Angiotensin Converting Enzyme Inhibitors Mitigate Collagen Synthesis Induced by a Single Dose of Radiation to the Whole Thorax

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Lung fibrosis/Mitigation/Angiotensin converting enzyme inhibitors.

Our long-term goal is to use angiotensin converting enzyme (ACE) inhibitors to mitigate the increase in lung collagen synthesis that is induced by irradiation to the lung, which could result from accidental exposure or radiological terrorism. Rats (WAG/RijCmcr) were given a single dose of 13 Gy (dose rate of 1.43 Gy/min) of X-irradiation to the thorax. Three structurally-different ACE inhibitors, captopril, enalapril and fosinopril were provided in drinking water beginning 1 week after irradiation. Rats that survived acute pneumonitis (at 6–12 weeks) were evaluated monthly for synthesis of lung collagen. Other endpoints included breathing rate, wet to dry lung weight ratio, and analysis of lung structure. Treatment with captopril (145–207 mg/m^2/day) or enalapril (19–28 mg/m^2/day), but not fosinopril (19–28 mg/m^2/day), decreased morbidity from acute pneumonitis. Lung collagen in the surviving irradiated rats was increased over that of controls by 7 months after irradiation. This increase in collagen synthesis was not observed in rats treated with any of the three ACE inhibitors. Analysis of the lung morphology at 7 months supports the efficacy of ACE inhibitors against radiation-induced fibrosis. The effectiveness of fosinopril against fibrosis, but not against acute pneumonitis, suggests that pulmonary fibrosis may not be a simple consequence of injury during acute pneumonitis. In summary, three structurally-different ACE inhibitors mitigate the increase in collagen synthesis 7 months following irradiation of the whole thorax and do so, even when therapy is started one week after irradiation.

INTRODUCTION

Radiological exposures can cause acute and chronic effects in many organ systems. In the event of a radiological attack or accident, high-dose upper-body exposure could occur that would damage the lung but not cause acute gastrointestinal or hematological mortality.[1] Radiation-induced pulmonary injury has been reported in many species including humans.[2,3] These injuries are primarily manifested in 2 phases; the first is an acute pneumonitis and the second a delayed pulmonary fibrosis.[2] Pneumonitis spontaneously regresses unless the severity is so great as to result in death. The hallmark *late* effect of radiation on lungs is interstitial pulmonary fibrosis.[4–6] The mechanism of induction of this fibrosis remains unclear.[2] While a number of agents have been reported to prevent pulmonary fibrosis when given prior to radiation[7–10] very few are effective when therapy is initiated after irradiation. Genistein is one such agent that reduces the extent of fibrosis in rats when therapy is started after irradiation.[11]

Modulators of the renin-angiotensin system (RAS) such as the angiotensin converting enzyme (ACE) inhibitors captopril and enalapril, and an angiotensin II type-1 receptor blocker (L-158,809) have been shown to decrease radiation-induced lung injury in rats exposed to a high dose of gamma rays to the hemithorax.[12] Rats exposed to total body irradiation (TBI) followed by bone marrow transplantation (BMT) and given ACE inhibitors or L-158,809 enjoyed protection against renal damage and lung fibrosis.[13] In that model the drugs were administered from 11 days prior to BMT to 56 days after BMT along with a conditioning regimen of cyclophosphamide to mimic clinical TBI-BMT regimens. Also pretreatment schedules are not relevant to unexpected exposures that might occur from a nuclear attack or accident.

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We have recently demonstrated that the ACE inhibitor captopril mitigates radiation-induced pneumonitis after whole-thoracic irradiation (WTI) even when therapy is started up to one week after irradiation.[14] Drug delivery was purposely delayed to provide a feasible window of time to reach victims of a radiological event. In the present work our goal was to test mitigation of collagen synthesis by ACE inhibitors[14] in animals with a model relevant to a single-dose exposure of the whole volume of the lung.[14–16] We determined the therapeutic potential of three ACE inhibitors. Since the efficacy of captopril in some previous studies was attributed to the thiol group of the drug,[12] we used three structurally-different ACE inhibitors: captopril (containing a reducing thiol group), enalapril (containing a dicarboxylate but no thiol group) and fosinopril (containing a phosphonate but no thiol group).

MATERIALS AND METHODS

The injury model

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical College of Wisconsin. Unanesthetized female rats (WAG/RijCmcr) were irradiated at 9–10 weeks of age at a weight of 120–140 grams.[14–17] The rats were placed in a Plexiglas jig and given 13 Gy of radiation limited to the thorax. A XRAD 320 orthovoltage system (Precision X-Ray, CT, USA) was used, with a 320-kVp beam, a half-value layer of 1.4 mm Cu, and a midline dose rate of 1.43 Gy/min. The absolute dose was measured with a calibrated Farmer-type ionization chamber. The radiation dose was delivered by two equally-weighted parallel-opposed lateral beams to improve the dose uniformity. A 30 mm (right-left) × 45 mm (up-down) collimator was used to define a radiation field that encompassed the whole thorax. The head, kidneys and GI tract were out of the field. However the heart and some portion of liver were exposed. Irradiated rats were randomized to different experimental groups immediately after irradiation and along with their age-matched controls were housed under identical conditions in a moderate-security barrier and studied for up to 7 months after irradiation. Endpoints from rats exposed to each drug treatment were compared to those of animals receiving 13 Gy WTI alone.

Based upon directives from the IACUC of the Medical College of Wisconsin, rats were considered morbid and euthanized if they met veterinarian's specified criteria. These included at least 3 of the following: i) greater than 10% loss in body weight; ii) inactivity on 2 consecutive days, defined as no movement unless actively stimulated; iii) lack of grooming that became worse after 24 hours iv) breathing rates of less than 60 or greater than 250 breaths per minute; v) hunched posture, death pose, on 2 consecutive days. Survival data are presented as percent morbidity over time based upon these criteria (see Fig. 1). Not all rats in the sur-

vival study were maintained for the remaining studies.

Delivery of modifiers of the renin-angiotensin system

Animals were provided with captopril (Sigma Chemicals, St. Louis, MO, USA), enalapril (gift from Merck Inc. or purchased from Sigma Chemicals) or fosinopril (Sequoia Research Products Ltd., Pangbourne, UK) added to the drinking water as described by us and others.[12–15,18] Bottles were checked regularly for adequate filling and replaced every week since captopril and enalapril are known to be stable in solution.[19–21] We tested the stability of fosinopril at room temperature for up to 8 days using high performance liquid chromatography. The drug was lost in solution very slowly, at 0.34% (fosinopril 40 mg/L) and 0.4% (fosinopril 100 mg/L) per day (Doctrow et al., personal communication).

Based on our measurements, rats consumed 20 ± 3.5 ml of water/day (mean $\pm$ SD, n = 12). This volume included loss while drinking or which incurred during cage movement. We estimated cage movement to account for only 1% of this loss. Captopril was made available to rats at the concentration of 300 mg/L. Considering water consumption, animal weights and values for rat body surface areas derived from the literature,[22] we calculated a delivered dose of 145–207 mg/m^2/day (one standard deviation above and below the mean). Enalapril or fosinopril were provided at 40 mg/L concentration, making the effective doses to be 19–28 mg/m^2/day. All drugs were started one week after irradiation and continued until study termination. This schedule of drug therapy, where therapy is started after irradiation but before there are pathophysiological signs of injury, is termed "mitigation".[23,24]
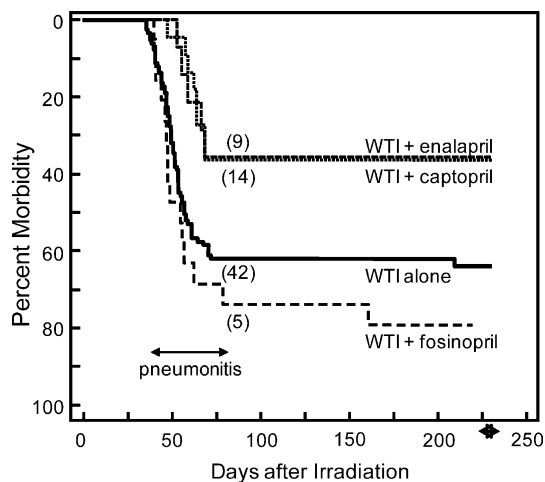

Fig. 1. Kaplan-Meier plot of morbidity after 13 Gy whole-thoracic irradiation (WTI). Drugs were started 1 week after irradiation. Numbers in the parentheses indicate the number of rats at risk after the acute pneumonitis phase (at 80 days). $P = 0.0008$ for 13 Gy vs. 13 Gy + captopril; $P = 0.0029$ for 13 Gy vs. 13 Gy + enalapril. The arrow (◆) along the X-axis represents the time at which rats were sacrificed for analyses of lung structure and function.

The doses of ACE inhibitors were shown to be biologically effective by demonstrating that they caused decreased blood pressure in normal WAG/RijCmcr rats of the same age and sex. Systolic blood pressures were measured with a tail cuff (IITC Life Science Inc., CA, USA). Non-irradiated rats were randomized and given captopril, enalapril or fosinopril for at least 3 weeks. Animals were pre-conditioned to the device and protocol before blood pressures were actually recorded. After conditioning, blood pressure measurements were made in triplicate on 3 separate days for each reading. The mean systolic pressure before drug was 118 ± 8.4 mm Hg (mean $\pm$ SD), after giving ACE inhibitors the pressures fell modestly, but significantly (all $p \leq 0.008$) to 94 ± 8 mm Hg (captopril), 97 ± 9 mm Hg (enalapril) and 105 ± 8 mm Hg (fosinopril).

Breathing rate assay and wet to dry weight ratio

The mean breathing rate (breaths per min) in some animals that survived to 7 months was measured as described in a previous publication.[14,15] Briefly, animals in a Plexiglas jig were placed in a transparent, airtight box connected to a differential pressure transducer. The mean breathing rate in each animal was calculated from a minimum of four steady regions of the recording lasting at least 15 seconds each. The breathing frequency was expressed as breaths per minute. The wet to dry weight ratio of lung was obtained as described previously.[15]

Histology

Lung sections were taken from randomized irradiated rats sacrificed at 7 months following 13 Gy WTI and from age-matched, non-irradiated animals. The left lungs were inflated by injecting 10% neutral buffered formalin (Fisher Scientific, CA, USA) and then fixed in the formalin solution. Sections were then processed and stained with Masson's trichrome (Newcomer Supply, WI, USA) or hematoxylin & eosin (Richard Allan-Thermo Scientific, MI, USA). All the histological work was done by the Children's Research Institute-Histology Core at the Medical College of Wisconsin.

Quantification of trichrome stained slides

All images from Trichrome stained left lungs were captured on the same day and under identical conditions. For quantification of collagen, a whole-mount section of lung from each rat was scanned independently by 2 operators for blue color typical of stained collagen (see Fig. 3). The operators were blinded to the identity of the samples. Since fibrosis was patchy, a numeric score based on the percent of alveoli with any blue color (e.g. marked by arrows in Figs. 3A–C) was given after estimation over the whole area of the section. This score estimated the frequency of blue foci in the section. A second score was based on a 4 point scale to estimate the extent of blue color in collagen stained foci. The product of the 2 scores given by each operator were aver-

aged to give a score for blue color in the parenchyma of the lungs. A third score was based on the percent of cells adjacent to the pleura only, that were stained blue, since pleural fibrosis is common in humans. Blue color associated with collagen present in the vasculature or airways were not considered for scoring.

Sircol collagen assay

Sircol collagen assay (Biocolor Ltd., Carrickfergus, Northern Ireland) was employed to measure the acid and pepsin soluble collagen content in rat lungs. This assay measures relatively newly synthesized collagen that is not yet covalently cross-linked. Minor modifications to the instructions in the kit were applied as described. The lower lobe of the right lung was excised, gently wiped, weighed and homogenized in 1 ml pepsin reagent (Accurate Chemical & Scientific Corp., NY, USA), then incubated over night at 4°C. The following day, homogenates were centrifuged at 15,000 X g for 10 min at room temperature. The supernatant was collected and filtered through 0.45 μm syringe filters (Thermo Fisher Scientific, PA, USA). The clear filtrate was then used for the collagen assay. Duplicate samples (5 μl) of the filtrate were dispensed into eppendorf tubes and 95 μl double distilled water added. Sircol reagent (200 μl) was added, then tubes were vortexed for 10 sec and incubated in an orbital shaker for 30 min. Next, samples were centrifuged at 10,000 X g for 10 min at room temperature. Supernatant was discarded and pellets were washed with ethanol. The pellets were air dried, dissolved in 200 μl of 0.5 M sodium hydroxide and the absorbance read in a microplate reader at 540 nm. The concentration of collagen in test samples was determined by comparison to a standard created by measuring absorbance of rat tail type I collagen (Biocolor Ltd.). Concentrations of collagen in test samples were normalized to milligram tissue wet weight.

Statistical analysis

The morbidity/mortality of rats following treatments was represented by Kaplan Maier survival plot and expressed as percent morbidity. The significance was analyzed by Peto Peto Wilcoxon test. The data for collagen content, arterial pressure, breathing rate and collagen assays for different treatment groups were compared by one-way analysis of variance. Multiple pairwise comparisons were done by Holm-Sidak or Dunnetts test, when permitted, to determine significance. Graph values are expressed as mean $\pm$ 95% confidence intervals (CI).

RESULTS

Captopril and enalapril but not fosinopril decrease morbidity through pneumonitis

Administration of captopril (p = 0.0008, Fig. 1) or enalapril (p = 0.0029, Fig. 1) decreased morbidity following 13

Gy WTI. While 62% morbidity (at 80 days) was observed in animals receiving 13 Gy WTI alone, the two groups of rats treated with captopril or enalapril exhibited only 36% morbidity. The captopril or enalapril therapy also delayed the onset of morbidity by about 10 days ($p < 0.02$, Fig. 1). In contrast, rats on fosinopril showed no decrease in the inci-
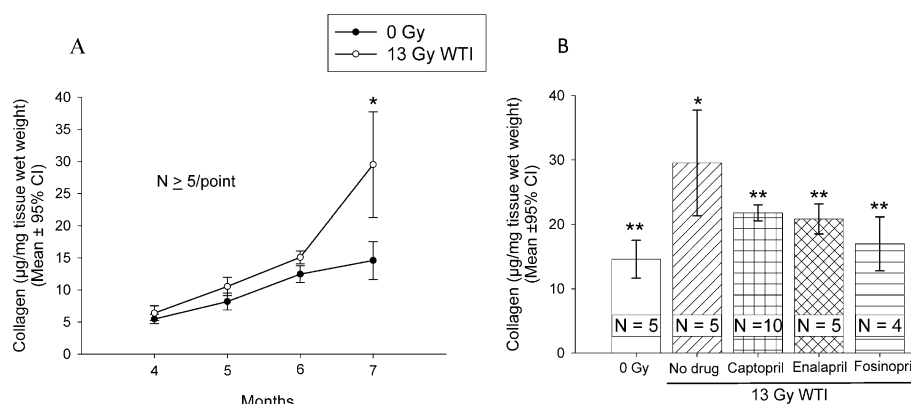

Fig. 2. Acid soluble collagen content (A). Newly synthesized collagen (acid and pepsin soluble) was measured in rat lungs each month at 4–7 months following exposure to 13 Gy whole thoracic irradiation (WTI) by the Sircol assay. N = 5–12 rats/point. (B): Collagen content following WTI and administration of ACE inhibitors at 7 months. *CI* indicates 95% confidence interval. '*' indicates $P < 0.05$ vs. age-matched normal & '**' indicates $P < 0.05$ vs.13 Gy WTI (no drug) at 7 months. N = the number of rats/group.
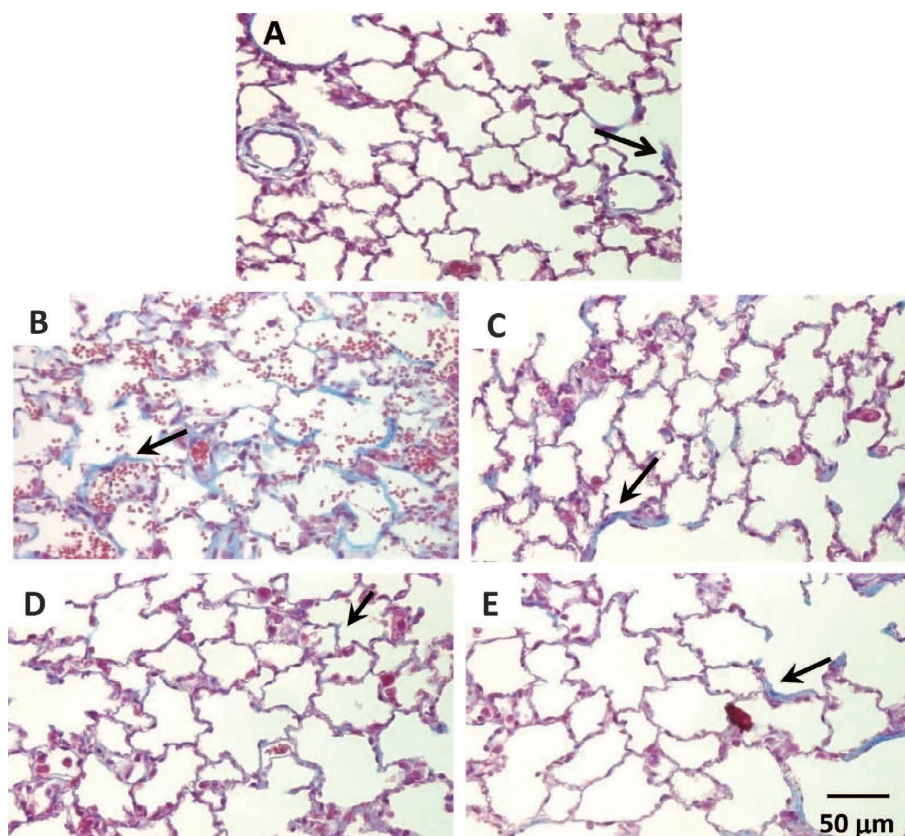

Fig. 3. Representative fields of Masson's trichrome stained slides of rat lungs 7 months after 13 Gy whole-thoracic irradiation (WTI). A: age-matched normal; B: 13 Gy WTI - no drug; C: 13 Gy WTI + captopril; D: 13 Gy WTI + enalapril; E: 13 Gy WTI + fosinopril. The arrow in (B) indicates deposition of collagen (stained in blue) in the alveolar walls. All sections are presented at the same magnification, represented by a scaled bar in E = 50 μm.

dence or timing of onset of morbidity at 40–80 days following irradiation.

ACE inhibitors protect against radiation-induced collagen synthesis

We assessed newly-synthesized collagen in control and irradiated rat lungs 4, 5, 6, and 7 months after 13 Gy WTI. We observed a progressive increase in lung collagen in both control and irradiated groups (Fig. 2A). A divergence was seen at 7 months where lungs from irradiated rats had twice the soluble collagen than those from age-matched controls (p < 0.05, Fig. 2A). At 7 months after irradiation, all ACE inhibitor-treated irradiated groups showed less collagen than the WTI-alone group, with no increase in collagen content in comparison to the age-matched normal rats (p < 0.05, Fig.

2B).

Trichrome stains of sections from irradiated rat lungs exhibited diffuse and patchy fibrosis (stained blue) in the alveolar wall or the interstitium (arrows in Figs. 3A–E) and adjacent to the pleura (not shown). Fibrosis was increased after 13 Gy (Fig. 3B) relative to that of age-matched control animals (Fig. 3A). Collagen deposition was decreased by ACE inhibitor therapy as seen in Figs. 3C–3E as compared to 13 Gy WTI alone (Fig. 3B).

The blue-color was quantified by scoring for the occurrence (see arrows in Fig. 3) and size of blue foci in the trichrome-stained slides (see Materials and Methods). This quantification (Fig. 4A) corroborated the histological changes observed in the lung parenchyma. Rats receiving irradiation alone had more collagen staining in the alveolar
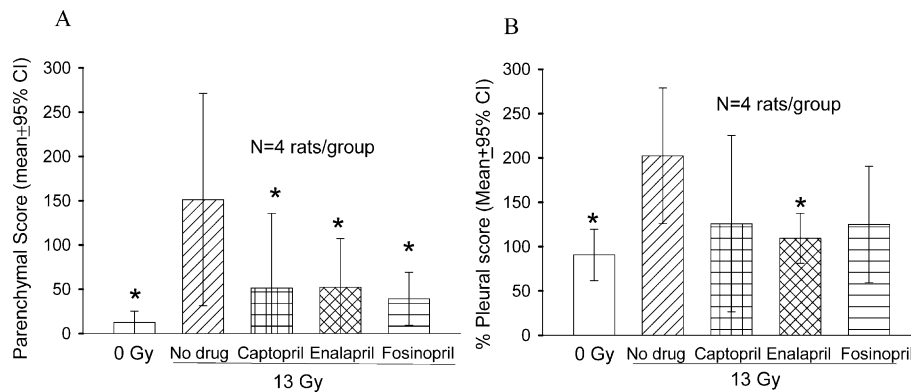

Fig. 4. Quantitation of trichrome staining. A: The blue stain over the whole area of the slide was quantitated in a blinded manner for occurrence and area of collagen in the interstitium (see arrows in Fig. 3), as described in the Material and Methods section. The product of these scores was used to represent collagen in the parenchyma. B: Quantification of blue area representing collagen associated with the pleura. *CI* indicates 95% confidence interval. '*' indicates *p* < 0.05 vs 13 Gy. (no drug) by ANOVA and Dunnett's test. Number of rats analyzed in each group = 4.
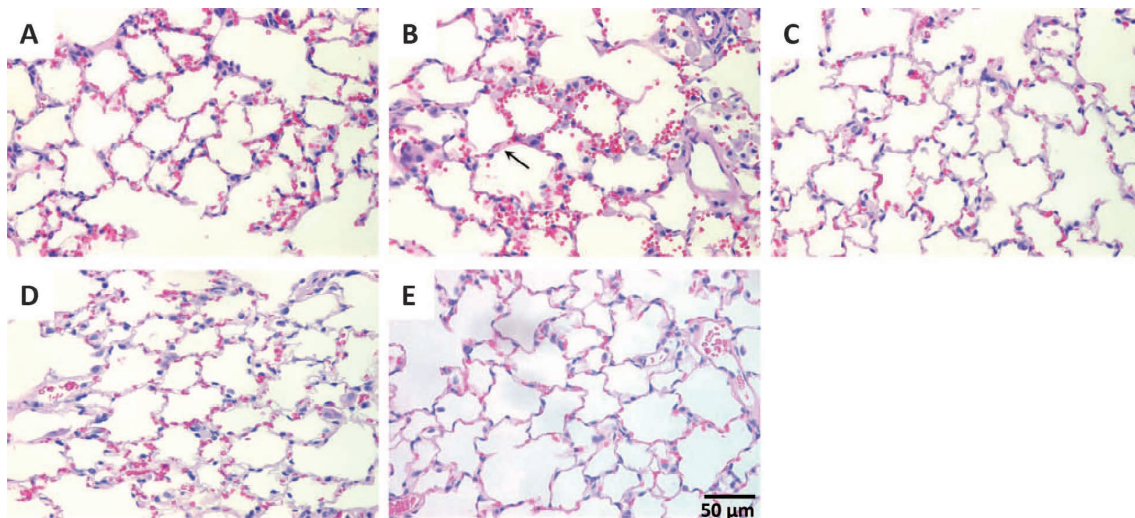

Fig. 5. Representative fields of hematoxylin & eosin (H & E) stained slides of rat lungs 7 months after 13 Gy whole-thoracic irradiation (WTI). A: Age-matched normal; B: 13 Gy WTI - no drug; C: 13 Gy WTI + captopril; D: 13 Gy WTI + enalapril; E: 13 Gy WTI + fosinopril. The arrow in (B) shows thickening of the alveolar wall. A–E are at the same magnification.
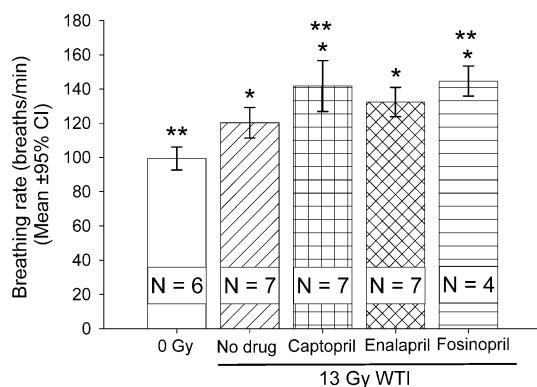
Fig. 6. Breathing rate at 7 months of rats that survived the acute pneumonitis phase after 13 Gy whole-thoracic irradiation (WTI), CI: 95% confidence interval. '*' indicates $p < 0.05$ vs. age-matched 0 Gy rats; '**' indicates $p < 0.05$ vs. 13 Gy alone. N = Number of rats studied in each group.

walls than age-matched normal animals (all $p < 0.05$, Fig. 4A). Groups treated with captopril, enalapril or fosinopril following WTI had less collagen staining than the rats receiving irradiation alone (all $p < 0.05$, Fig. 3). Collagen staining around the periphery (pleura) of the lung was greater in rats receiving irradiation alone than in irradiated rats treated with enalapril or in unirradiated controls (both $p < 0.05$, Fig. 4B).

Effect of radiation and ACE inhibitors on lung structure

There was no difference in the wet to dry lung weight ratio between unirradiated animals 4.6 (4.3–4.9) and 13 Gy WTI groups 4.0 (3.3–4.7) at 7 months. Animals treated with ACE inhibitors showed no difference in wet to dry lung weight ratio when compared to control or irradiated groups. Hematoxylin and eosin staining of the rat lungs at 7 months following 13 Gy WTI shows modest thickening of the interstitium (see arrow) in lungs from irradiated rats which was not as prominent with ACE inhibitors (Fig. 5C–E).

Effects of ACE inhibitors on lung function as assayed by measurement of breathing rates

At 7 months after WTI, breathing rates among surviving rats in all irradiated groups were higher than in unirradiated controls (Fig. 6). In addition, surviving rats treated with captopril or fosinopril had a slight but significant elevation in breathing rates compared to that of animals receiving irradiation alone.

DISCUSSION

In this study we show that irradiation of the total lung volumes of WAG/RijCmcr rats with a single dose of 13 Gy results in severe acute pneumonitis followed by increase in

lung collagen after 7 months. We further show that when therapy with ACE inhibitors is started 1 week after irradiation, captopril and enalapril but not fosinopril improve survival through the acute pneumonitis phase. All three ACE inhibitors mitigate late radiation-induced collagen deposition and positive trichrome staining in the parenchyma (structural markers of fibrosis).

Mitigators which are efficacious when the start of therapy is delayed until one week after exposure would provide a significant advantage after a mass casualty event. Because three structurally different ACE inhibitors were effective, in this regard the mechanism of mitigation against radiation-induced collagen synthesis should be a shared property of these drugs. Fibrosis in the lung is characterized by deposition of collagen[25,26] and is reported to be mediated by the AT_1 and AT_2 receptors present on fibroblasts.[27] ACE inhibitors block synthesis of angiotensin II, the peptide ligand for these receptors. This explains why all 3 inhibitors attenuate the synthesis of collagen. Radiation pneumonitis is a different injury that is caused by vascular and parenchymal damage to the lung and accumulation of an inflammatory infiltrate.[14–17,26,28] Mitigation of radiation pneumonitis by ACE inhibitors captopril and enalapril has been described, but the mechanism for this is not known.[12–14] To our knowledge we are the first to test fosinopril as a mitigator of radiation pneumonitis and we observe no mitigation of the early injury, but interestingly detect a decrease in collagen synthesis, a late effect of radiation to the lung.

Captopril is no longer a widely used ACE inhibitor because it has a short half-life and must be given more than twice a day. Enalapril and fosinopril are now more commonly prescribed because they are longer lasting and can be given only once daily. Therefore enalapril and fosinopril are attractive, readily assessable agents which could be used following radiation exposure. Enalapril is preferred over fosinopril since it is effective against both acute pneumonitis (Fig. 1) and delayed collagen synthesis (Figs. 2–4).

Several agents have been demonstrated to decrease morbidity of radiation to the thorax.[29,30] However, most have been started before or immediately after radiation or have been studied only after fractionated doses of radiation. Of interest, treatment with captopril in doses similar to those we employed in this study was reported not to decrease vascular permeability associated with the pneumonitis phase of WTI in rats.[31]

In our model, pulmonary fibrosis as evidenced by increased collagen content was not evident until 7 months after irradiation. Wet to dry weights were not increased in our cohorts, suggesting that the wet to dry weight ratio is not as sensitive an indicator for pulmonary fibrosis as our other endpoint. Other investigators have reported that increased hydroxyproline concentrations, histological evidence of fibrosis and tachypnea in WAG/Rij rats receiving WTI appears after 76 weeks.[25,32] Increases in soluble collagen

and trichrome stain in Sprague Dawley rat lungs are evident 35 weeks after a single WTI dose of 18 Gy.[11] Fractionated radiation[25] or higher doses to smaller portions of lung are more efficient than WTI in producing fibrosis, the latter attributable at least in part to lesser acute-morbidity because some portion of the lung is spared. Perhaps most relevant to the present studies, captopril, enalapril or losartan started immediately following radiation to a single hemithorax are reported to decrease collagen and histological evidence of fibrosis in Sprague Dawley rats 6 months later.[12]

The doses of captopril (145–207 mg/m²/day), enalapril (19–28 mg/m²/day) and fosinopril (19–28 mg/m²/day) used for our experiments are within the range administered for treatment of hypertension and other illness in humans. These doses demonstrated roughly equivalent biological activity as observed by the decrease in blood pressure and have been described by other investigators to inhibit ACE activity in unirradiated rats.[33]

All irradiated rats had a slightly higher breathing rates than age-matched controls at 7 months after irradiation (Fig. 6). These observations are in accord with Calveley *et al.* (2010)[11] who reported a second peak in the breathing rate at 14–26 weeks in rats receiving WTI and those of van Rongen *et al.*,[32] who observed an increase in breathing rate correlated to an increase in hydroxyproline at 76 weeks after radiation. Although captopril, enalapril or fosinopril showed mitigation of radiation-induced lung fibrosis at 7 months (Figs. 2–4), there was no apparent reduction in the breathing rate by the ACE inhibitors. Rather, we observed a slight increase in breathing rate in these rats compared to rats receiving irradiation alone (Fig. 6), though this was very modest as compared to the increase observed during pneumonitis.[14,15] Similarly the structural changes in lung collagen are not severe enough that they would be expected to substantially increase the breathing rate or alter lung function.

In summary, we observed 2 stages of injury to the lung after exposure to whole-thorax irradiation: the acute pneumonitis phase occurring from 40–80 days (Fig. 1) and collagen deposition after 7 months (Figs. 2–4). The ACE inhibitors captopril and enalapril but not fosinopril, decreased morbidity during the pneumonitis phase while all three mitigated radiation-induced pulmonary fibrosis at 7 months. These agents afford protection even when therapy is not started until one week after irradiation. Because three structurally-different ACE inhibitors are effective against pulmonary fibrosis, the mechanism of mitigation could be explained by suppression of the renin angiotensin system and not structural side groups of different ACE inhibitors. Understanding the mechanism(s) of action of ACE inhibitors will boost their potential for use against radiation-induced multiple organ injuries. Our results also suggest the severity of pneumonitis may not affect development of fibrosis. A similar observation has been made in strains of mice, where the primary lethal end point for the C3H/HeN mice is alveolitis while the C57/BL/6 strain exhibit mild pneumonitis but a strong late fibrosis.[34–36] Therefore we suggest the two stages of lung injury occur by different mechanisms. Further studies are needed to elucidate this.

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