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Enalapril Mitigates Radiation-Induced Pneumonitis and Pulmonary Fibrosis if Started 35 Days after Whole-Thorax Irradiation

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Abstract

Victims of a radiological attack or nuclear accident may receive high-dose, heterogeneous exposures from radiation to the chest that lead to lung damage. Our goal is to develop countermeasures to mitigate such injuries. We used WAG/RijCmcr rats receiving 13 Gy to the whole thorax to induce pulmonary fibrosis within 210 days. The angiotensin converting enzyme (ACE) inhibitor enalapril was evaluated as a mitigator of these injuries at two doses (18 and 36 mg/m²/day) and 8 schedules: starting at 7, 35, 70, 105 and 140 days and continuing to 210 days or starting at 7 days and stopping at 30, 60 or 90 days after whole-thorax irradiation. The earliest start date at 7 days after irradiation would provide an adequate window of time for triage and dosimetry. Survival after 35 days, as permitted by our Institutional Animal Care and Use Committee (IACUC) was also recorded as a primary end point of pneumonitis. Pulmonary fibrosis was evaluated using the Sircol biochemical assay to measure lung collagen. Our results indicated that a short course of either dose of enalapril from 7–90 days improved survival. However, pulmonary fibrosis was only mitigated by the higher dose of enalapril (36 mg/m²/day). The latest effective start date for the drug was 35 days after irradiation. These results indicate that ACE inhibitors can be started at least a month after irradiation for mitigation of pneumonitis and/or pulmonary fibrosis.

INTRODUCTION

The angiotensin converting enzyme (ACE) inhibitors are commonly used to treat hypertension and cardiac disease. In the past 20 years, a number of laboratories including our own have demonstrated efficacy of this class of drugs for the management of radiation-

induced lung damage in rats (1–4). The studies have tested the compounds given before, during and after fractionated irradiation to one or both lungs (1, 2) or after whole-thorax irradiation (WTI) in rats (3–5). The rat WTI model induces two waves of injury, pneumonitis at ~35–70 days after irradiation and fibrosis that can be detected only after 210 days (4). Both injuries are also observed in human lungs (6). More recently there have been reports of decreased risk of clinical radiation pneumonitis with incidental use of ACE inhibitors and thoracic radiation therapy (7–9). These studies are some of only a few examples we are aware of in which drug therapy improves the outcome of radiation injury in human lungs. However, details of the dosage and schedules of the ACE inhibitors in the clinical studies are not mentioned and the dose and schedule of radiation is different from that expected for an unanticipated radiological event.

In the past decade, the National Institute of Allergy and Infectious Diseases (NIAID) has pioneered a research program to develop mitigators of radiation-induced injuries (10) relevant to an attack by a dirty bomb or nuclear detonation. Mitigation is defined as an intervention(s) that reduces tissue damage when started after irradiation but before the development of symptoms (11). Mitigators have the potential to reduce radiation effects after unexpected and single exposures. Ideal mitigators would retain efficacy if started as late as possible *after* exposure to ionizing radiation (12). Countermeasures developed as mitigators will have to gain regulatory approval by the FDA animal rule since appropriate clinical trials would be unethical (13). Countermeasures that are known to be safe in irradiated humans will have an easier path to approval. ACE inhibitors are therefore ideal candidates for development as mitigators of radiation-induced pneumonitis and pulmonary fibrosis. Our long-term goal is to repurpose this class of drugs for these indications by successful navigation of the animal rule.

After a nuclear accident or attack some individuals will be expected to have total-body irradiation (TBI), while others will sustain less than TBI. In the current study we have focused attention on localized injury to the thorax only, but we are extending our investigation to include the effects of TBI. Use of ACE inhibitors after radiation is safe and the drug captopril used alone demonstrated no effect on pulmonary structure or function in unirradiated rats (5). To advance the use of ACE inhibitors as mitigators, the shortest time of treatment with these agents must be described. Most published reports have started treatment within hours and days after radiation and continued for the duration of the studies. We were funded by the Biomedical Advanced Research and Development Authority (BARDA) by NIAID to investigate efficacious doses and schedules of the ACE inhibitor enalapril, for mitigation of radiation lung injury. Two doses of the drug were started at different times after irradiation and stopped at termination of the study, or were started at 7 days after WTI and stopped at varying intervals before 210 days. Our results using enalapril indicate that the drug can be started as late as 35 days after irradiation to mitigate both the phases of radiation lung injury: acute pneumonitis as well as delayed pulmonary fibrosis. Stopping the drug as early as 90 days after irradiation supported mitigation of pneumonitis but not fibrosis.

MATERIALS AND METHODS

Animal Care

This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical College of Wisconsin. Female rats (WAG/RijCmcr) were maintained in a moderate security barrier as described previously (4). Based upon directives from the IACUC of the Medical College of Wisconsin, rats were considered morbid if they met veterinarian's specified criteria. These included at least 3 of the following: (1) greater than 20% loss in body weight; (2) inactivity on 2 consecutive days, defined as no movement unless actively stimulated; (3) poor grooming that became worse after 24 h; (4) breathing

rates of less than 60 or greater than 250 breaths per minute; and (5) hunched posture, death pose, on 2 consecutive days. Rats that were morbid were euthanized while those that did not meet the criteria continued in the study as “survivors”.

Injury Model

Unanesthetized 9- to 10-week-old rats weighing approximately 140 g were immobilized in a plastic jig and irradiated with a 320-kVp orthovoltage X-ray system, with a half-value layer (HVL) of 1.4 mm Cu. Rats were treated with a single dose of 13 Gy to the whole thorax at a dose rate of 1.43 Gy/min (4). The radiation dose was delivered by two equally weighted lateral beams to improve uniformity. The whole lung, heart and a small amount of liver were in the field. The irradiated rats and their age-matched controls were sacrificed after 210 days at which time the experiment was terminated. One group of rats was not irradiated (unirradiated controls or 0 Gy group) but maintained under identical conditions.

Administration of Drug

Randomly selected animals were treated with enalapril (gift from Merck Inc. or catalogue no. E6888, Sigma Chemicals, St. Louis, MO) added to the drinking water (30 and 60 mg/L) as described by us and others (2, 4). From previously reported studies (4), this concentration delivers 12.5–42 mg/m²/day (1 standard deviation above and below the mean). For simplicity the mean dose of enalapril is given as 18 mg/m²/day and 36 mg/m²/day corresponding to 30 and 60 mg/L, respectively in drinking water in the remainder of this report.

Drug start times—The drug was started either at 7, 35, 70, 105 or 140 days after irradiation as mentioned in each experiment.

Drug stop times—The drug was stopped after irradiation at 30, 60, 90 or 210 days as mentioned in each experiment.

For each experiment there were 3 groups of rats treated at the same time for comparisons; (1) 13 Gy WTI alone, (2) 13 Gy + enalapril (18 mg/m²/day), (3) 13 Gy + enalapril (36 mg/m²/day). The numbers of rats at the start of the study in each drug group are given in Table 1, while rats that were not treated with drug (matched irradiated controls) for each drug schedule were: 7–210 days, n = 20; 35–210 days, n = 17; 70–210 days, n = 43; 105–210 days, n = 63; 140–210 days, n = 65; 7–30 days, n = 17; 7–60 days, n = 14; 7–90 days, n = 18. A larger number of rats were included for studies with drug start times after pneumonitis due to the high morbidity during pneumonitis after 13 Gy WTI. Sufficient numbers of rats were needed at the start to provide enough numbers for randomization to the treatment groups after pneumonitis and to progress to fibrosis.

Sircol Collagen Assay

The Sircol collagen assay was used to measure collagen as a marker of fibrosis. The assay was performed as described previously with minor modification (Biocolor Ltd., Carrickfergus, Northern Ireland) (4). Collagen content in test samples (µg/mg protein) were compared to values measured in unirradiated rats (0 Gy) and expressed as percentage of 0 Gy. The control groups consisted of pooled data from rats used in parallel with each group receiving enalapril.

Histology

Inflated lungs from unirradiated and irradiated rats as shown in Fig. 3 were fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburg, PA), and then embedded in paraffin.

Whole mount sections were cut (4 μm), processed and stained with Masson's trichrome (Newcomer Supply, Middletown, WI). Representative images were captured at the same magnification from all samples. All histological work was performed by the Children's Research Institute-Histology Core at the Medical College of Wisconsin.

Statistical Analysis

The morbidity/mortality of rats after treatments was evaluated by a Kaplan-Meier survival plot and expressed as percentage morbidity. The significance was analyzed by the Peto and Peto Wilcoxon test. Results for Sircol collagen assays were compared by one-way analysis of variance (ANOVA). All pairwise multiple comparisons were done by the Holm-Sidak test to determine significance.

RESULTS

1. Enalapril Improves Survival When Started after Irradiation but Before Pneumonitis

Two doses of enalapril were used, a low dose (18 $\text{mg}/\text{m}^2/\text{day}$) and a high dose (36 $\text{mg}/\text{m}^2/\text{day}$) as described in Table 1. The high dose decreased morbidity from 83% to 42% after 13 Gy WTI when started 7 days after irradiation and continued to 210 days. The low dose at the same schedule (Table 1) delayed morbidity rather than decrease the number of morbid rats after 13 Gy WTI (data not shown). Next we tested the two doses starting at 35 days after irradiation. The Kaplan-Meier plot in Fig. 1A shows that both doses reduced morbidity after 13 Gy WTI. Survival improved from 18% in the absence of drug to 53% with enalapril at 18 $\text{mg}/\text{m}^2/\text{day}$ and 75% with enalapril at 36 $\text{mg}/\text{m}^2/\text{day}$. The area shaded in gray represents the time of treatment with enalapril. We did not expect a difference in morbidity if the drug was started 70 days or later after WTI (Table 1) since there is a minimal increase in morbidity past 70 days i.e., after pneumonitis with 13 Gy WTI alone (Fig. 1A and B).

2. Enalapril Improves Survival Through Pneumonitis When Started at 7 Days and Stopped at 90 Days after Irradiation

To study the earliest time to stop enalapril we started the drug (18 or 36 $\text{mg}/\text{m}^2/\text{day}$) at 7 days and stopped at 30, 60 or 90 days after 13 Gy WTI. Morbidity was not altered if the drug was stopped at 30 or 60 days after 13 Gy (Table 1). However, a schedule of 7–90 days decreased morbidity at both doses of the drug (Fig. 1B).

3. Enalapril Mitigates Increase in Lung Collagen if Started at 7 or 35 Days after Irradiation

Newly synthesized collagen was assayed in lungs at 210 days after irradiation, from the groups of rats described in Table 1. Most groups included 4 rats that survived to this time, except the low dose of enalapril (18 $\text{mg}/\text{m}^2/\text{day}$) given from 7–210 days (not shown). Lung collagen was not decreased in any group given the lower dose of enalapril (18 $\text{mg}/\text{m}^2/\text{day}$) (Fig. 2A and B) or if the drug was stopped before 210 days (Fig. 2D). The higher dose of enalapril (36 $\text{mg}/\text{m}^2/\text{day}$) mitigated the increase in lung collagen if started at 7 or 35 days after 13 Gy WTI (Fig. 2C). Figure 3 demonstrates representative trichrome stained sections from control rats and those irradiated with and without treatments with enalapril. All sections were prepared from lungs harvested at termination (210 days). Interstitial collagen (stained blue) and alveolar wall thickening was visible in lungs after 13 Gy alone (Fig. 3B) or 13 Gy with enalapril (70–210 days, Fig. 3E) compared to unirradiated lungs (Fig. 3A) or those derived from rats treated with enalapril from 7–210 (Fig. 3C) or 35–210 days (Fig. 3D).

DISCUSSION

We previously observed mitigation of morbidity during pneumonitis and pulmonary fibrosis by enalapril (average 24 mg/m²/day or 40 mg/L in drinking water) given from 7–210 days (4) after 13 Gy WTI. Even though our goal in that and this current study was to mitigate the increase in newly synthesized collagen that would result in pulmonary fibrosis, we determined considerable survival advantage of enalapril through radiation-pneumonitis. Our results now demonstrate that a lower dose of enalapril (18 mg/m²/day or 3.4 mg/kg/day in drinking water) and different schedules of the drug also improved survival and mitigated pulmonary fibrosis. More importantly for the first time we show that the drug is effective when started as late as 35 days after irradiation and continued. Previous studies have not assessed such a delayed start of ACE inhibitors to reduce radiation-induced pulmonary fibrosis (2, 4). Thirty-five days was selected assuming that in the event of a mass casualty the longer the time given to identify and triage the better the chances of reaching a maximum number of potential victims. Research to identify organ specific biomarkers and more sensitive biodosimetry is ongoing. By 35 days, a treatment for pneumonitis is still classified as a “mitigator” since symptoms of this disorder do not begin prior to this time in the model we have used. Most importantly our study demonstrates that a short course of low or high doses of the drug (18 or 36 mg/m²/day) from 7–90 days after irradiation was effective in improving survival after WTI (Fig. 1B). The efficacy of a short course of an ACE inhibitor for mitigation of radiation pneumonitis has also not been described previously. This result is important since withdrawal of the drug does not increase morbidity from pneumonitis, though it may be continued to improve fibrotic outcome in the irradiated lung.

Fibrosis was mitigated in this study by the higher, but not the lower dose of the drug. The high dose of enalapril could be started as late as 35 days after irradiation but only mitigated pulmonary fibrosis if it was continued past 90 days. Later treatments starting after 70 days were selected to assess the potential of enalapril to mitigate against pulmonary fibrosis in *survivors* of pneumonitis. Pulmonary fibrosis is a delayed injury that takes months to years to develop. These delayed starts did not demonstrate efficacy of enalapril assessed at 210 days. It is possible that evaluation at time points later than 210 days may be improved by starting treatment at 70 days or later. We could not use morbidity as an end point for fibrosis as these rats do not die of fibrosis by 210 days. Higher doses of radiation to the whole thorax that may induce robust fibrosis would kill all rats during pneumonitis. These results are new and exciting because they allow a large window of time (35 days) for biodosimetry and/or other analyses to be completed before commencing a mitigating schedule of enalapril. This will also permit use of other drugs to mitigate or treat acute effects that may not be compatible with enalapril. There is no difference in survival between the higher dose of enalapril started at 7 or 35 days ($P = 0.420$). Earlier start times have also shown efficacy (3) and in general we believe ACE inhibitors are more effective the earlier they are started (results not shown). However, a delayed start from the time of exposure is a requirement for mitigators developed in the NIAID program. We believe 7 days is a safe minimum time to consider mitigation of radiation pneumonitis and fibrosis, since prodromal GI effects are resolving which will permit ACE inhibitors to be given orally.

Our results advance our goal of developing enalapril as a radiomitigator by providing encouraging evidence of dosing schedules optimal for the NIAID/BARDA program. Besides ACE inhibitors there are a number of mitigators for radiation lung injury such as statins, glutathione variants, nutraceuticals and antioxidants that have been funded by NIAID for development against radiation damage to the lung (12, 14).

Insight from the treatment window we used in this study suggest a few mechanisms that may account for mitigation from radiation injury by enalapril. The first is protection against remodeling of the heart secondary to pneumonitis and hypoxemia. After irradiation, vascular dysfunction develops over the first month (1, 15–18). The peak increase in pulmonary vascular resistance and fall in pulmonary artery distensibility, arterial reactivity and peripheral pulmonary artery density are observed from 6–8 weeks after exposure. Right heart remodeling is promoted by the increase in vascular resistance in the lungs that eventually leads to pulmonary edema and lethal disruption of alveolar gas exchange.

Increase in pulmonary vascular pressure between 4–6 weeks may be reduced by suppressing levels of angiotensin II with an ACE inhibitor. Therefore enalapril may mitigate pneumonitis when started at this critical time after one month after 13 Gy. Several investigators have published protection by ACE inhibitors or angiotensin receptor antagonists from hypoxia induced ventricular remodeling (interstitial fibrosis and hypertrophy of the cardiomyocytes) (19). Our published data support reversible pneumonitis and an increase in right ventricular weight in rats after whole-thorax irradiation with 10 Gy (16). In this model, cardiac remodeling is not present at 1 month, but is at 2 months (the peak of pneumonitis); by 5 months coincident with the resolution of pneumonitis, cardiac remodeling is gone as well.

Second and third mechanisms for mitigation by ACE inhibitors started after 35 days may be by protection against remodeling in the lung vasculature or a lung parenchymal inflammatory response. Chronic hypoxia such as may be expected with radiation induced pneumonitis induces structural changes in pulmonary arteries, which have been previously summarized by Stenmark *et al.* (20). Resident vascular cell types (including endothelial, smooth muscle, adventitial fibroblast) undergo site- and time-dependent alterations in proliferation, matrix protein production, expression of growth factors, cytokines and receptors. Hypoxic exposure *in vivo* also induces an inflammatory response within the vessel wall, and the recruited cells contribute to the structural remodeling of the pulmonary circulation. Infiltrating lymphocytes, macrophages and mast cells express mediators of the renin-angiotensin system (21, 22). Suppression of these signals by ACE inhibitors may also help to reduce pneumonitis.

Because mitigation of radiation lung injury is common to more than one structurally different ACE inhibitor (captopril and enalapril) it does not appear to be attributable to the different side chains in these molecules.

Based upon tachypnea, increase in right heart size and histological inflammation at the peak of pneumonitis, we strongly believe rats receiving 13 Gy to the thorax die a respiratory death. With reversal of these endpoints in rats treated with ACE inhibitors, we link the survival benefit of these agents to reduced severity of pneumonitis. Because ACE inhibitors started as late as 35 days after irradiation are efficacious in enhancing survival through pneumonitis we can conclude that the mechanism of protection is not prevention of free oxygen radicals generated after irradiation. Because continuation of the enalapril through 90 days is required to mitigate pulmonary fibrosis, cellular and signaling mechanisms underlying radiation-induced remodeling of the lungs appear to be sensitive to the effects of enalapril until the host recovers from this inflammatory response. These observations together favor (though do not prove) enalapril-associated improvement in survival and lung structure due to mitigation of radiation induced effects on lung parenchyma or vasculature, remodeling or inflammatory effects that are sustained after exposure to radiation.

Of the mitigators currently being developed, broad spectrum catalytic antioxidants such as the Eukarion series of compounds (23, 24) and manganese porphyrins (25, 26) have reported

considerable progress. Both EUK and MnTE-2-PyP⁵⁺ were delivered by subcutaneous injection in these studies. EUK 207 mitigated lung damage when started one week after irradiation and stopped after 4 weeks (23). Similar to enalapril, EUK 207 improved survival, a preferred primary end point for the NIAID program, after irradiation to the lungs. The latest start date for treatment of lung injury of rats given the porphyrin MnTE-2-PyP⁵⁺ was 8 weeks after irradiation (25, 26) representing a starting time after pneumonitis. Such a regimen is termed as “treatment” since the drug is started after symptoms develop. The MnTE-2-PyP⁵⁺ study (25) tested after 28 Gy hemithoracic irradiation, which was not lethal to the rats, so survival could not be used as an end point. MnTE-2-PyP⁵⁺ mitigated oxidative stress and overall lung damage in this model. To the best of our knowledge both antioxidants in this class have not been described for efficacy to reduce lung injury in irradiated humans.

In summary, our results demonstrate another advantage for development of ACE inhibitors as mitigators of radiation-induced lung injury: a delayed (up to 35 days) start date for efficacy against both radiation-induced pneumonitis and pulmonary fibrosis. This will allow time for accurate biodosimetry and recovery from acute prodromal gastrointestinal toxicity. Other advantages of ACE inhibitors include oral availability, chemical stability, once a day dosing, affordable pricing and proven efficacy in multiple organ systems (27, 28). These drugs have a long history of safe use in humans and clinical trials showed that incidental use was effective against radiation injury to the lung and kidneys (7, 9, 27). Our results are very encouraging for forwarding ACE inhibitors to FDA licensure.

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FIG. 1.

Panel A: Kaplan-Meier plot of morbidity up to 210 days after 13 Gy whole thoracic irradiation (WTI) with enalapril started after 35 days WTI. Enalapril was started at 35 days after WTI and continued as marked by the gray rectangle. Numbers in parenthesis indicate the number of rats at risk at 100 days. The numbers of rats in each group at the start of the drug are shown in Table 1. Enalapril mitigated morbidity due to radiation pneumonitis as analyzed by the Peto and Peto Wilcoxon test. Panel B: Kaplan-Meier plot of morbidity up to 210 days after 13 Gy whole thoracic irradiation (WTI) with enalapril from 7–90 days. Enalapril was started at 7 days after WTI and continued to 90 days as marked by the gray rectangle. Numbers in parenthesis indicate the number of rats at risk at 100 days. The numbers of rats in each group at the start of the experiment are shown in Table 1. A short course of enalapril (7–90 days after WTI) mitigated morbidity due to radiation pneumonitis as analyzed by the Peto and Peto Wilcoxon test.

FIG. 2.

Mitigation of lung collagen by different schedules of enalapril after 13 Gy whole thoracic irradiation (WTI). Acid and pepsin soluble collagen content representing newly synthesized collagen was measured in rat lungs at 210 days after WTI by the Sircol assay. Results are presented as percentage of collagen ($\mu\text{g}/\text{mg}$) in unirradiated rats (Y axis). Panel A: Comparisons of lung collagen in rats given enalapril (18 $\text{mg}/\text{m}^2/\text{day}$) starting at 35, 70, 105 or 140 days after irradiation and continued to 210 days. Only unirradiated rats had lower lung collagen than rats receiving 13 Gy WTI alone. Enalapril (18 $\text{mg}/\text{m}^2/\text{day}$) did not lower lung collagen in any of the schedules shown in this figure. Panel B: Enalapril (18 $\text{mg}/\text{m}^2/\text{day}$) starting at 7 days after irradiation and stopping at 30, 60 or 90 days did not lower lung collagen. Panel C: Comparisons of lung collagen from rats given enalapril (36 $\text{mg}/\text{m}^2/\text{day}$) starting at 7, 35, 70, 105 or 140 days after irradiation and continued to 210 days. Unirradiated rats and those given enalapril from 7–210 or 35–210 days had lower lung collagen than rats receiving 13 Gy WTI alone. Panel D: Enalapril (36 $\text{mg}/\text{m}^2/\text{day}$) starting at 7 days after irradiation and stopping at 30, 60 or 90 days did not lower lung collagen. There were 4 rats in each group (mean 11 rats/group) except after enalapril (18 $\text{mg}/\text{m}^2/\text{day}$) from 7–210 days, which was therefore not represented in panel A. Results for Sircol collagen

assays were compared by one-way analysis of variance (ANOVA) with all pairwise multiple comparisons done by the Holm-Sidak test.

FIG. 3.

Histology of representative lung sections at termination (210 days after 13 Gy WTI). Lung sections were stained with Masson's trichrome as follows: Panel A: No irradiation; panel B: 13 Gy WTI without drug; panel C: 13 Gy WTI with enalapril (36 mg/m²/day) given from 7–210 days. Panel D: 13 Gy WTI with enalapril (36 mg/m²/day) given from 35–210 days. Panel E: 13 Gy WTI with enalapril (36 mg/m²/day) given from 70–210 days. Note increased collagen (blue stain) and alveolar wall thickening in lungs from rats in panel B (13 Gy WTI, no drug) and panel E (13 Gy WTI + enalapril (36 mg/m²/day) given from 70–210 days).

TABLE 1

Results of Survival of Rats up to 210 Days after 13 Gy WTI

Dose of enalapril (mg/m ² /day)	Drug start (days after 13 Gy WTI)	Drug stop (days after 13 Gy WTI)	Number of rats (n) at drug start	P value vs. 13 Gy alone
18	7	210	19	0.037
36	7	210	19	>0.001
18	35	210	19	0.016
36	35	210	16	0.001
18	70	210	9	>0.600*
36	70	210	9	>0.600*
18	105	210	11	>0.600*
36	105	210	10	>0.600*
18	140	210	10	>0.600*
36	140	210	11	>0.600*
18	7	30	20	0.166
36	7	30	20	>0.600
18	7	60	22	0.170
36	7	60	22	0.125
18	7	90	16	0.041
36	7	90	19	0.009

* The drug was started after pneumonitis after which additional animals were seldom lost.