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Renin-Angiotensin System Suppression Mitigates Experimental Radiation Pneumonitis

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Abstract

Purpose—To find mitigators of pneumonitis induced by moderate doses of thoracic radiation (10–15 Gy).

Materials and Methods—Unanesthetized WAG/RijCmcr female rats received single doses of X-irradiation (10, 12 or 15 Gy at 1.615 Gy/minute) to the thorax. Captopril (an angiotensin converting enzyme inhibitor) or losartan (an angiotensin receptor blocker) was administered in drinking water after irradiation. Pulmonary structure and function were assessed after 8 weeks in randomly selected rats by evaluating breathing rate, *ex vivo* vascular reactivity and histopathology. Survival analysis was undertaken on all animals except those scheduled for sacrifice.

Results—Survival following a dose of 10 Gy to the thorax was not different from unirradiated rats up to one year. Survival decreased to less than 50%, by 45 weeks after 12 Gy and by 8–9 weeks after 15 Gy. Captopril (17–56 mg/kg/day) improved survival and reduced radiation-induced increases in breathing rate, changes in vascular reactivity and histopathological evidence of injury. Radiation-induced increases in breathing rate were prevented even if captopril was started 1 week following irradiation or if it was discontinued after 5 weeks. Losartan, though effective in reducing mortality was not as efficacious as captopril in mitigating radiation-induced increases in breathing rate or altered vasoreactivity.

Conclusions—In rats, a moderate thoracic dose of radiation induced pneumonitis and morbidity. These injuries were mitigated by captopril even when it was commenced 1 week after irradiation or

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It is declared that none of the authors have any commercial associations that might give rise to a conflict of interest in connection with the article.

if discontinued after 5 weeks following exposure. Losartan was less effective in protecting against radiation-induced changes in vascular reactivity or tachypnea.

Keywords

Radiation injury; mitigation; lung function; angiotensin converting enzyme (ACE) inhibitors

INTRODUCTION

The lung is a sensitive target for injuries that develop months to years after exposure to ionizing radiation (1). Preclinical research indicates that radiation-induced injuries may be treatable, though no therapies are currently FDA-approved (2). Our interest is to develop drugs for mitigation (agents given after irradiation, but before symptoms manifest) of lung dysfunction that may occur in survivors of radiation accidents or radiological attacks (3). The relevance of such studies are of increasing value due to the rising demand for nuclear energy and threats of radiological terrorism (2,3). In this regard we have developed a rat model of pulmonary injury after single non-lethal doses of radiation (4). To minimize effects on other organs (except the heart) irradiation was localized to the thorax. Rats were housed in a barrier to minimize infections. These animals developed pneumonitis at 4–12 weeks following a dose of 10 Gy, but not after 5 Gy (4). Structural and functional analyses of the lung revealed histopathological changes that were accompanied by an increase in the breathing rate and a loss of vascular reactivity (measured *ex vivo*).

Since radiation exposures to humans at volumes encompassing both lungs are rare, development of agents for mitigating the effects of such irradiation must largely be restricted to animal models. Free radical scavengers (5), antioxidants (6), blockers of growth factors and inhibitors of the renin angiotensin system (RAS) (7) have demonstrated efficacy for mitigation of radiation injuries in animal models (2,8). These studies are preliminary and do not explicitly address radiation accident or terrorism scenarios (9,11). For example, in previous studies, ACE inhibitors captopril or enalapril, and the AT1 receptor blocker L158,809, were administered *before* exposure to radiation. In addition, the doses of radiation were *fractionated*, rendering them of uncertain relevance to single-dose exposures. Radiation was often applied to the hemithorax, which may not reflect the consequences of simultaneous injury in both lungs. Finally, many of the studies were conducted to evaluate the effect of the total body irradiation for bone marrow transplantation which included a preparatory course of cyclophosphamide. We chose to study two FDA-approved RAS inhibitors in our model of rat radiation pneumonitis: the angiotensin converting enzyme (ACE) inhibitor captopril, and the angiotensin II, type-1, (AT1) blocker, losartan.

METHODS

Animal Model

All protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. Rats (female WAG/RijCmcr) were housed in a moderate security barrier. After exposure to radiation, the drinking water of preselected groups were supplemented with pharmaceutical-grade captopril (Sigma) or losartan (Merck) immediately, or after a delay of 1 or 2 weeks. Drugs were delivered in drinking water at concentrations from 100–500 mg/L (delivering 11–56 mg/kg/day). The drug doses were chosen within range, on a mg/m²/day basis, of those used in humans for treatment of hypertension and other clinical applications.

All animals were followed until morbid except those randomly selected for assays at 8 weeks after irradiation. Rats were euthanized if they appeared distressed and were not eating or

drinking, inactive, hunched, and ungroomed. Over 300 rats were included in the study. Five rats were lost to follow-up prior to 3 weeks after irradiation (2 controls, 1 at 12 Gy and 2 at 15 Gy). These rats were excluded from the study as their cause of death was not believed to be due to irradiation but from congenital conditions like hydrocephalus.

Unanesthetized 9–10 week-old rats weighing approximately 140 grams were given whole-thorax irradiation with a single dose of 10, 12 or 15 Gy of X-rays as previously described (4).

One group was maintained as unirradiated controls and housed under identical conditions. Changes in structure and function were assessed at 7–8 weeks after irradiation unless otherwise specified.

Doses of radiation and drug schedules

Rats treated with 10 Gy received 17 or 56 mg/kg/day of captopril or 11 or 56 mg/kg/day losartan. Rats given 12 or 15 Gy received 34 or 56 mg/kg/day of captopril or losartan, respectively. These drugs were started immediately after radiation and continued for 8 weeks till assay. Rats treated with 12 Gy received drugs in 3 additional schedules, (i) starting one week and up to 8 weeks (ii) 2 weeks up to 8 weeks (iii) starting immediately after radiation and discontinued after 5 weeks.

Lung Injury Endpoints

Breathing rate was measured as described previously (4) for all animals in the study that survived to 8 weeks after exposure.

Vasoactivity studies were carried out as previously published (4,12). Briefly, the lungs were harvested for microdissection of pulmonary arteries (PAs) and histological examination from rats randomly selected from control and irradiated groups. Dissected pulmonary artery rings (1 mm in diameter) were contracted with either Ang II (10^{-10} M to 10^{-7} M, Sigma catalogue # A-9525, St. Louis, MO, USA) or U46619 (10^{-8} M to 10^{-7} M, Biomol International, Catalogue # PG023-0001, Plymouth Meeting, PA, USA). All rings were washed till they returned to basal state and recontracted with KCl (80 mM).

For relaxation studies, rings were precontracted with U46619 (10^{-7} M) and then treated with 10^{-6} M acetylcholine to induce endothelial-dependent relaxation (13), washed and then treated with KCl. Vasorelaxation was calculated as [tensionU46619-tensionacetylcholine/tension U46619].

For histopathology, lung tissue ~100 mg in weight, was fixed in zinc formalin for 24 to 48 hr and stained with hematoxylin and eosin (H&E) or Massons trichrome as described previously (4).

After staining, the samples were evaluated in a blinded manner (14).

Statistical Analyses

Data for animal mortality were graphically represented as Kaplan-Meier survival plots. Animals that were scheduled for sacrifice at 8 weeks were treated as lost to follow up if sacrificed on schedule at 8 weeks, and as morbid if they needed to be euthanized prior to that time. Significance was assessed by the Peto-Peto-Wilcoxon Test. Data for breathing rates and changes in vasoreactivity were calculated as mean $\pm$ sem (standard error of the means). Values from the test groups (irradiated, age-matched un-irradiated and animals treated with and without captopril or losartan) were compared using a one way ANOVA with all pairwise multiple comparisons computed by the Holm Sidak method. Differences between groups were

considered significant if the p value was below the critical level defined by the test (always < 0.05). In order to account for bias due to attrition, rats that died of radiation lung injury before assay (8 weeks; 12 Gy, 6/33 rats; 15 Gy, 7/22 rats) were assigned a sub-maximal breaths per minute value, and statistical differences reconfirmed using the non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks test.

RESULTS

Prosurvival actions of captopril and losartan

We have previously described that thoracic irradiation to a dose of 10 Gy did not affect acute survival of rats and also did not generate fibrotic injury to the lung (4). Figure 1A shows morbidity after whole thoracic doses of 10, 12 and 15 Gy. Survival after 15 Gy was reduced to less than 50% by 8–9 weeks after irradiation (Figure 1A), but survival was increased to above 80% by treatment with captopril at 56 mg/kg/day started immediately after radiation (Figure 1B). In further studies, we used a lower dose of radiation (12 Gy), to minimize the morbidity and allow longer follow-up. At 12 Gy there was less than 20% mortality by 8 weeks following radiation, and this was completely reversed by administration of captopril at doses as low as 25 mg/kg/day (result not shown) or 34 mg/kg/day (Figure 1C) or by losartan (34 mg/kg/day).

Mitigation of lung injury by captopril and losartan

We previously assessed lung structure by histology and function by measuring breathing rate from 3 days to one year after radiation. We observed that pneumonitis peaks around 7–8 weeks (4). We therefore tested the two drugs, captopril and losartan, for mitigation starting soon after radiation and assaying for three end points, breathing rate, vascular reactivity and histology at 8 weeks.

(i) Functional recovery of breathing rate—Breathing rates were not significantly increased after 10 Gy at 8 weeks as compared to unirradiated rats (data not shown). At 12 Gy there was a significant elevation in breathing rates (Figure 2A). Captopril or losartan (34 mg/kg/day) were effective in limiting this increase (Figure 2A). Captopril and losartan (56 mg/kg/day), started immediately after irradiation also attenuated the increase in breathing rate caused by 15 Gy (Figure 2B).

(ii) Recovery of vasocontraction—As previously reported (4), at 8 weeks after 10 Gy irradiation, there was a decrease in contraction of PAs to Ang II, to only 30% of the tension developed in unirradiated controls (Figure 3A). Administration of captopril (17–56 mg/kg/day), starting within hours after irradiation mitigated this loss in reactivity (Figure 3A&B). In contrast losartan (11 mg/kg/day) at a clinically-equivalent dose did not improve vascular reactivity (Figures 3A&B). Similar trends were observed if the PA rings were contracted with high concentrations of potassium (80 mM KCl) (Figure 3C).

Vascular contraction in response to Ang II was attenuated in rats irradiated with 15 Gy as compared to unirradiated controls (Figures 3D and E). Treatment with a high dose of captopril (56 mg/kg/day) immediately after 15 Gy improved the loss of vasoactivity to Ang II or KCl (Figures 3 D and E). Losartan at the same dose and in the same schedule was less effective.

However, after 12 Gy the decrease in contraction to KCl was attenuated by captopril and losartan (Figure 4B). For rats receiving 12 Gy we tested a different vasoconstrictor, the thromboxane mimetic U46619, to enable us to study vasorelaxation (described next). We did not observe a change in contraction to U46619 in animals given 12 Gy (Figures 4A) and

treatments with captopril and losartan at 34 mg/kg/day did not change vasocontraction to this agent (Figure 4A).

(iii) Mitigation of vasorelaxation (endothelial function)—We tested the PAs precontracted with U46619 for relaxation by an endothelial-dependent vasodilator acetylcholine (Figure 4C), as this is a well-known method for monitoring the status of the vascular endothelium (15). There was a significant attenuation (~25%) in relaxation of PAs from rats irradiated with 12 Gy; this was mitigated in animals treated with captopril at 34 mg/kg/day for 8 weeks. Losartan at the same dose was less effective (Figure 4C).

(iv) Mitigation of histopathological changes—To conserve animal numbers, rat lungs that were used for dissection to harvest PAs were also used for histology (Figure 5). Thus lungs were fixed in an uninflated state. One of the pathologies in these uninflated lungs by 8 weeks after exposure to 10 Gy was mild fibrosis around vessels and airways (see arrow in Figure 5B) (4) which appeared to be decreased by treatment with captopril (Figure 5E). Irradiation at 12 Gy resulted in changes that were more marked than those observed at 10 Gy, and these also appeared to be mitigated by captopril (Figure 5C&F). 15 Gy treatment showed an increase in the infiltration of inflammatory cells and vessel wall thickening (Figure 5D) in addition to the pathologies observed with 10 and 12 Gy. These changes were less severe in rats treated with captopril (Figure 5G). The degree of mitigation by losartan for all doses of radiation was observed to be less than that noted with equivalent doses of captopril (data not shown).

(v) Optimization of mitigation schedules—To determine an optimal schedule for effective mitigation, a dose of 34 mg/kg/day was selected with 12 Gy since that dose of captopril mitigated most functional injuries at 12 Gy. Because it is not expected that therapy will be available to mass casualty victims for as much as 1–2 weeks following an attack, we assessed the effect of delaying administration of the drugs by 1 or 2 weeks. Commencement of captopril 1 week but not 2 weeks after radiation decreased the breathing rate to values close to unirradiated controls (Figure 6D). Captopril started immediately after radiation but discontinued after 5 weeks was also effective while losartan started after 1 week was not. Captopril started after 2 weeks decreased contraction to U46619 after 12 Gy, an effect that was not altered in any other groups (Figure 6A). As shown in Figure 4B, 12 Gy decreased constriction to KCl, and this decrease was less pronounced with captopril begun after 1 week (Figure 6B). The decrease in relaxation of precontracted PAs by acetylcholine after 12 Gy was only improved by captopril started after 1 week (Figure 6C).

DISCUSSION

Our long-term goal is to identify mitigators and treatments for radiation-induced lung injury after a radiological attack or radiation accident (2,3,16).

In the present study we tested two FDA-approved RAS suppression agents (captopril and losartan) for mitigation efficacy 8 weeks after radiation when pneumonitis peaked. The drug doses were chosen to match, on a mg/m²/day basis, those used in the clinic. Some of these drug doses are effective for mitigation and treatment of radiation-induced renal injury in the same strain of rat (7,17,18). Captopril (34mg/kg/day) and losartan (34 mg/kg/day) rescued rats from morbidity induced by 12 Gy to the thorax for up to 10 weeks (Figure 1C). Our results demonstrate that captopril at a dose as low as 17 mg/kg/day via drinking water mitigated the loss of vasoreactivity after 10 Gy while losartan at a clinically-equivalent dose (11 mg/kg/day) was not effective.

After 12 Gy irradiation, a higher dose of captopril (34 mg/kg/day) was effective in mitigating radiation-induced effects on breathing rate, reactivity to KCl and vasocontraction; and this

occurred even when drug therapy was not started until one week after radiation. We substituted U 46619 for Ang II for vasoconstriction to facilitate relaxation studies with acetylcholine. Vasorelaxation, an important determinant of vascular endothelial function was altered by 12 Gy and mitigated by captopril (34 mg/kg/day) started up to 1 but not 2 weeks after radiation; the same dose of losartan did not have this mitigating effect (Figures 4C and 6C).

After 15 Gy irradiation, a higher dose of captopril (56 mg/kg/day) only partially mitigated the increase in breathing rate and the loss of vasoreactivity while losartan at the same dose was less effective. After 15 Gy, this high dose of captopril decreased structural injury and increased animal survival. Necropsies performed on rats that were morbid or died after exposure to 15 Gy showed pericardial effusion and cardiac hypertrophy in most animals, with pleural effusion or obvious lung injury present in some subjects. Therefore morbidity in these rats could be due to injuries to the heart as well as the lungs.

The vascular injuries we observed would be expected to adversely affect lung function, since the ability of pulmonary arteries to contract to different agonists by receptor-dependent (Ang II or U46619) or independent (KCl) mechanisms, is vital to adjust blood flow in the lung during altered physiological states such as hypoxia and inflammation. Endothelial-mediated vasorelaxation with acetylcholine is an important indicator of vascular disease (19-21).

An unexpected result was the superior mitigation by the ACE inhibitor captopril as compared to the AT1 blocker losartan. This was surprising because the AT1 blocker L-158,809 was more effective than captopril for mitigation of radiation nephropathy (7). Other investigators have noted that ACE inhibitors and an AT1 receptor blocker were effective in mitigation and treatment of lung fibrosis (9). These results stress the importance of testing the effect of each drug on known pathologies in individual organs. Our results also suggests that mitigation of lung injury by captopril is not mediated solely by suppression of Ang II activity via the AT1 receptor, because captopril (which decreases generation of Ang II) was more effective than losartan, which blocks the response of Ang II on the AT1 receptor. The AT1 receptor mediates the proapoptotic, inflammatory and profibrotic actions of Ang II that can contribute to vascular remodeling (22,23). AT1 receptors in vascular tissue can also induce TGF- β 1 (24) and generate reactive oxygen species, which could exacerbate lung injury. In fact a small molecular inhibitor of TGF- β protects against radiation induced-lung injury (25). In addition, activation of the AT1 receptor acutely promotes vasoconstriction (23). It is therefore logical to anticipate that blocking AT1, whether by reducing Ang II or preventing stimulation of AT1, would benefit pulmonary function. The mortality after 12 Gy was effectively prevented by captopril or losartan, indicating that lung injury may not be the only cause for mortality in a thoracic irradiation model.

There are a number of other signaling pathways that could be affected by inhibition of ACE but not AT1 receptors such as AT2 receptors and RAS-independent proteolytic activities such as degradation of the vasodilator and growth inhibitor bradykinin (26). Thus ACE inhibitors could act by directly stimulating bradykinin type 1 receptors (27,28) or via cross talk with bradykinin type 2 receptors. Finally, captopril is a thiol-containing ACE-inhibitor and this side group may act as an antioxidant to reduce inflammatory ROS and thus mitigate pneumonitis. Because of these multiple possibilities, it is beyond the scope of this investigation to resolve how captopril acts as a mitigator. Nevertheless, delineating mechanisms of action of captopril will be needed to gain approval for its use as a countermeasure against radiation injury under the FDA "animal efficacy rule".

In conclusion, we have shown that the ACE inhibitor captopril is an effective mitigator of pulmonary dysfunction caused by survivable doses of radiation. To our knowledge, this is the first demonstration that lung injury caused by a single whole-thoracic dose of radiation can be

mitigated by an FDA-approved agent. At doses (on a mg/m²/day basis) approved by the FDA for use in humans, captopril improves vascular, functional and structural derangements that develop in the rat lung by 8 weeks after a single dose of radiation. Inhibition of ACE is also able to mitigate radiation nephropathy (7), radiation injury to the central nervous system (29) and to lower the incidence of radiation-induced neoplasms (30). Our studies indicate that initiation of captopril therapy after a delay of 1 week following injury also has mitigating properties. All these advantages make it imperative to continue investigations on a promising drug that can reduce morbidity after exposure to radiation.

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Fig. 1.

Actuarial survival curves after thoracic radiation. Plots are a summary of morbidity/mortality. Animals at risk are in parenthesis. (A) Survival curves following 10, 12 and 15 Gy thoracic radiation. $P < 0.0001$ for 10 Gy vs 12 Gy; $P < 0.0001$ for 10 Gy vs 15 Gy, $P = 0.0008$ for 12 Gy vs 15 Gy. (B) 15 Gy with and without treatment with captopril (56 mg/kg/day) started a few hours after radiation and continued for the duration. $P = 0.0286$ for 15 Gy vs 15 Gy with captopril. (C) 12 Gy with and without captopril or losartan (34 mg/kg/day) started a few hours after radiation and continued for the duration; $P < 0.02$ for 12 Gy vs 12 Gy with captopril; $p = 0.03$ for 12 Gy vs 12 Gy with losartan.

Fig. 2.

Radiation-induced increase in breathing rate and mitigation by captopril and losartan. All assessments were at 7–8 weeks after irradiation. Captopril and losartan were started immediately after irradiation at doses of 34–56 mg/kg/day and continued for 7–8 weeks: (A) 12 Gy (B) 15 Gy. Values are mean breaths per min $\pm$ sem; n=number of animals tested; * indicates $p \leq 0.05$ vs unirradiated controls.

Fig. 3.

Loss of vascular reactivity of pulmonary arteries. Captopril (17 or 56 mg/kg/day) and losartan (11 mg/kg/day) were started immediately after irradiation and continued for the duration. (A) Representative line graph showing increased contraction by increasing concentrations of Ang II in pulmonary artery rings, and the mitigating effect of captopril (17 mg/kg/day) but not losartan (11 mg/kg/day). (B) Vascular tension to Ang II (10^{-7} M) after 10 Gy with and without drug interventions. (C) Vascular tension to KCl after 10 Gy with and without drug interventions. (D) Vascular tension to Ang II after 15 Gy with and without drug interventions. (E) Vascular tension to KCl after 15 Gy with and without drug interventions. All data are mean $\pm$ sem and expressed as % of the baseline tension. * indicates $p \leq 0.05$ vs unirradiated controls; # indicates $p < 0.01$ vs 10 Gy; n = number of rings (no more than 4 rings were tested from each rat). Panels A-C include data from rats described in Zhang *et al* 2008 (4).

Fig. 4.

Loss of reactivity of pulmonary arteries at 8 weeks after 12 Gy. Captopril and losartan were started immediately after irradiation at 34 mg/kg/day and continued for the duration. (A) Vascular tension after treatment with 10^{-7} M U46619 with and without drug interventions. There was no difference in vasoconstriction between any groups (B) Vascular tension to KCl (80 mM) with and without drug interventions. Both captopril and losartan improved the radiation induced loss of vasoconstriction by KCl, (C) Relaxation of pulmonary arteries precontracted with 10^{-7} M U46619 and then treated with 10^{-6} M acetylcholine. All data are mean $\pm$ sem and basal tension expressed as % of the baseline tension; n= number of rings (no more than 4 rings were tested from each rat); * indicates $p \leq 0.05$ vs unirradiated controls.

Fig. 5.

Representative sections (5 μm thick) of hematoxylin-eosin stained, uninflated lung tissue: (A) unirradiated rats; (B) 10 Gy, with mild fibrosis around airways and vessels marked with arrows; (C) 12 Gy with solid arrow marking interstitial widening and dotted arrow marking mononuclear cell infiltration. (D) 15 Gy, with solid arrow pointing to vessel wall thickening and dotted arrow marking inflammatory cell infiltration. (E) 10 Gy and treated with captopril started immediately after irradiation at 56 mg/kg/day and continued for the duration, which attenuated radiation-induced injury. (F) 12 Gy and treated with captopril started immediately after irradiation at 34 mg/kg/day and continued for the duration. These lungs demonstrated less injury than observed in lungs after only 12 Gy irradiation. (G) 15 Gy and treated with captopril started immediately after irradiation at 56 mg/kg/day and continued for the duration. Lungs from rats treated with captopril did not manifest as much injury as untreated rats. The scale bars represents 200 micrometers.

Fig. 6.

Effect of different schedules of captopril or losartan (34 mg/kg/day) after 12 Gy. (A) Vascular tension after treatment of pulmonary arteries with U46619 with and without treatment with captopril or losartan (34 mg/kg/day) for different times of intervention (B) Vascular tension after treatment of pulmonary arteries with KCl (80 mM) (C) Relaxation of pulmonary arteries precontracted with 10^{-7} M U46619 and then treated with 10^{-6} M acetylcholine. N= number of rings (≤ 4 rings/rat); * indicates $p \leq 0.05$ vs unirradiated controls. (D) Radiation-induced increase in breathing rate after 12 Gy and mitigation by captopril (34 mg/kg/day) for different schedules of intervention as labeled. Treatments with captopril but not losartan mitigated the increase in breathing rate. Values are mean breaths per min $\pm$ sem; n=number of animals tested; * indicates $p \leq 0.05$ vs unirradiated controls.