. SigmaStat™ software, version 2.03 (SPSS Inc., Chicago, IL, USA) was used. For the pathway analysis in IPA, we used the recommended activation z-score >2.0 to indicate significantly activated transcription regulators.

Results

Cytokine levels in brain tissue. Minocycline treatment, radiation or both had a significant impact on 5 of the 12 cytokines that were detectable on day 4 (Figure 1). Although radiation generally decreased IL-9 ($p<0.05$), only the 2- and 3-Gy, non-drug treated groups were significantly different from 0 Gy controls in post-hoc Tukey analysis ($p<0.05$). There was also a main effect of radiation on IP-10 ($p<0.05$). This was likely due to slight decreases noted at 1 Gy and increases at 3 Gy, although these differences did not reach significance in post-hoc analysis. Similarly, although minocycline generally decreased IP-10 compared to their non-treated counterparts, resulting in the main effect of drug ($p<0.05$), there were no drug-associated differences in post-hoc analysis. In contrast, there were drug-associated increases in IL-10, IL-15 and VEGF ($p<0.05$ for a main effect of minocycline). Although the radiation-induced decreases in IL-10 and VEGF were relatively slight, reduction in IL-10 was significant in two of the irradiated groups ($p<0.05$ *vs*. 0 Gy without drug). However, all three irradiated groups that received minocycline had high IL-10 compared to their counterparts that did not receive drug ($p<0.05$). For VEGF, the drug-induced enhancement was noted for the 1-Gy and 2-Gy groups. This divergent response resulted in a significant drug x radiation interaction for both IL-10 and VEGF ($p<0.05$). In contrast, minocycline-induced increases in IL-15 did result in a main effect of drug ($p<0.05$), but reached significance only in the 1-Gy-treated mice ($p<0.05$ *vs*. 1 Gy without drug) and the interaction was not enough to reach significance. The drug and/or radiation had no effect on the following cytokines that were detectable at day 4 of assessment: IFN-γ (20.0±0.5 to 21.5±0.7 pg/ml), KC (23.1±0.7 to 31.2±7.3 pg/ml), IL-1α (49.4±1.4 to 53.3±3.2 pg/ml), IL-4 (3.4±0.03 to 3.5±0.03 pg/ml), IL-7 (12.7±1.9 to 17.8±1.9 pg/ml), IL-13 (11.5±2.5 to 17.2±3.7 pg/ml) and MMP-9 (0.16±0.02 to 0.3±0.06 pg/ml).

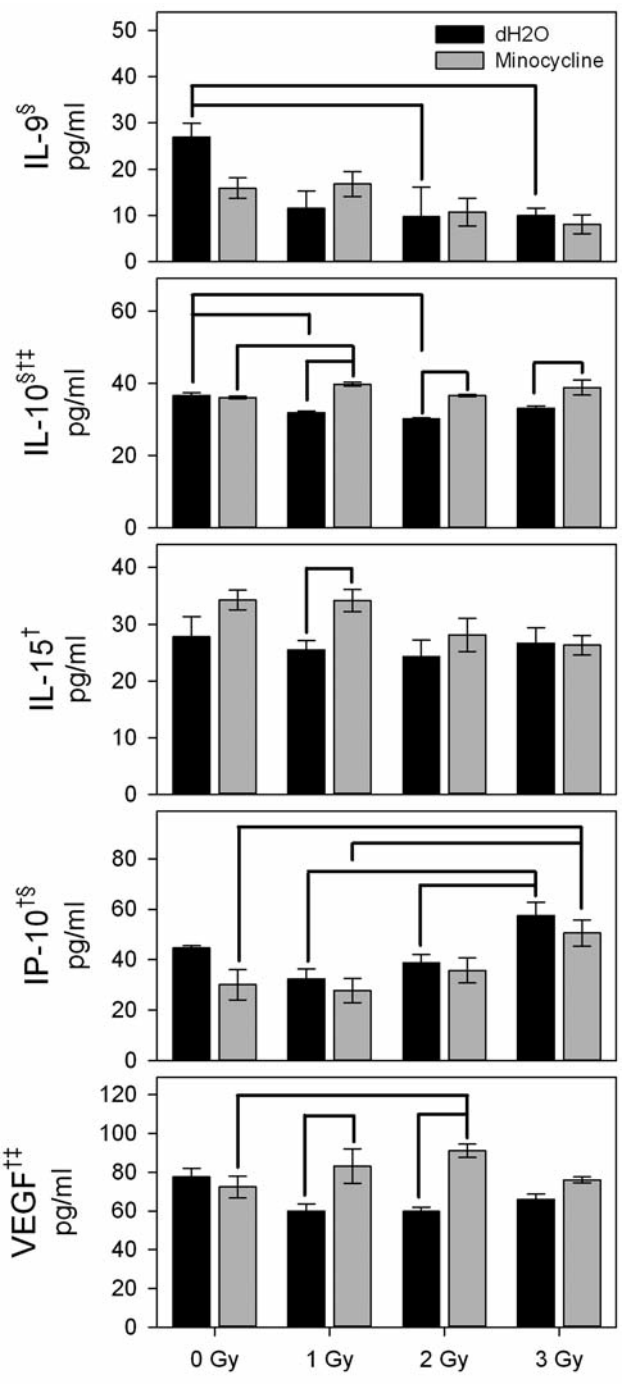

Figure 1. *Cytokines in brain at 4 days after irradiation. Each bar represents the mean±SEM for n=4-5 mice/group. Two-way ANOVA (p<0.05): §, Main effect of radiation; †, main effect of drug; ‡, drug x radiation interaction. Tukey test: Lines above bars indicate the groups that differ significantly, p<0.05.*

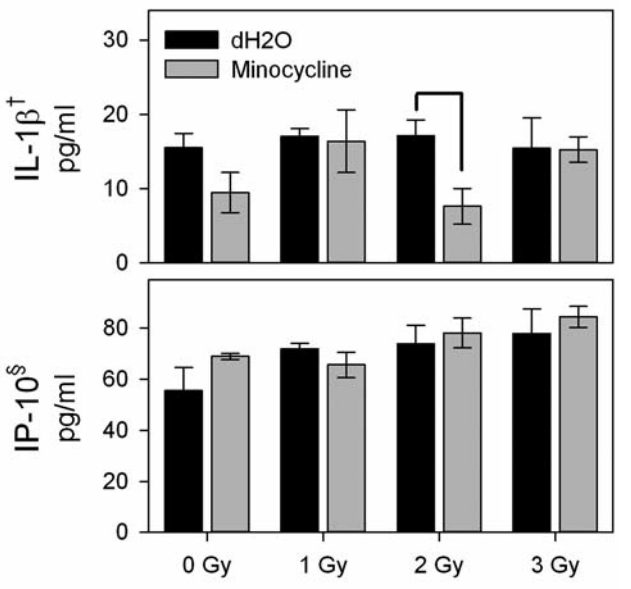

Figure 2. *Cytokines in brain at 32 days after irradiation. Each bar represents the mean±SEM for n=4-5 mice/group. Two-way ANOVA (p<0.05): §, Main effect of radiation; †, main effect of drug. Tukey test: Lines above bars indicate the groups that differ significantly, p<0.05.*

On day 32 after irradiation, 10 cytokines were detectable. However, significance was obtained for only two of these, *i.e.* IL-1β and IP-10 (Figure 2). The main drug effect on IL-1β ($p<0.05$) was most likely due to the relatively low levels at 0 and 2 Gy compared to non-drug-treated counterparts. This drug effect reached significance in post-hoc analysis at 2 Gy ($p<0.05$). There were no main effects or interactions involving radiation for IL-1β. Although, radiation generally increased IP-10 ($p<0.05$ for a main effect of radiation), minocycline had no effect and there were no significant differences between any groups in *post-hoc* analysis. The range for each of the detectable cytokines that were not affected by either drug or radiation were as follows: G-CSF (8.4±0.2 to 9.2±0.3 pg/ml), IL-6 (6.6±0.4 to 8.6±0.3 pg/ml), IL-9 (34.1±10.9 to 57.1±7.7 pg/ml), IL-15 (13.6±3.9 to 23.5±2.3 pg/ml), KC (17.4±4.5 to 24.0±0.1 pg/ml), MIP-1α (21.6±5.7 to 34.2±2.3 pg/ml), MMP-9 (0.1±0.01 to 0.2±0.02 pg/ml) and VEGF (77.8±4.2 to 104.6±15.8 pg/ml).

Cytokine and neurotoxicity gene expression in the brain. These analyses were performed on the 0-Gy and 3-Gy groups (with and without drug) only on day 32 post-irradiation. To be considered significant, the changes in gene expression had to meet the criteria of $p<0.05$ and fold-change ≥1.5 *vs.* 0 Gy (no drug).

Figure 3 shows results from the cytokine gene array. Exposure to 3-Gy radiation alone increased the expression of 23 genes: *Csf2, Ctf1, Il15, Inha, Il11, Il17c, Il20, Tnfrsf11b, Tnfsf12, Gdf1, Ifnb1, Gdf15, Il12b, Il1f6, Il1f8, Il24, Il1f10,*

Table I. *Cytokine-related genes in brain tissue that were significantly modulated on day 32 after irradiation compared to dH_2O + 0 Gy.*

Gene	Gene description
Bmp5	Bone morphogenetic protein 5
Bmp6	Bone morphogenetic protein 6
Bmp7	Bone morphogenetic protein 7
Bmp10	Bone morphogenetic protein 10
Cd70	CD70 antigen
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
Ctf1	Cardiotrophin 1
Gdf1	Growth differentiation factor 1
Gdf2	Growth differentiation factor 2
Gdf5	Growth differentiation factor 5
Gdf10	Growth differentiation factor 10
Gdf15	Growth differentiation factor 15
Ifnb1	Interferon beta 1, fibroblast
Il1f6	Interleukin 1 family, member 6
Il1f8	Interleukin 1 family, member 8
Il1f9	Interleukin 1 family, member 9
Il1f10	Interleukin 1 family, member 10
Il1rn	Interleukin 1 receptor antagonist
Il3	Interleukin 3
Il11	Interleukin 11
Il12b	Interleukin 12B
Il15	Interleukin 15
Il17c	Interleukin 17C
Il20	Interleukin 20
Il24	Interleukin 24
Inha	Inhibin alpha
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10
Tnfsf11	Tumor necrosis factor (ligand) superfamily, member 11
Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12

These genes had fold-change ≥1.5 and $p<0.05$ compared to the control group that received no minocycline treatment and no radiation.

Table II. *Neurotoxicity-associated genes in brain tissue that were significantly modulated on day 32 after irradiation compared to dH_2O + 0 Gy.*

Gene	Gene description
Atf4	Activating transcription factor-4
Bik	Bcl2-interacting killer
Birc2	Baculoviral IAP repeat-containing 2
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A
Ereg	Epiregulin
Fas	Fas (TNF receptor superfamily member 6)
Hspa5	Heat shock protein 5
Nostrin	Nitric oxide synthase trafficker
Pappa	Pregnancy-associated plasma protein A
Tacr1	Tachykinin receptor-1
Txnip	Thioredoxin interacting protein
Tyrp1	Tyrosinase-related protein 1

These genes had fold-change ≥1.5 and $p<0.05$ compared to the control group that received no minocycline treatment and no radiation.

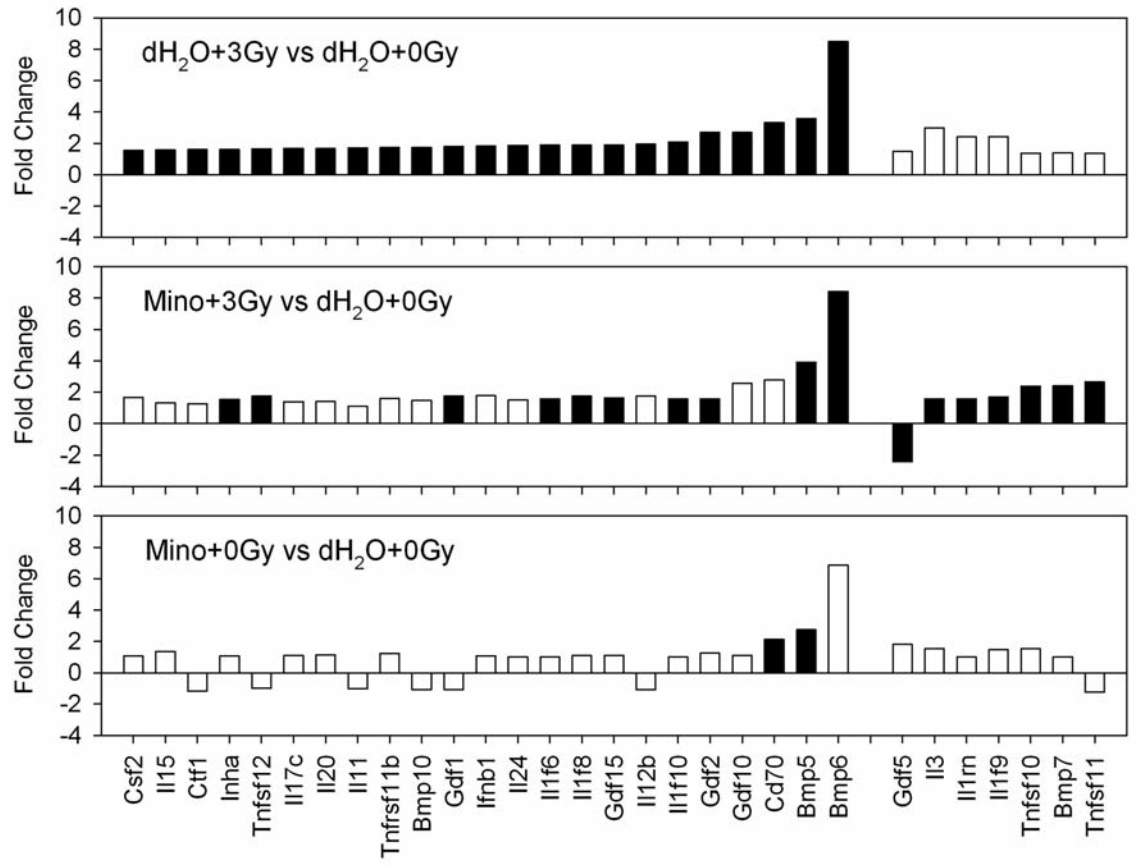

Figure 3. *Fold-change in cytokine-related genes on day 32 post-irradiation. Data were obtained using an 84-gene microarray and quantitative real-time polymerase chain reaction. Black bars: p<0.05.*

Gdf10, Gdf2, Cd70, Bmp5, Bmp6 and *Bmp10*. In the 3-Gy group that also received minocycline, the expression of 13 of these genes was equivalent to controls. In addition, the combined treatment resulted in down-regulation of *Gdf5* and up-regulation of *Il1rn, Il3, Il1f9, Bmp7, Tnfsf10* and *Tnfsf11*, genes that were not modulated by either radiation or drug alone. The effect of drug alone was very minimal, *i.e. Cd70* and *Bmp5* had increased expression *versus* 0 Gy (no drug). Table I presents a brief description of the 30/84 cytokine-related genes with significantly modified expression compared to 0 Gy (no drug).

Figure 4 presents results from the neurotoxicity array. Exposure to 3 Gy radiation alone increased the expression of 10 genes: *Ereg, Hspa5, Nostrin, Birc2, Txpnip, Atf4, Fas*, *Bik, Cidea* and *Pappa*. The increase in five of these genes was no longer present in the irradiated group that was treated with minocycline. As with the cytokine array, drug alone had very little effect; only *Tryp1* was up-regulated *versus* 0 Gy (no drug). Overall, the expression of 12/84 neurotoxicity genes was significantly modified compared to 0 Gy (no drug). A brief description of these genes is presented in Table II.

Biological pathways. Due to the nature of the assessed genes, the limited number of significant changes, and the fact that "functionally-related" genes (as defined by SABiosciences) are not necessarily all part of the same biological pathway, we focused the analysis on upstream transcription regulators. Based on this analysis, two transcription regulators were significantly activated by one or both treatments: CTNNB1 and TP53 (Figure 5). Although we did not characterize the expression of the *CTNNB1* gene itself, based on the IPA analysis of downstream activity, CTNNB1-dependent regulation was predicted to be significantly activated (activation z-score=2.2) in the dH_2O + 3-Gy group compared to the dH_2O + 0-Gy controls. This regulation was no longer significant when the mice were also treated with minocycline (Figure 5). In contrast, TP53-dependent transcription regulation was significantly activated only in the Minocycline+3-Gy group compared to dH_2O+0-Gy controls (activation z-score=2.1). Neither of these transcription regulators were predicted to be activated in the Minocycline + 0-Gy group.

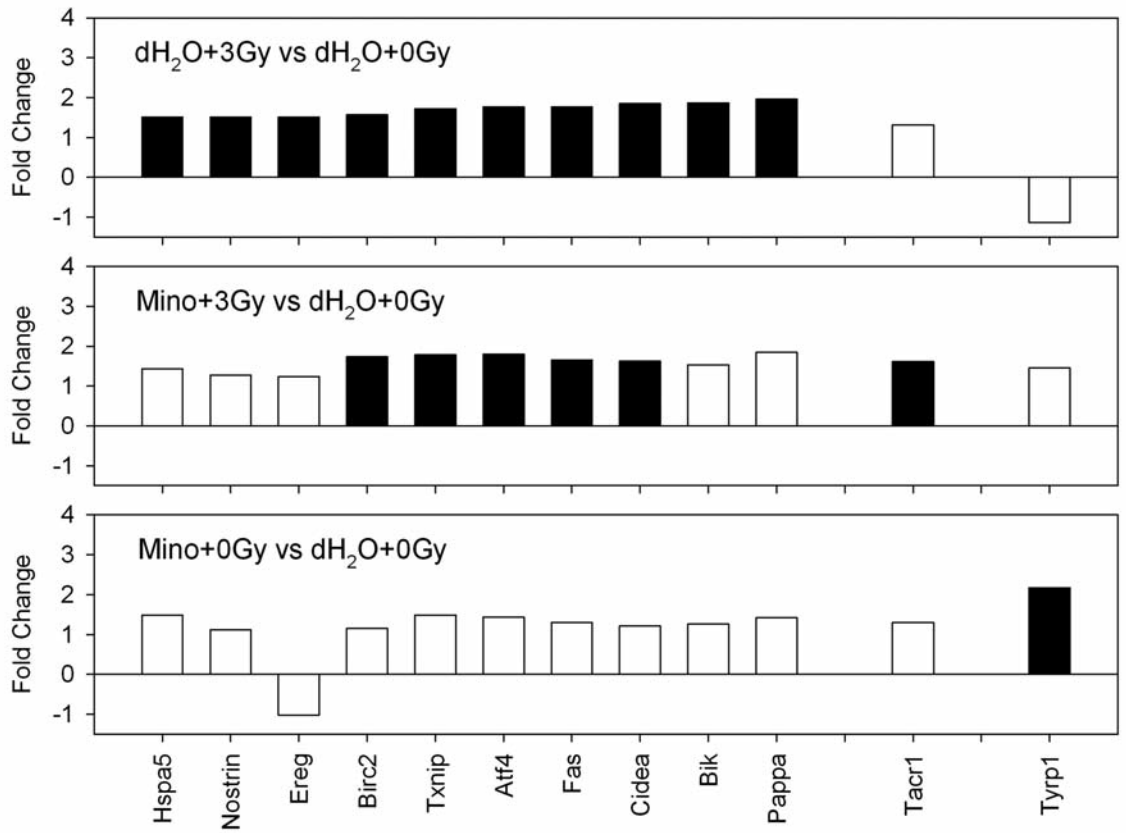

Figure 4. *Fold-change in neurotoxicity-related genes on day 32 post-irradiation. Data were obtained using an 84-gene microarray and quantitative real-time polymerase chain reaction. Black bars: p<0.05.*

Discussion

A number of differences were noted in brain cytokine levels, especially at the early time point of assessment. On day 4, minocycline increased IL-10 significantly in all irradiated groups compared to their irradiated counterparts that did not receive the drug (Figure 1). In studies of traumatic spinal cord injury and neurogenic hypertension, an increase in IL-10 upon treatment with minocycline has also been reported (29, 30). This cytokine is produced by leukocytes involved in both adaptive and innate immunity, *e.g.* $CD4^+$ T-cells, neutrophils, macrophages and others (31, 32). It has potent anti-inflammatory effects and is currently being evaluated in clinical trials for pathologies that include inflammation (33). IL-10 also possesses neuroprotective properties that have been attributed to its ability to suppress pro-apoptotic proteins (34, 35). This is an important characteristic, since apoptosis is among the most significant responses of the brain to damage caused by ionizing radiation (11).

Minocycline increased VEGF in the 1- and 2-Gy irradiated groups on day 4 (Figure 1), but not on day 32. Similar to IL-10, an increase did not occur in the 0-Gy group. This cytokine is produced under hypoxic conditions in many body compartments by cells that include astrocytes, macrophages and fibroblasts (36-38). However, although an increase in VEGF in response to minocycline has been previously reported (39, 40), to our knowledge this is the first study to show that this occurs in brains of irradiated animals. VEGF can be neuroprotective under a variety of conditions that include radiation damage. For example, it has been demonstrated that deficiency in hippocampal neurogenesis after low radiation doses can be reversed when VEGF is increased (41). Although we did not determine the cell type(s) producing VEGF, astrocytes are a likely source. Others have noted that minocycline increases astrocyte viability in a mouse model of ischemic stroke (42). In our study, the drug may simply have arrested the astrocytes in a relatively radioresistant phase of the cell cycle. It is important to note, however, that astrocytes have numerous activities in the CNS, some of which do result in neuron protection, whereas others can be destructive (43).

Minocycline increased the levels of IL-15 in brains of mice exposed to 1 Gy (day 4; Figure 1), but did not modify

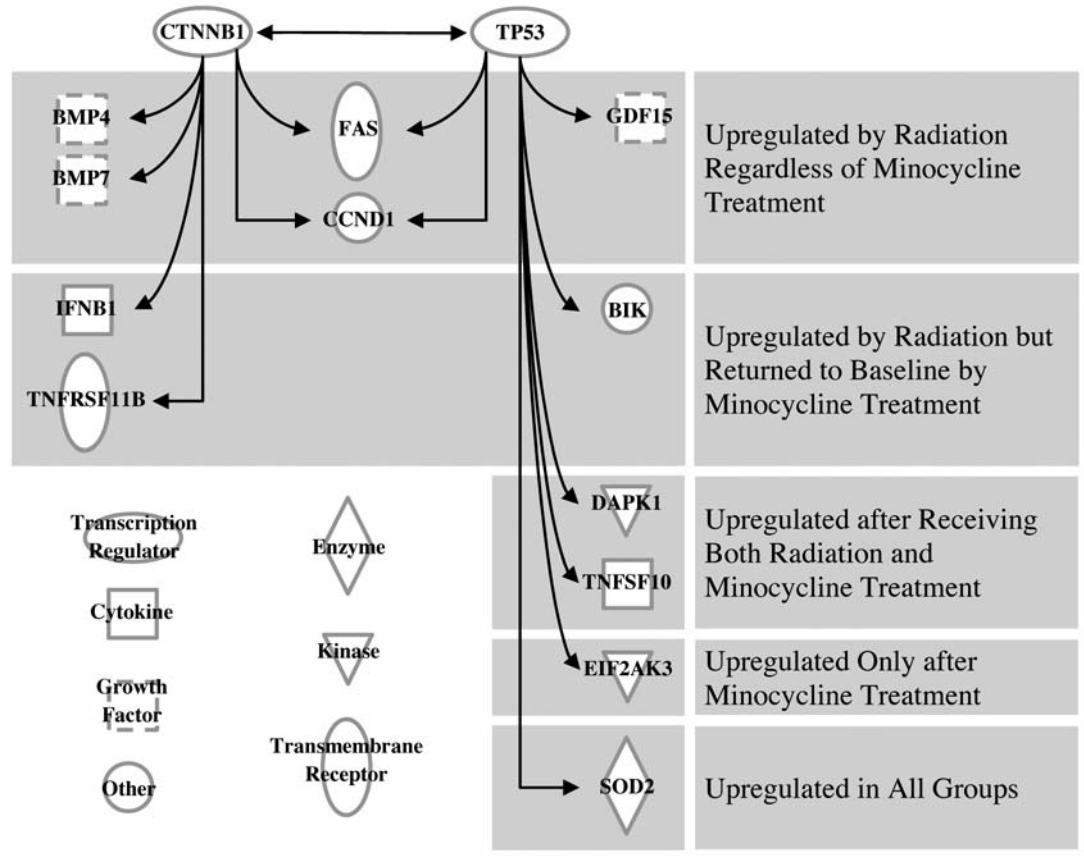

Figure 5. *Transcription regulators at day 32 post-irradiation. Upstream Regulator Analysis using IPA indicated that the activity of two transcription regulators was significantly impacted by the treatments. CTNNB1: Activation z-score=2.2 in the dH_2O + 3 Gy group vs. dH_2O + 0 Gy group. TP53: activation z-score=2.1 in the Minocycline + 3Gy group vs. dH2O + 0 Gy group.*

the levels of the cytokine in the other irradiated groups. This suggests that the drug had an impact on the production of IL-15 only when the radiation dose was relatively low. This cytokine is produced by many cell types, including macrophages, and can cross the BBB (44, 45). Although IL-15 has usually been reported to be pro-inflammatory, some studies have suggested an anti-inflammatory role for this molecule (46). An additional potentially beneficial effect of IL-15 is facilitation of neurogenesis (47).

IP-10, also known as C-X-C motif chemokine 10 (CXCL10), was the only cytokine on which radiation had a main effect at both time points of analysis (Figures 1 and 2). Furthermore, the level of IP-10 was consistently lower on day 4 in the drug-treated groups, regardless of radiation. This could be a positive finding, since the chemokine is implicated in pathologies that include injury and inflammation (48). IP-10 is constitutively expressed in lymphoid organs, but its production can be induced in a wide variety of cells, including T-lymphocytes and endothelial cells (49, 50). The drug-related decrease, however, was no longer evident on day 32. Beneficial health effect, if any, related to the early drug-induced decrease in IP-10 remains to be determined.

Minocycline reduced the level of IL-1β in the brain on day 32 (Figure 2). Although this decrease was especially pronounced in the 2-Gy irradiated group, relatively low values were noted in the 0-Gy group as well. The somewhat sporadic nature of IL-1β level in the groups that received minocycline plus varying doses of radiation may be related to lack of a strict dose response, *i.e.* discontinuous dose-dependency, as has been previously reported for cells that secrete this cytokine (51). Another possibility may be related to the fact that cytokine secretion has a cyclic pattern after radiation exposure (52). Thus, it seems possible that different radiation doses together with minocycline may have resulted in dose-dependent shifts in the cycling cascade that involves this cytokine. IL-1β is well-known to be produced in large amounts by cells of the monocyte-macrophage lineage that include microglia. Minocycline-induced reduction of IL-1β in the brain has been observed by other researchers in

models of Alzheimer's disease and excitotoxicity (53, 54). Since this cytokine has been implicated in numerous neurodegenerative diseases and is reported to be neurotoxic (55-57), its reduction may be beneficial. Furthermore, studies have shown that IL-1β (as well as IL-1α) produced by microglia contributes to neurodegeneration (58) and neuron loss has been associated with a simultaneous increase in activated microglia (59). Thus, our findings are consistent with reports that minocycline suppresses microglial production of certain cytokines such as IL-1β (60).

It is worthy to note that we have previously found, under the same conditions as the ones used here, that minocycline enhanced the numbers and/or percentages of certain leukocyte populations in the blood and spleen, when combined with radiation (26, 27). The enhancement was especially pronounced for splenic granulocytes on day 4 post-irradiation. We also found that drug treatment resulted in high levels of G-CSF and GM-CSF that are well-known to facilitate granulocyte recovery after radiation exposure. However, since the high levels were found in spleen supernatants after activation of T-lymphocytes with immobilized antibody against CD3, direct comparison with brain cytokines in the present study is not possible.

Neutrophils, the most abundant granulocyte type in blood, are rapidly recruited to injured sites to begin repair of the damage. When activated, these cells release a range of cytokines, *e.g.*, IL-6, IL-8, IL-12, TGF-β and TNF-α (61, 62). Neutrophils can induce T-cell migration and influence the activities of endothelial cells that comprise the BBB, microglia and astrocytes, all of which can also produce cytokines. In addition, some cytokines can be transported directly across the BBB (63). Given the complexity of actions and interactions among cytokine-secreting cells, it is not possible, at this time, to identify any one specific cell type as the source responsible for the minocycline-induced changes in the present study.

Microarray analyses of brains from mice on day 32 showed that the expression of 42 out of a total of 168 evaluated genes was significantly different from the 0 Gy (no drug) control group (all panels in Figures 3 and 4). Thirty-three of these genes were modified by 3 Gy radiation alone, *i.e.*, 23 cytokine-related genes (Figures 3 top panel) plus 10 neurotoxicity-related genes (Fig. 4 top panel). This is not entirely surprising, since other investigators have reported radiation-induced aberrations in the brain a long time after whole-body exposure (64, 65). With the combined treatment (minocycline + 3 Gy), the expression of many genes altered by radiation alone, was no longer different from normal and some genes that were not modified by radiation alone were affected (Figures 3 and 4 middle panels). Because so many genes were affected, further discussion must be limited to only a few.

Expression of *Gdf10* was up-regulated (2.7-fold) by radiation-alone, but was equivalent to normal when minocycline treatment was included (Figure 3 top two panels). Since some Gdf are reported to be neuroprotective (66), a reason why *Gdf10* expression was not increased in the presence of the drug could be due to less damage and hence also less need to promote recovery. In support of this possibility is that radiation did enhance the expression of several cytokines that contribute to brain inflammation, but not when minocycline treatment was added. In addition, *Il12b* (up-regulated by radiation alone, but not in either of the minocycline groups; Figure 3 all panels) encodes a segment of the pro-inflammatory IL-12 cytokine. *Il17c*, another gene that encodes a potent cytokine important in brain inflammation, was no longer up-regulated when drug treatment was included.

Expression of *Tnfrsf11b*, a member of the TNF superfamily, was enhanced when radiation was used alone, but not when combined with minocycline (Figure 3 top two panels). Many diseases with an inflammatory component are associated with over-production of factors in this superfamily (67). Minocycline-induced reduction of TNF-α in the brain has been previously reported (68), thus further supporting the possibility that the drug may reduce the risk for neurocognitive deficits. However, the drug had no effect on the radiation-induced increase in *Tnfsf12* and both *Tnfsf10* and *Tnfsf11* were significantly up-regulated only in the combined-treatment group (Figure 3 middle panel).

Radiation and minocycline increased the expression of *Bmp5* by >2.5-fold, regardless of whether they were administered alone or in combination (Figure 3 all panels). It has been suggested that the protein derived from *Bmp5* may ameliorate Parkinson's disease, a condition that includes brain inflammation (69). Overall, the most affected gene was *Bmp6*; a >8-fold increase in expression occurred in the 3-Gy irradiated groups (with and without minocycline; Figure 3 top two panels). Thus far, this and the other *Bmp* genes have been studied primarily in the context of bone regeneration. Much more research is needed to clarify the roles that these genes play under conditions of radiation-induced oxidative stress in the brain.

It is interesting to note that many of the cytokine genes that were up-regulated in the irradiated mice are typically released by antigen-presenting cells, including dendritic cells (*Il12b, Il15, Il20* and *Ifnb1*) and macrophages (*Il1f10, Il36a* and *Il36b*). Because we did not isolate any specific cell type from the brain tissue for the gene expression analysis, we cannot be certain if the cytokines were up-regulated in resident or infiltrating cell populations that present antigens (70). Migration of these cell types from peripheral sites into the brain has been linked to neurodegeneration and a variety of CNS diseases (71, 72). The fact that the expression of many of these genes was no longer enhanced in mice treated with minocycline further suggests that the drug, indeed, has a significant radioprotective role.

Data from the neurotoxicity array (day 32) showed that radiation alone up-regulated expression of five genes whose expression was equivalent to normal when combined with minocycline (Figure 4 top two panels). These genes included Bcl-2 interacting killer (*Bik*), nitric oxide synthase trafficker (*Nostrin*) and heat shock 70 kDa protein 5 (*Hspa5*). *Bik* encodes a pro-apoptotic protein while the protein derived from *Nostrin* induces the synthesis of nitric oxide. Reduction in the expression of *Nostrin* is consistent with reports showing that minocycline inhibits nitric oxide (73, 74).

After characterization of the 168 functionally-related genes, we were able to identify two transcription regulators that were likely to have been activated in one or more of our treatment conditions (Figure 5). Although we did not directly measure expression of the CTNNB1 gene that encodes catenin-β1, upstream regulator analysis indicated that its activity was increased in mouse brains sometime after exposure to 3 Gy (activation z score=2.2). This increased activity is indicated by the up-regulation of BMP4, BMP7, IFNB1, TNFRSF11B, FAS and CCND1 expression. The increase in CTNNB1-regulated activity was no longer present when mice were also treated with minocycline, primarily due to decreases in IFNB1 and TNFRSF11B activity. CTNNB1 is an important part of the cadherin adhesion complex that plays a major role in the canonical Wnt signaling pathway (75). Radiation-induced activation of the Wnt/catenin-β signaling cascade has been previously reported in the hippocampus of mice by Wei *et al*. (76). The study also demonstrated that a low radiation dose (0.3 Gy) reduced apoptotic death of neuronal stem cells.

Furthermore, TP53-dependent activity was significantly enhanced in brains from mice that received both radiation and minocycline (activation z score=2.1), based on increases in GDF15, DAPK1, TNFSF10, SOD2, FAS and CCND1 expression (Figure 5). TP53, a well-known tumor suppressor (77), plays a key role in determining whether DNA repair or apoptotic cell death takes place. Since it also functions in DNA repair and recombination independently of its transcriptional properties (78), it seems possible that enhancement of its activity by minocycline in the irradiated mice may be beneficial. This possibility is supported by the drug-induced return to baseline in BCL-2 interacting killer (BIK) that is pro-apoptotic. However, it must be noted that TP53-dependent regulation could up-regulate DAPK1 (death-associated protein kinase) that facilitates inflammasome formation (79) and TNFSF10 (tumor necrosis factor-related apoptosis-inducing ligand, also known as TRAIL) that may promote vascular calcification (80).

Overall, our data increase knowledge on the potential of minocycline as a radioprotectant in a radiation setting that involves the CNS. A major promising finding is that the drug increased the production of cytokines that are anti-inflammatory, neuroprotective and/or neurogenic in an intact mammalian model. Although in some cases, *e.g.*, IL-10 and VEGF, the drug-associated increases were relatively small, a beneficial effect seems possible in the context of the microenvironment within which they were produced. Cytokines are highly potent proteins that are capable of mediating biological effects at concentrations ranging from pM to nM (52). In addition, the drug "normalized" expression of genes that were up-regulated by radiation-alone and may preserve viability of cells such as astrocytes. IPA upstream analysis indicated that two transcription regulators were likely to have been activated under one or more of our treatment conditions, but confirmation requires additional work, *e.g.* western blot and immunoprecipitation assays. It would also be interesting to evaluate other tissues to determine if the observed changes are specific to the brain. Finally, it should be emphasized that firm conclusions cannot yet be made. Further studies with minocycline are needed to confirm its full potential as a radioprotective agent of the CNS and whether it can reduce radiation-induced cognitive decline. Understanding the mechanisms by which the drug modifies radiation response is also essential in order to optimize its utilization in a clinical setting.

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