Published in final edited form as:
Int J Radiat Oncol Biol Phys. 2014 July 15; 89(4): 722–728. doi:10.1016/j.ijrobp.2014.03.048.

Targeting the renin-angiotensin system combined with an antioxidant is highly effective in mitigating radiation-induced lung damage

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Abstract

Purpose—We investigated the outcome of suppression of renin angiotensin system (RAS) using Captopril combined with an antioxidant (EUKarion-207) for mitigation of radiation-induced lung damage in rats.

Materials and Methods—The thoracic cavity of female Sprague-Dawley (SD) rats was irradiated with single dose of 11 Gy. Treatment with Captopril at a dose of 40 mg/kg/day in drinking water and EUK-207 given by subcutaneous injection (8 mg/kg daily) was started 1 week (wk) post-irradiation (PI) and continuing until 14 wks PI. Breathing rate was monitored until the rats were sacrificed at 32 wks PI when lung fibrosis was assessed by lung hydroxyproline content. Lung levels of the cytokine, Transforming Growth Factor (TGF)-β1, and macrophage activation were analyzed by immunohistochemistry. Oxidative DNA damage was assessed by 8-hydroxy-2-deoxyguanosine (8-OHdG) levels and lipid peroxidation was measured by a T-BARS assay.

Results—The increase in breathing rate in the irradiated rats was significantly reduced by the drug treatments. The drug treatment also significantly decreased the hydroxyproline content, 8-OHdG and malondialdehyde levels, and levels of activated macrophages and the cytokine TGF-β1

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at 32 wks. Almost complete mitigation of these radiation effects was observed by combining Captopril and EUK-207.

Conclusion—Captopril and EUK-207 can provide mitigation of radiation-induced lung damage out to at least 32 wks PI following treatment given 1–14 wks PI. Overall the combination of Captopril and EUK-207 was more effective than the individual drugs used alone.

Keywords

Radiation; Lung; Mitigation; Captopril; EUK-207

Introduction

There is significant concern regarding accidental overexposure to radiation but our knowledge of the best clinical management of such exposures remains uncertain[1]. Radiation injury to the lung is one of the major concerns due to its sensitivity. Mechanisms are still poorly understood, although several studies have implicated a prolonged inflammatory response, the upregulation of pro-inflammatory cytokines and reactive oxygen species (ROS), hypoxia and loss of alveolar epithelial cells as well as arterioles and influx of fibrocytes as major factors in causing pneumonitis and fibrosis in irradiated lung[2–4]. Effective measures to mitigate or treat such exposures are currently very limited and no agent has yet been approved by the US Federal Drug Agency (FDA) for "radiation mitigation" to lung when administered after radiation[5–7].

Classes of drugs studied for protection/mitigation of radiation-induced late injuries include suppressors of the renin-angiotensin system (RAS) and anti-oxidants. Initial studies reported promise with RAS inhibitors as protectors[8] and more recent work has reported that various angiotensin converting enzyme (ACE) inhibitors can also act as mitigators of radiation damage in lung[4,9–11]. Anti-oxidants have also shown promise as potential mitigators of radiation damage and acute pneumonitis can be partially mitigated with salen-Mn complexes such as the compound, EUK-207 with administration initiated at 1–2 weeks (wks) after radiation exposure[12–14]. Salen-Mn complexes are a class of synthetic low molecular weight agents that mimic the antioxidant enzymes superoxide dismutase (SOD) and catalase, scavenging superoxide and hydrogen peroxide, respectively[15,16]. EUK-207 is well-tolerated, suggesting low toxicity[17]. In this study, we examined the combination of the ACE inhibitor Captopril and EUK-207 in SD rats, a well-established model for radiation-induced lung damage. Captopril has widespread clinical use, is orally available and is well tolerated.

Materials and Methods

Animal treatments

Female SD rats (Charles River Laboratories International, Inc., Wilmington, MA, USA) aged 7–8 wks and weighing 150–160g were used in all experiments. They were housed in animal facilities accredited by the Canadian Council on Animal Care and treated in accordance with approved protocols. This strain was chosen for the study because they tend to show early increases in breathing rate associated with acute pneumonitis and robust radiation-induced fibrosis at late time points following irradiation[13]. The experimental

groups of rats (8 rats per group) included an untreated control group maintained throughout the study, whole thoracic radiation alone (WTR), radiation with EUK-207 (WTR+EUK), radiation with Captopril (WTR+CAPT), radiation with Captopril+EUK-207 (WTR+Capt +EUK) and Captopril and EUK-207 without radiation (Capt+EUK). All surviving rats were euthanized at 32 weeks post irradiation (PI).

Drug treatments

Drug treatments were initiated 1 week PI and terminated 14 weeks PI. EUK-207 was custom-synthesized and characterized as described previously[18]. The animals received a dose of 8 mg/kg/day in saline by daily subcutaneous (sbc) injection as reported previously[13]. EUK-207 is water-soluble and given by sbc injection results in readily detectable plasma levels that persist for several hours[19]. Pharmaceutical-grade captopril (Sigma, USA) was dissolved in the drinking water at 300 mg/L, which is expected to deliver ~40 mg/kg/day or 236 mg/m^2/day to a rat, a dose in the approved range for use of captopril in humans[9].

Irradiation

The thoracic cavity in each animal was irradiated using an image-guided small animal-irradiator (CX-Rad-225, Precision X-ray Inc. USA). The description and calibration of this unit has been described previously[13, 20]. The dose rate at 225kVp, 13mA (HVL: 0.93mm Cu, added filtration: 0.3mm Cu) was estimated as 3.51 Gy/min at the depth of 1.5cm in solid water for the whole thorax irradiation. Each rat was set up individually for irradiation, using the imaging capability of the irradiator, and given a dose of 11Gy to the whole thorax (WTR) using an anterior and posterior beam.

Breathing Rate

We measured the breathing frequency of rats using a respiration rate monitor (Columbus Instruments, Columbus, OH, USA) as described previously[13,21].

Tissue Analysis

After euthanasia of the animals at 32 weeks the lobes of the right lung were flash frozen and kept for biochemical analyses and the left lobe of the lung was used for histopathology. A volume (0.5–1.0 ml) of 10% formalin was injected into the left lobe to expand the alveoli and the lobes were placed in 10% formalin for fixation. The antibodies used for the immunohistochemical staining and Masson's Trichrome staining have been described previously[13,21,22]. Sections were cut from the left lobe of the lung in the coronal plane. We analyzed five sections from the lungs of different surviving rats. Following staining, the slides were scanned using the ScanScope XT (Aperio Technologies, USA). The images were then viewed using ImageScope (Aperio Technologies) for quantitative analysis of the positive staining (the ratio of positive pixels/total number of positive and negative pixels, with air spaces excluded, as described previously[13, 21]). Since the staining in these sections is primarily cellular the quantitative analysis gives an estimate of the density of stained cells over the whole section. Tissue Studio (Definiens AG, Munich, Germany) was also used to measure percent of cells stained with TGF-β1 (at different levels) in the whole tissue section. Nuclei were identified (hematoxylin staining) and a circle with a radius of 3 μm

greater than the nucleus was drawn to define the cell area. Four levels of cell staining were defined (high, medium, low, none).

Lipid peroxidation

Lipid peroxidation was assessed by measuring Malondialdehyde (MDA) levels in part of the right lung (25 mg wet wt.) of each rat using a T-BARS assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The flash-frozen sample was thawed, sonicated for 15s in RIPA buffer with protease inhibitors (Roche Applied Science, Laval, Quebec), and then centrifuged (1600g). The supernatant containing the lipid fraction was used for the assay as described previously[13,21].

Hydroxyproline estimation

Fibrosis was assessed by analyzing part (100 mg wet wt.) of the upper right lung for hydroxyproline content using a colorimetric assay as described previously[13,21].

Statistical analysis

ANOVA and Tukey's method for the adjustment of least square means in multiple comparisons were used for analysis of the data sets. The WTR-only group was set as the primary comparison group for these analyses since the purpose of the study was to determine the efficacy of treatments relative to this group. Linear mixed effects modeling was used to examine trends in the breathing rate data over fixed time windows of 0–6, 6–10, 10–14 and 14–32 weeks. We also did a spot time point comparisons at 32 weeks.

Results

Figure 1 shows the average breathing rate for the rats following irradiation. For the control rats and those given the combined drug treatment alone, the breathing rate stayed at approximately 130–150 breaths per minutes (bpm), over the whole 32 wks. The rats given WTR alone demonstrated an initial increase in bpm over 2–14 wks which peaked at approximately 240 bpm at 6–10 wks PI. The bpm of this group of rats declined at later times but remained significantly ($p < 0.01$) above the control level out to 32 weeks. The rats given WTR plus captopril, or EUK-207 or their combination all showed a significant reduction ($p<0.01$) in bpm relative to the WTR group in all time windows. The combination drug treatment showed levels significantly lower than EUK treatment alone in the 6–10 and 14–32 wk time windows ($p<0.05$ and $p<0.01$) but was significantly different from Captopril treatment alone only during the 6–10 wk time window ($p<0.05$). At 32 weeks only the WTR group was significantly different from the other groups.

Irradiation affected the increase in body weight of the rats (Figure 2). The two non-irradiated groups showed a steady increase until about 28 weeks. The WTR group showed a 3–4 wk delay and this was similar for the WTR + EUK and WTR+ captopril groups. However, the rats given WTR + captopril + EUK-207 were similar to the unirradiated groups and significantly better than WTR plus either drug treatment alone ($p<0.01$). That the dose of 11Gy (225kVp X-rays) is close to the lung tolerance limit for the SD strain of rats is illustrated by the fact that at the peak of the breathing rate increase (6–10 wks PI) we

observed some morbidity in the WTR group (2 rats), the WTR + Captopril group (1 rat) and the WTR + EUK-207 group (1 rat) which required euthanasia. No other rats died during the course of the experiment.

We assessed fibrosis in the lungs of the rats using both Masson-Trichrome (M-T) staining and hydroxyproline levels. Images of M-T staining in the lungs (see Supplementary Figure S1) showed large increases in staining for rats given WTR alone. These staining levels were substantially reduced in the rats given the drug treatments. Consistent with the M-T levels there was a large increase in hydroxyproline levels in the WTR only rats (Figure 3A) relative to both unirradiated groups ($p<0.01$). All the drug treatment groups had significantly ($p<0.01$) lower hydroxyproline content than the WTR group but there was no significant differences among the groups. The drug combination showed a trend as the most effective treatment.

TGF-β1 is believed to play an important role in stimulating collagen deposition. Images of TGF-β1 staining are shown in Figures S2 and S3. There were increased levels of both localized and regional staining in the irradiated animals. We analysed the staining levels for this cytokine using both Image Scope (Figure 3B and S2) and Tissue Studio (Figures 3C and S3) to assess both regional and local cellular staining levels. Both analyses give very similar results. In the WTR group there was a significant ($p<0.01$) increase in levels of TGF-β1 expression at 32 weeks post-irradiation. All the drug treatment groups showed a significant ($p<0.01$) reduction in TGF-β1 staining to levels similar to those observed in the two unirradiated groups but differences between the drug treatment groups were not significant.

The presence of oxidative damage was measured using two markers of oxidative lesions. In the DNA we assessed 8-hydroxy-2-deoxyguanosine (8-OHdG) staining (using Image Scope), and in the tissue we measured levels of malondialdehyde (MDA) to determine lipid peroxidation. Quantitative analysis of the lung sections stained for 8-OHdG (see Figure S4) showed that the WTR group demonstrated a significant ($p < 0.01$) increase relative to the control (Figure 4A). All the drug treatment groups showed significant reduction in 8-OHdG levels ($p<0.01$), and the WTR+Capt+EUK group showed a significantly greater decrease ($p<0.05$) than the WTR+Capt group. Relative to the WTR+EUK group the greater decrease with the combination treatment was on the borderline of significance ($p = 0.066$).

The T-BARS assay used to quantify the concentrations of MDA in the lung (Figure 4B) showed a significant ($p<0.01$) increase in the WTR group relative to the unirradiated groups and all the drug treatment groups showed significant ($p<0.01$) reduction of MDA concentration to levels similar to those in the control. There were no significant differences between the three treatment groups but again the combination drug treatment group showed the lowest value.

We measured overall levels of the inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α in irradiated lung using Image Scope (see Figure S5). IL-1β, IL-6 and TNF-α levels were significantly ($p<0.01$) increased at 32 weeks post-irradiation relative to the unirradiated controls but only IL-1β was significantly ($p<0.01$) affected by drug treatment and only in the groups treated with Captopril. Levels of activated macrophages (ED-1 staining assessed

using Image Scope) are shown in Figure 5 (and Figure S6). In the WTR group ED-1 staining was significantly ($p<0.05$) elevated relative to the non-irradiated groups. The WTR + drug treatment groups showed reduced levels but only that for the combined drug treatment group was significant ($p<0.05$). The difference between the combination and the individual drug treatment groups was not significant.

Discussion

The lung is one of the most susceptible organs to radiation toxicity and after radiation accidents lung injury may play an important role in multi-organ failure[7]. There remains a need to develop effective mitigators against radiation-induced lung damage. The ideal such agents must be easy to deliver, inexpensive and relatively non-toxic. Our current study suggests a significant step in this direction. The combination of EUK-207 and Captopril gave almost complete mitigation of acute pneumonitis and fibrosis using a treatment schedule that started 1 week after radiation exposure (PI) and was continued through the pneumonitis phase but stopped at 14 weeks PI, well before the measurements (at 32 wks) of fibrosis.

Our current experiments were based on recent work which demonstrated that different agents with anti-oxidant properties, such as EUK-207 and genistein, are potential mitigators of radiation-induced lung damage in animal models[12,13,22]. Similar results have been reported using chronic administration of the novel catalytic antioxidant, AEOL 10150[23]. Various renin angiotensin converting enzyme inhibitors have also been found to mitigate various aspects of radiation-induced lung damage including improvement of pulmonary vascular hemodynamics altered by radiation injury, even when treatment was started 2 weeks after radiation exposure[4, 9–11]. Furthermore the increase in lung collagen at 30 weeks after a single dose of 13 Gy X-rays to the whole thorax of WAG/RijCmcr rats was mitigated by ACE inhibitors, enalapril, captopril and fosinopril using a different strain of rats (Wistar) than in the current study[9]. Both captopril and enalapril improved survival during pneumonitis between 6–10 weeks when drug treatment was started 1 week after irradiation.

In radiation-induced lung damage, prolonged expression of pro- and anti-inflammatory cytokines and ROS production together with epithelial cell death and potential influx of fibrocytes from the bone marrow are believed to form the basis for the inflammatory and fibrogenic processes. Recent studies investigating the effect of lipopolysaccharide (LPS) treatment on radiation-induced lung response in mice knocked-out for various components of the Tumour Necrosis Factor (TNF) alpha pathway have suggested a role for this cytokine pathway[24]. It is thus of interest that in the current study we observed increased levels of TNF-alpha and IL-6 expression at 32 weeks post irradiation despite observing almost complete mitigation of fibrosis. Levels of activated macrophages were reduced, indicating that other cells within the lung may be maintaining the high levels of these cytokines, as has been suggested by others[2,25]. While it is well recognized that expression of inflammatory cytokines is temporally variant after irradiation, our findings suggest the possibility that prolonged expression of certain inflammatory cytokines may not be directly associated with the development of radiation-induced fibrosis in all animal models, consistent with suggestions from a recent review of other syndromes resulting in lung fibrosis[26]. Notably,

the cytokine most closely associated with radiation-induced fibrosis TGF-β1[27–29] was significantly increased in WTR group but was clearly reduced in the drug treated groups in parallel with reduced fibrosis.

It is possible that the two mitigating agents are acting directly on the oxidative stress associated with the inflammatory response. In this context both EUK-207 and captopril alone suppressed oxidative stress, based on staining of 8-OHdG and on MDA levels, in the irradiated lung. This suggests that the mitigating effects of captopril may involve some antioxidant mechanism(s). Angiotensin II is known to increase activation of NADPH oxidases[30] so this is one possible mechanism by which an ACE inhibitor might have an antioxidant effect during an injury scenario. A direct antioxidant effect, while possible and not without precedent, seems less likely[31]. Captopril has a free SH group and, therefore, potential redox modulating properties, but enalapril does not, and, as discussed above, it is also a potent mitigator of radiation lung injury. As noted by Kma et al.[9], collagen deposition is reported to be mediated by the AT1 and AT2 receptors present on fibroblasts. ACE inhibitors block synthesis of angiotensin II, the peptide ligand for these receptors, potentially attenuating fibrosis. These agents may thus work effectively together because EUK-207 (with help from Captopril) attenuates the early inflammation, while Captopril can also help to block collagen production.

Conclusion

In this study, we found significant mitigation of radiation-induced lung damage using a short schedule of Captopril and EUK-207 individually or their combinations in S-D rats. The combination of the two drugs, at the doses used, demonstrated effects that were generally greater than either drug alone suggesting strong potential for mitigating of radiation-induced lung damage following exposure in an event like terrorist attack or nuclear accident or for future clinical trial of these drugs among patients. The window of one week before starting of drug treatment should allow biodosimetry and other necessary evaluation in a mass casualty event. Further studies relating to the exact mechanism of action of these drugs in mitigating radiation-induced lung damage, including detailed cytokine analysis, remain necessary, as do studies of whether such treatment would increase the tolerated dose. Similarly, studies with fractionated treatments will be required to assess the therapeutic potential of this approach for reducing lung injury in cancer patients receiving thoracic irradiation, although reports on radiation therapy patients taking ACE inhibitors suggest they may reduce incidence of lung complications[32–34].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Melania Pintilie for help analyzing the breathing rate data. This work was supported by funds from an NIAID/NIH U19 program (U19 AI-067734) and by funds from the Canadian Institutes of Health Research. Partial support was also provided by the Princess Margaret Foundation and the Ontario Ministry of Health and Long Term Care. The views expressed do not necessarily reflect those of OMHLTC.

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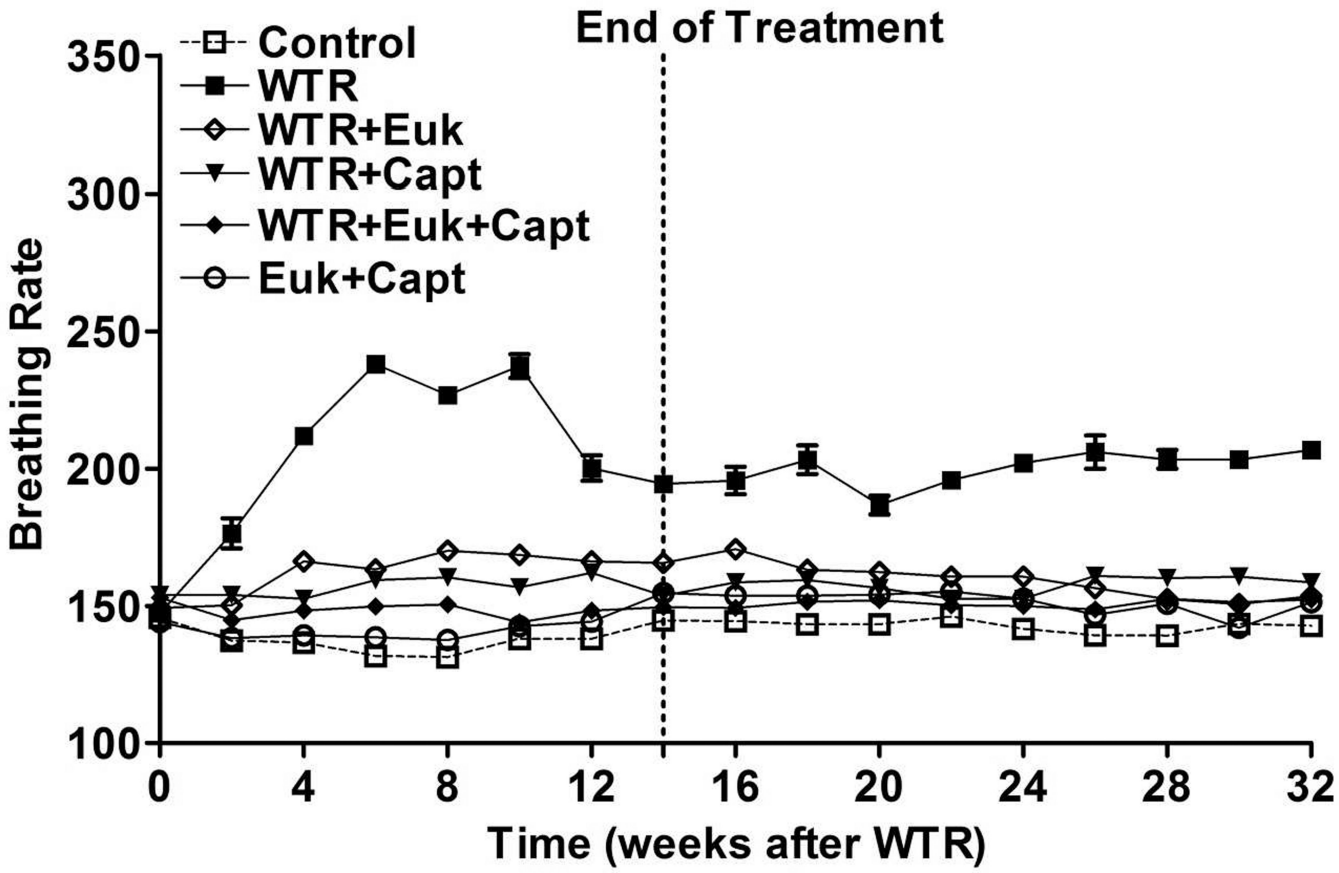

Figure 1.

Breathing rate (mean breaths per minute) as a function of time after irradiation. Control = No irradiation; WTR = whole thorax irradiation (11Gy); WTR+Euk = whole thorax irradiation + Euk-207; WTR+Capt = whole thorax irradiation + Captopril; WTR+Euk+Capt = whole thorax irradiation + Euk-207 + Captopril; Euk+Capt = Euk-207 + Captopril. The drug treatments were started 1 week post-irradiation (PI) and terminated at 14 weeks PI. The rats were euthanized at 32 weeks PI. Each point represents the mean (±SEM) for all rats available for analysis at the different times. All groups contained 8 rats at the start of the experiment. At 8 weeks this was reduced to 6 rats for the WTR group and to 7 rats for the WTR + Euk-207 group and the WTR + Capt group due to morbidity requiring euthanasia. These numbers remained constant out to sacrifice at 32 weeks.

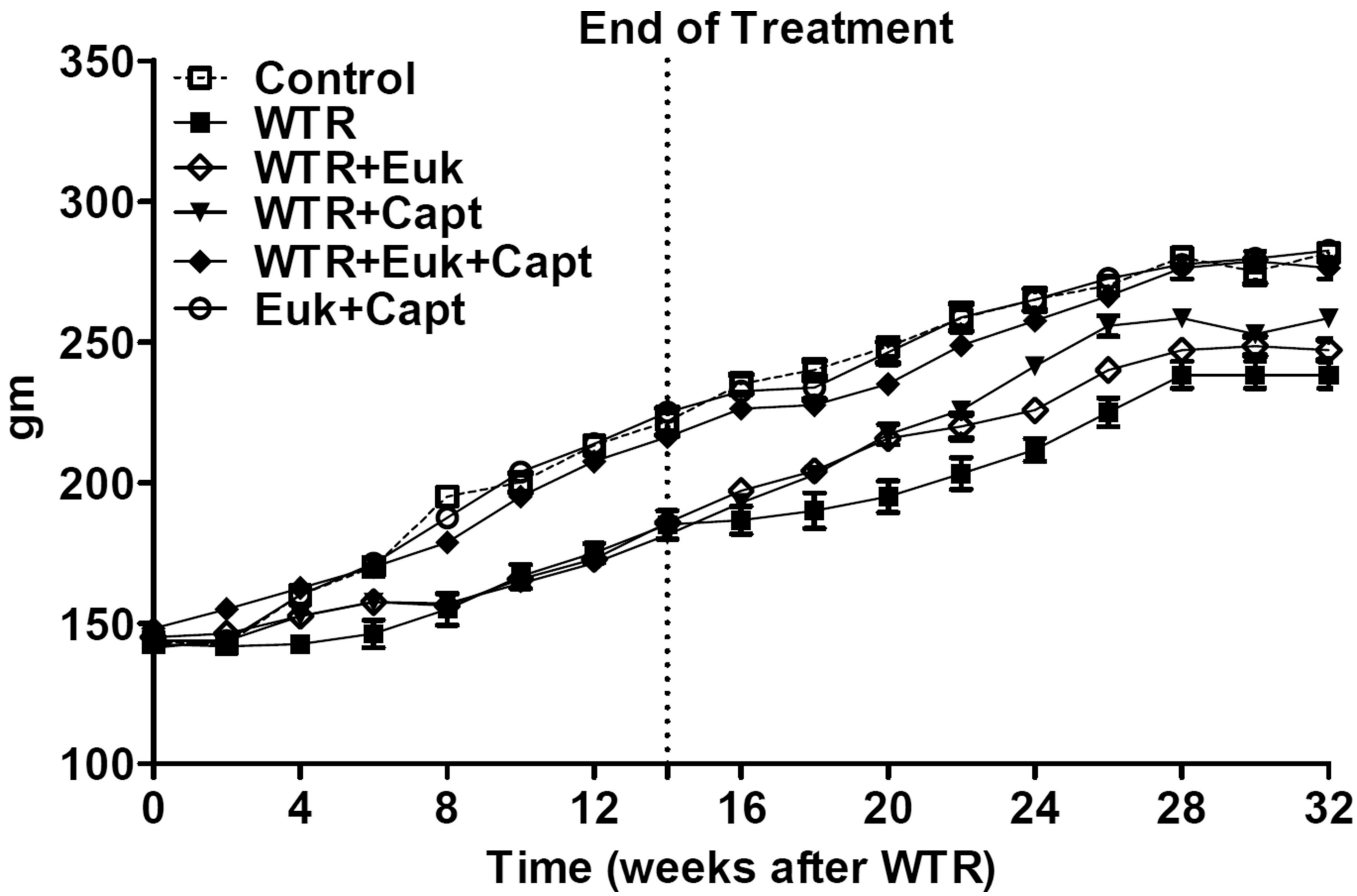

Figure 2.

Body weight (mean in grams) as a function of time after irradiation. Labeling of the treatment groups (and number of rats per group) as indicated in the legend to Figure 1. Each point represents the mean (±SEM) for all rats available for analysis at the different times.

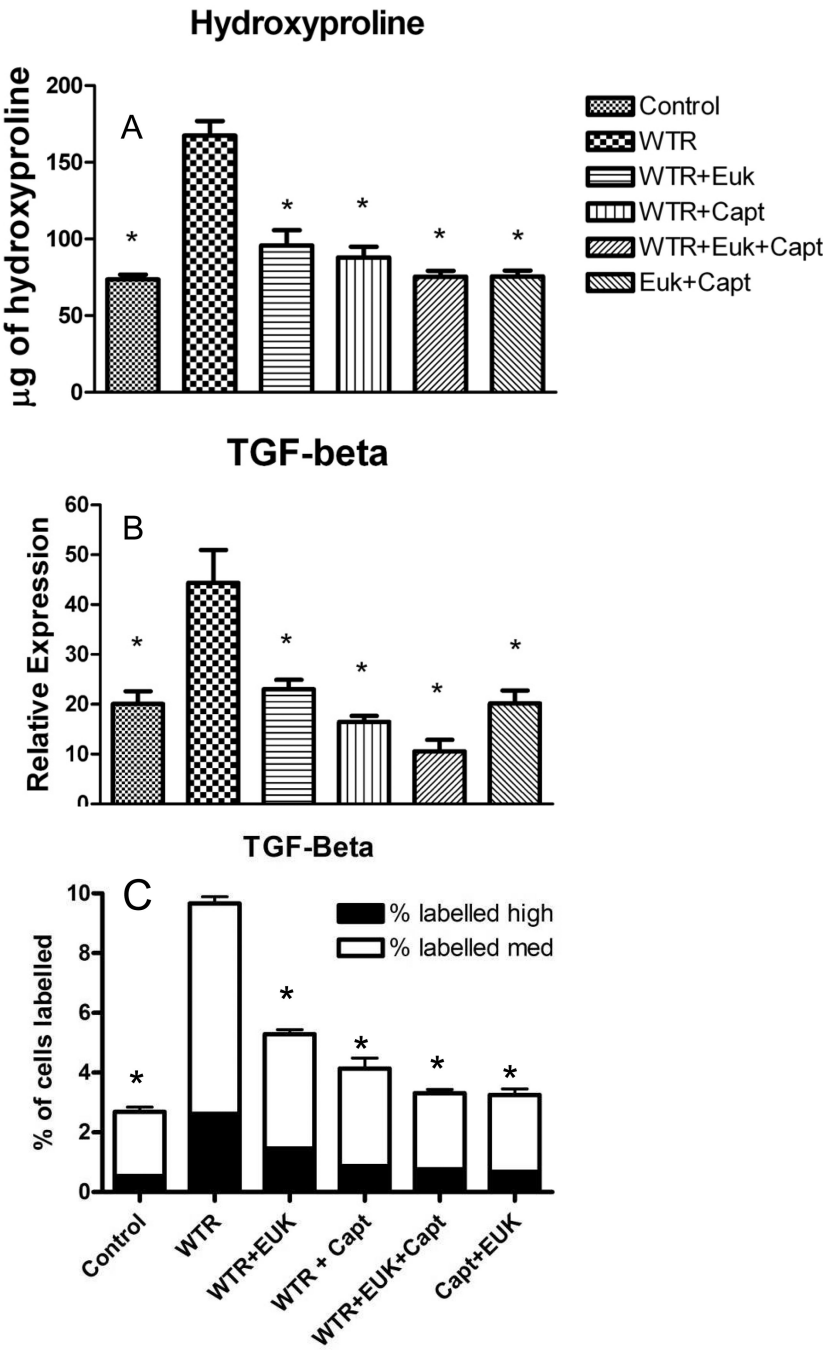

Figure 3.

A) Hydroxyproline (µg of hydroxyproline/100mg of wet lung tissue) content of the lung tissue at 32 weeks after irradiation; B) Aperio analysis of TGF-beta staining (percent positivity) at 32 weeks after irradiation. C) Definiens analysis of TGF-beta staining (percent of cells stained). Each bar represents the mean (±SEM) for all rats available for analysis. Labeling of the treatment groups (and number of rats per group) as indicated in the legend to Figure 1. The asterisks indicate groups that are significantly different from the WTR group in each panel.

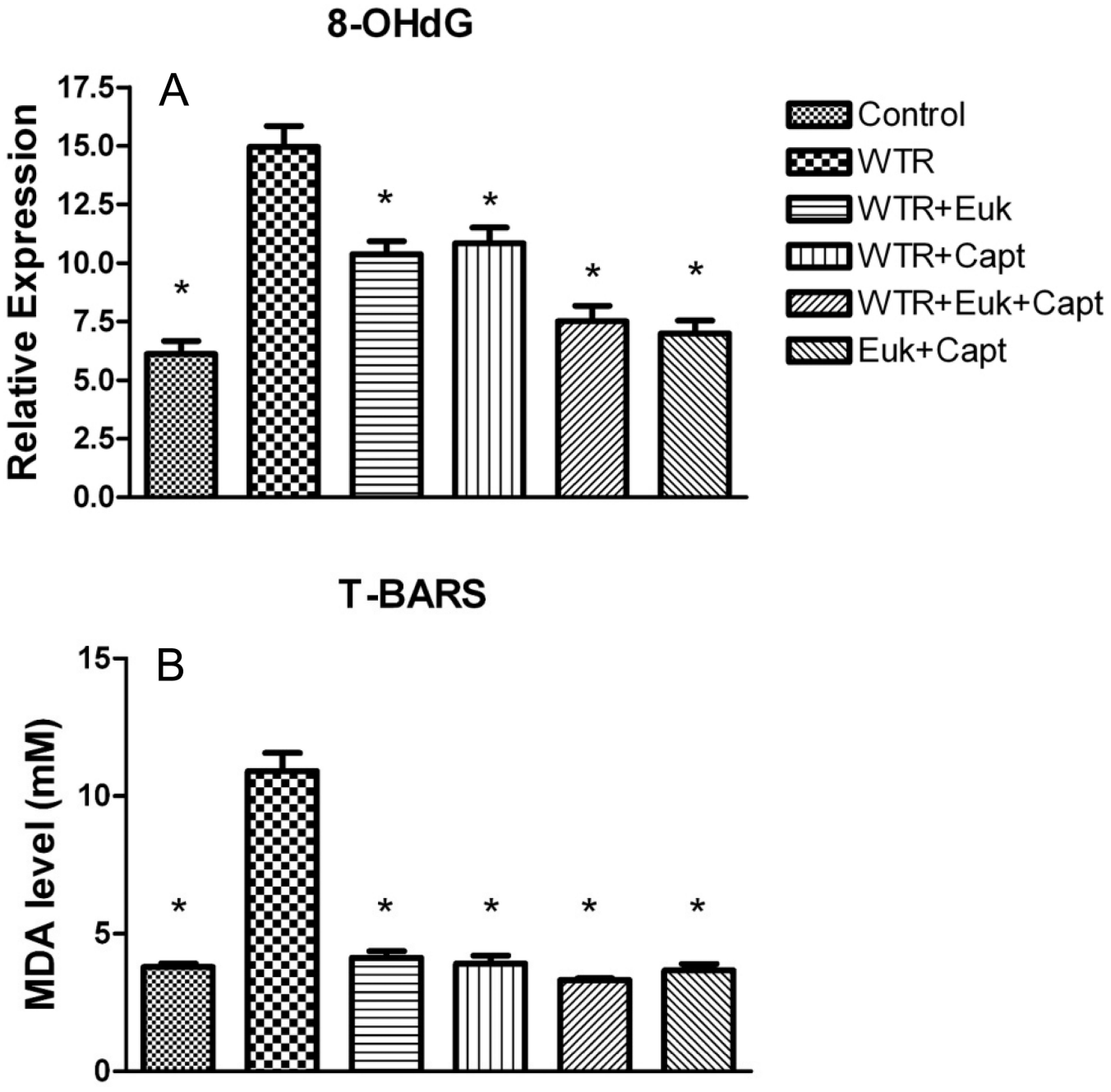

Figure 4.

A) Analysis of 8-OHdG staining (percent positivity assessed using Tissue Scope) at 32 weeks after irradiation; B) Analysis of MDA levels (mM) in the lung tissue at 32 weeks after irradiation. Each bar represents the mean (±SEM) for all rats available for analysis. Labeling of the treatment groups (and number of rats per group) as indicated in the legend to Figure 1. The asterisks indicate groups that are significantly different from the WTR group in each panel.

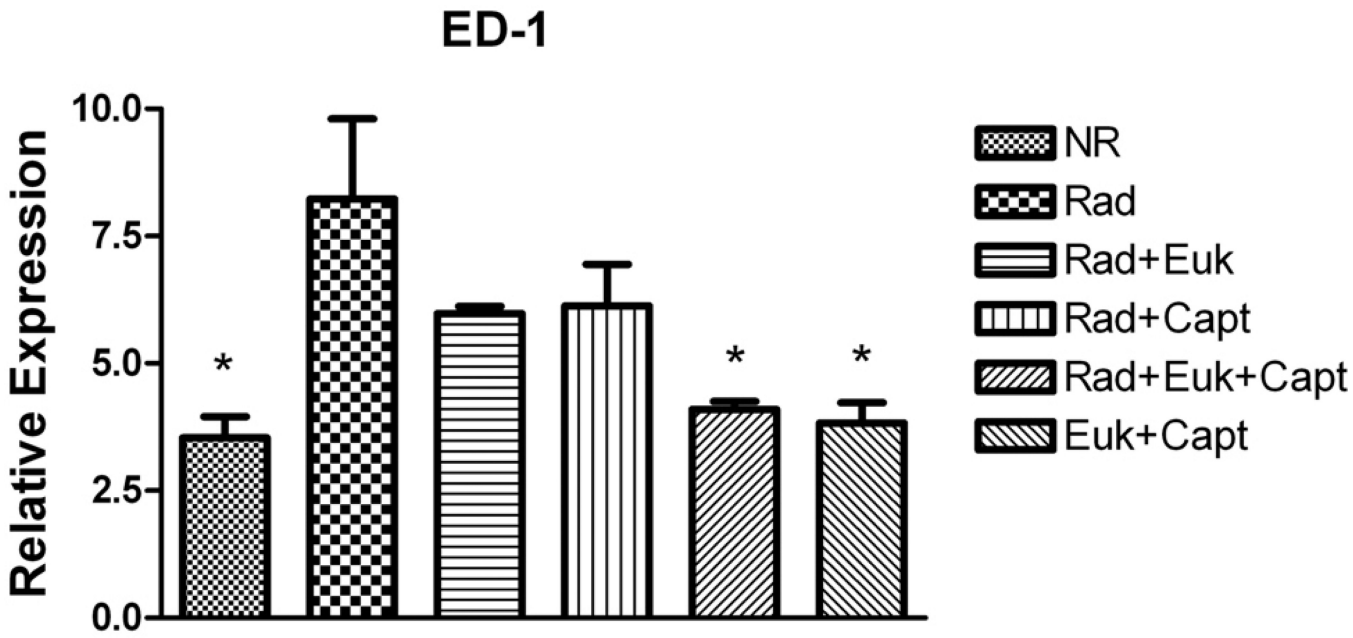

Figure 5.

Analysis of macrophage activation (ED-1 staining) at 32 weeks after irradiation as percent positivity (assessed using Tissue Scope). Each bar represents the mean (±SEM). Labeling of the treatment groups (and number of rats per group) as indicated in the legend to Figure 1. The asterisks indicate groups that are significantly different from the WTR group.