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Combined radiation and burn injury results in exaggerated early pulmonary inflammation

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Abstract

Events such as a nuclear meltdown accident or nuclear attack have potential for severe radiation injuries. Radiation injury frequently occurs in combination with other forms of trauma, most often burns. Thus far, combined injury studies have focused mainly on skin wound healing and damage to the gut. Since both radiation exposure and remote burn have pulmonary consequences, we examined the early effects of combined injury on the lung. C57BL/6 male mice were subjected to 5 Gy of total body irradiation followed by a 15% total body surface area scald burn. Lungs from surviving animals were examined for evidence of inflammation and pneumonitis. At 48 hours post-injury, pathology of the lungs from combined injury mice showed greater inflammation compared to all other treatment groups, with marked red blood cell and leukocyte congestion of the pulmonary vasculature. There was excessive leukocyte accumulation, primarily neutrophils, in the vasculature and interstitium, with occasional cells in the alveolar space. At 24 and 48 hours post-injury, myeloperoxidase levels in lungs of mice given combined injury were elevated compared to all other treatment groups ($p < 0.01$), confirming histological evidence of neutrophil accumulation. Pulmonary levels of the neutrophil chemoattractant KC (CXCL1) were 3 times above that of either injury alone ($p < 0.05$). Further, monocyte chemotactic protein-1 (MCP-1, CCL2) was increased 2-fold and 3-fold compared to burn injury or radiation injury, respectively ($p < 0.05$). Together, these data suggest that combined radiation and burn injury augments early pulmonary congestion and inflammation. Currently, countermeasures for this unique type of injury are extremely limited. Further research is needed to elucidate the mechanisms behind the synergistic effects of combined injury in order to develop appropriate treatments.

Keywords

radiation injury; burn injury; severe combined injury; cytokines; pneumonitis

INTRODUCTION

The threat of a nuclear event resulting in mass casualties is ever-present and of great concern (1–3). In nuclear accidents or attacks, radiation exposure is likely to be coupled with other forms of injury, such as burns, blast injury, wounds, blunt trauma, and infectious complications. It has been predicted that 65–70% of casualties in a realistic nuclear emergency will sustain some form of traumatic injury in addition to radiation exposure, while only 15–20% will be affected by radiation alone (4). Reports from the nuclear explosions in Hiroshima and Nagasaki, as well as the Chernobyl reactor nuclear accident, showed that over 60% of radiation victims also sustained a traumatic injury of some kind, most often burn injury (4, 5). Clinical data from these incidents show that victims of combined radiation injury (CRI) suffer more severe post-injury complications than those patients with a single type of injury. Individuals that died shortly after these incidents sustained injuries to nearly every organ system, and their injuries were further complicated by infections (6).

Combined radiation and burn injuries cause aberrant wound healing and severe hematopoietic impairment (7, 8). Burn injury alone, even in the absence of smoke inhalation, often causes damage to lung tissue (9). Radiation exposure also has negative effects on lung tissue and lung function. The lung is a highly radiosensitive organ, and pulmonary injury has been reported in over 50% of victims of radiation accidents (10). Following the Chernobyl incident, two of seven patients that were diagnosed with pneumonitis died. Six other patients that survived the initial hematopoietic and gastrointestinal injury, died later on with varying degrees of respiratory disorders (11). More recent nuclear accidents in Japan, Tokaimura and Fukushima, demonstrate that there is an imperative need to learn how to best treat victims of these types of disasters with readily available, safe, and efficacious mitigators. Data from Tokaimura have shown that medical efforts were effective in the treatment of acute radiation syndrome (ARS), but did not prevent the progressive development of radiation-induced lung injury such as radiation pneumonitis and/or pulmonary fibrosis (12, 13). It remains to be seen how those who were accidentally exposed to radiation in the Fukushima Daichii disaster of 2011 will respond over time.

While substantial information is available relative to how radiation exposure affects lung tissue from both animal research models and data collected from victims exposed to radiation, very little is known about how CRI might alter clinical outcomes. To examine mechanisms behind organ system damage and failure following CRI, we developed a mouse model of radiation and scald burn injury and first examined inflammatory mediators and leukocyte distribution in the blood. In our model, we previously reported 30% mortality within 48 hours of the injury. At 48 hours post-injury, the surviving animals exhibited severe leukopenia and increased circulating pro-inflammatory cytokines (14). We demonstrate here that early pulmonary inflammatory events (within 48 hours) after injury are enhanced in the combined injury compared to either insult alone. Thus, combined injuries would likely require special consideration in a clinical setting.

METHODS

Animals

Eight to ten week-old male C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN). This strain was chosen for our combined injury model since the majority of our historical burn injury data is from experiments using C57BL/6 male mice. Also, many genetically knocked-out strains are available on the C57BL/6 background which allows us to examine the role of specific molecules in the inflammatory response after the combined injury (such as IL-6 and TLR4), so it is a convenient strain to use for this purpose. All animals were housed with food and water available ad libitum at the Loyola University Medical Center Animal Facility. In this barrier facility, temperature, humidity, and a 12/12-hour light/dark cycle are controlled. All animal studies described here were approved and performed with accordance to the guidelines established by the Loyola University Institutional Animal Care and Use Committee.

Combined irradiation and burn injury

Combined injury was performed as previously described by our laboratory. Combined with burn, 5 Gy yields a severe enough injury to cause 30–50% mortality within the first several days following injury, yet still leaves us animals leftover to study (14). Mice were subjected to 5 Gy of total body ionizing radiation by exposure to a ^{137}Cs source in a Gammacell 40 irradiator (MDS Nordion, Ottawa, ON, Canada). The emission rate of the Gammacell was 74.3 cGy/minute. Mice were not anesthetized for the radiation procedure and were held in place using ventilated plastic “pie” caging to separate them from one another (Braintree Scientific, Braintree, MA). Sham (0 Gy) irradiation mice were placed into the irradiator for matched amounts of time, but without exposure to the source. Approximately one hour following radiation injury, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p., and their dorsal surfaces were shaved with animal clippers. Mice were then placed into a plastic template with an opening allowing 15% TBSA on their dorsum to be exposed, as calculated by previously described methods (15). Full-thickness scald injury was achieved by immersing the animals in a 95°C water bath for 7 seconds as previously described (16, 17). Immediately following exposure to the water, animals were dried to prevent any further scalding. Sham animals were anesthetized, shaved, and immersed in room temperature water. To compensate for fluid loss, all animals received 1.0 ml of warmed 0.9% normal saline i.p. immediately after the burn injury. Body temperature was maintained by placing the cages on heating pads while the animals recovered from anesthesia.

Histopathology of lungs

Lung tissue was collected immediately after sacrifice. The upper left lobe was formalin-inflated and stored in 10% buffered formalin as previously described (18). Fixed tissue was embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin (H&E) as previously described (19). Lungs were examined by light microscopy and imaged using a digital camera attached to the microscope (Zeiss Axioskop 40, Zeiss, Carlsbad, CA). A pathologist who was blinded to the study examined and scored the lungs for presence of neutrophils per alveolus. The number of neutrophils present per alveolus in 10 high power

fields (400x) were scored for each animal, and then averaged by treatment group as a marker of inflammation (18).

Alveolar wall thickness was assessed using Image J software. Photographs were taken in a blinded fashion of 10 high power fields (400x) per animal and analyzed using the Java-based imaging program ImageJ (National Institutes of Health, Bethesda, MD). The images were converted to binary to differentiate lung tissue from air space and then analyzed for the percent area covered by lung tissue in each field of view as described (20, 21).

Myeloperoxidase content assay

Lung tissue was snap frozen in liquid nitrogen and stored at -80°C . Tissue was prepared and assayed for MPO content as previously described (22). Briefly, frozen lung samples were homogenized in potassium phosphate buffer with 0.05% hexadecyltrimethylammonium bromide (HETAB) at a pH of 6.0, followed by brief sonication. Samples were then incubated with hydrogen peroxide and o-dianisidine-dihydrochloride. MPO content was determined by reading the optical density of samples and the MPO standard (Sigma Aldrich, St. Louis, MO) in parallel on a Spectramax plate reader at 460 nm (Molecular Devices, Sunnyvale, CA). Results were normalized against the total protein content of each sample and are reported as Units MPO/mg protein.

Cytokine and chemokine content measurement

Lung lobes were snap frozen immediately after removal and stored at -80°C until further analysis. Frozen lung tissue was homogenized in Bio-Rad cell lysis buffer according to manufacturer's instructions, and analyzed using multiplex bead array on a Bio-Plex instrument (Bio-Rad, Hercules, CA). The following analytes were measured via multiplex: interleukin-1 beta (IL-1b), IL-6, IL-10, tumor necrosis factor-alpha (TNF α) and monocyte chemotactic protein-1 (MCP-1, CCL2). KC (CXCL1) and macrophage inflammatory protein-alpha (MIP-2, CXCL2) were measured by ELISA following manufacturer's instructions (R&D Systems, Minneapolis, MN). The total protein in each tissue homogenate sample was measured using the Bio-Rad protein assay based on the methods of Bradford (23). All multiplex and ELISA results were normalized against the total protein present in each sample and are reported as pg/mg protein.

Statistical analysis

Statistical differences were conducted using analysis of variance (ANOVA) followed with a Tukey-Kramer Multiple Comparisons post-hoc test. Treatment groups were considered significantly different at $p<0.05$.

RESULTS

Changes in lung histopathology after combined injury

Lungs stained with hematoxylin and eosin (H&E) were examined in a blinded manner. Histologically, lungs from sham injured animals looked very similar to those of unmanipulated mice (data not shown). 48 hours after injury, radiation injury lungs were not markedly different from sham (Figure 1, A and C). At this time point, burn injury alone

caused increased leukocyte congestion. Lungs of CRI animals exhibited greater overall inflammation compared to all other groups at 48 hours post-injury (Figure 1, D). This consisted of marked leukocyte and red blood cell congestion of the pulmonary vasculature. Excessive leukocyte congestion (primarily neutrophils) was noted in both the vasculature and interstitium, with occasional cells scattered in the alveolar space. While lungs from CRI animals showed markedly increased leukocyte congestion, interestingly, we have reported 48 hours to have lowest levels of circulating white blood cells in our model of combined injury (14).

Lungs from both burn injury alone and CRI animals also appeared to have thickened alveolar walls, which could indicate evidence of edema and/or increased cellularity. To quantify this observation, alveolar wall thickness and cellularity were measured using imaging software to measure the area of lung tissue present in 10 high power fields per animal which is reported as a percentage of the entire field of view. An increase in tissue area, which corresponds to a relative decrease in air space, was found after both burn only and CRI at 24 and 48 hours, and was significantly greater than sham or radiation only at both time points (Figure 2).

Neutrophil accumulation is increased in lungs after combined injury

Pulmonary neutrophils were counted and found to be most numerous in CRI lungs at 48 hours following injury, $p < 0.01$ (Figure 3). Other combined insult burn (aging and burn injury) studies in our laboratory have shown drastic increases in pulmonary neutrophils at early time points, so this was not surprising (19). Interestingly, radiation injury alone induced a small increase in pulmonary neutrophil numbers, which could synergize with the inflammatory response caused from the scald burn injury. Additionally, lung myeloperoxidase (MPO) content was significantly increased as compared to radiation alone at 24 hours ($p < 0.05$, Figure 4). At 24 hours, burn injury only MPO levels were also significantly elevated above sham and radiation only groups ($p < 0.05$). However, similar to neutrophil counts at 48 hours after injury, pulmonary MPO levels in the CRI group continued to increase and were higher than all other groups ($p < 0.01$). This provides further evidence for a more prolonged and abundant neutrophil presence in the lungs of animals that received combined injury.

Pulmonary inflammatory response

To further elucidate the pulmonary effects of this dynamic injury, we measured several key pro-inflammatory mediators using multiplex bead array and ELISA assays on lung homogenates. Pulmonary IL-6 was found to be only slightly elevated in CRI at 48 hours following injury (data now shown). KC (CXCL1) levels from CRI animals were significantly elevated over all other groups at 48 hours, $p < 0.05$ (Figure 5). Pulmonary MCP-1 (CCL2) was also increased above all other groups at 48 hours, $p < 0.05$ (Figure 6). There was slightly more MCP-1 present in samples from radiated animals, but the differences were not statistically significant. There was not a global elevation of cytokines and chemokines, others were measured without any notable differences among groups or change from sham levels, including IL-1b, IL-10, and TNF α (data not shown). Interestingly, macrophage inflammatory protein-2 (MIP-2, CXCL2) was not elevated above

sham in any treatment group at either time point (data not shown). This early overproduction of key pro-inflammatory cytokines could contribute to permanent tissue damage to vascular organs such as the lung after combined injury, which could worsen over time and contribute to higher morbidity and mortality in CRI.

DISCUSSION

In order to further investigate the effects of CRI on various organ systems, we developed a murine model of radiation injury and burn, two injuries that are commonly encountered together after a nuclear or radiological event. This severe and unique combination of injuries often results in multiple organ failure and death. The lung is particularly radiosensitive and is often among the first organs to fail after burn injury alone, even in the absence of inhalation injury (24, 25). To date, there are no easily deliverable agents for the treatment of combined radiation injuries in humans, so there is an urgent need to identify and develop effective therapies. To do so, we must better understand what is changing in the critical hours and days following this dynamic and poorly understood injury.

Radiation-induced pneumonitis has been fairly well characterized in humans and animal models (26, 27). However, the progression to radiation-induced fibrosis is not as well understood. It is thought that early increased vascular permeability allows cells and protein into the interstitial space, leading to cellular proliferation and fibrosis (28, 29). Studies of radiation-induced pulmonary late effects have shown a progression from very persistent, chronic inflammation to increased cellular matrix deposition leading to fibrosis (27, 30). Data suggest that the release of cytokines and growth factors in the early period after radiation injury directly influences the chronic inflammation and eventual development of fibrosis (31, 32). In humans, high doses of radiation can induce pneumonitis (within the first couple of months), and pulmonary fibrosis can follow in months to years after exposure (10). Murine models of radiation injury have a similar course of pulmonary consequences, divided into two phases. Radiation-induced pneumonitis occurs within weeks, and fibrosis develops at around six months after irradiation (33).

Radiation exposure induces pneumonitis, respiratory dysfunction, and fibrosis (34, 35). Radiation-induced pneumonitis (or alveolitis) is characterized by acute development of inflammation to resident epithelial cells and endothelial cells (36–38). Burn injury can cause exacerbated inflammatory responses globally as well as locally, in organs such as the lung (9, 39–41). The inflammatory cascade triggered by local burn injury leads to lung edema within the first 24 hours (25, 42), and impaired pulmonary function (24, 25), leading to acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS). Continual burn wound inflammation is sufficient to initiate a systemic inflammatory response syndrome, which can lead to ALI/ARDS in the absence of any infection (43, 44). Abating inflammation, or hastening the resolution of the early inflammatory response could be of great benefit to combined radiation and burn injury victims. Early time points post-injury were examined in this study to determine pulmonary changes within the critical period in which interventions might be of benefit to victims. It is possible that these early events could drastically modulate the immune response and healing response and outcome later on.

In the present study, there was an over-exuberant inflammatory response in lungs from animals that received CRI within 48 hours of injury. Radiation and burn injury appear to have somewhat of an additive effect on the lungs, as mice that received the combined injury displayed histological evidence for marked pulmonary inflammation, greater than either injury alone. This type of inflammation could trigger pneumonitis and persistent inflammation, leading to eventual scarring and fibrosis. Leukocyte and red blood cell congestion of the vasculature was observed in the combined injury group. There were increased numbers of neutrophils in the lungs of CRI animals as compared to either injury alone, and higher levels of myeloperoxidase were also present in the lungs of CRI mice as further evidence of heightened neutrophil activity and inflammation. Neutrophils might infiltrate the lungs after combined injury as a result of endothelial barrier breakdown and altered vascular permeability, but this has not yet been determined in our model. The additive effects of radiation and burn injury on pulmonary inflammation are similar to previous studies of “two-hit” injury models done in our laboratory. Experiments with ethanol exposure prior to scald burn injury, as well as aging combined with burn have demonstrated increased levels of neutrophil accumulation, edema, and cytokine production in the lungs (18, 19, 45–47).

Increased cytokine production following lung irradiation has been reported in various studies. IL-1 α , IL-1 β , IL-6, TNF α , and transforming growth factor-beta (TGF β) have all been shown to be inducible within one day following irradiation in rodent models (26, 30, 48). Other combined insult models such as wounding combined with radiation, and burn injury combined with infection, have also demonstrated increased cytokine and chemokine expression in serum (45, 49–52). Cytokine expression has been shown to follow a recurring pattern, beginning almost immediately in rats, with more expression occurring in a cyclical pattern out to 16 weeks after exposure (53). Mouse models also have shown an initial early cytokine induction, followed by another wave of cytokine production between weeks 4–10 post-irradiation (26, 48, 54).

Since we observed increased numbers of neutrophils and macrophages in the lungs of CRI animals, we next look for relevant cytokines and chemokines that might contribute to this phenomenon. KC, a neutrophil chemoattractant, was increased 2-fold in CRI mice as compared to burn alone and 5-fold compared to radiation injury alone and sham. This is consistent with histological observations and could certainly account for more neutrophils flooding into the pulmonary vasculature and interstitium of CRI animals, and remaining there after inflammation is normally resolving. Interestingly, MIP-2 was not elevated at all above sham in any injury group (data not shown). Blocking this early pulmonary neutrophil influx could be an attractive therapeutic target. Fox et al. reported a reduction in radiation-induced alveolitis using antagonists against CXC receptor 1 and 2 (55). MCP-1 is a potent macrophage chemokine that was significantly increased above all other treatment groups at 48 hours. Differential blood counts reported previously in our combined injury mouse model show that leukocyte counts are at their lowest 48 hours post-injury (14), so it is interesting that this is seen despite major global depletion of white blood cells, with numerous neutrophils present in lung tissue of injured animals. We did not observe consistent changes in other pro-inflammatory cytokines such as IL-1 β or IL-6 in lungs, but previously reported early changes in serum IL-1 β and IL-6 in our model (14).

The literature has shown mouse and rat strain-dependent differences in susceptibility to radiation-induced pneumonitis (33, 38, 56–60). C57BL/6 mice are commonly used in research models (and were used for the studies described here), although they have been recently noted to have a less defined period of pneumonitis than other strains such as CBA and C57L mice (38). However, C57BL/6 mice are known to develop radiation-induced focal fibrosis. It has also been shown that despite strain differences in the severity or duration of pneumonitis, most strains tested were similar in the underlying radiosensitivity of their lung cells (33, 61).

The studies herein are only a snapshot of early pulmonary events after combined injury. Multiple organ systems are getting affected at the same time, which adds to the negative consequences of this type of injury. It is possible that enhanced early lung inflammation could be used as an indicator of the severity of overall combined injury. However, early lung inflammation is also quite robust after burn injury alone (at least in the parameters measured here). CRI negatively impacts multiple organ systems, more so than either injury alone. For indicators of severity of damage, it is likely that factors from multiple organ systems will need to be assessed and compared to predict outcomes. We are expanding our experiments to follow these animals long term, for weeks and months after injury, in order to determine if the early inflammation and pneumonitis symptoms reported here contribute to permanent organ damage, such as scarring, lung fibrosis, and diminished lung function and failure.

The critical early timeframe studied here is desirable for intervention to mitigate the downstream effects of the excessive inflammatory response observed after CRI. Thus, describing underlying mechanisms early after the injury could provide attractive targets for effective therapies. Early cytokine and chemokine levels could also provide valuable biomarkers to indicate the severity and extent of radiation exposure and combined injury. Moreover, the ability to mitigate these early inflammatory effects could improve late pulmonary outcomes. These experiments should provide valuable information about the mechanisms that might cause elevated tissue damage after the combined insult of radiation exposure and burn injury in the lung, and give insight into potential therapeutic interventions that might diminish the extensive inflammatory response, organ damage and failure, susceptibility to infections, and other complications associated with this unique type of injury.

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Figure 1. Lung histopathology after combined injury

Lungs were sectioned and stained with hematoxylin and eosin (H&E). These images are representative of the treatment groups at 48 hours post-injury. (A) sham (B) burn injury only (C) 5 Gy only (D) 5 Gy with burn injury. Panel D demonstrates the increased red blood cell congestion, neutrophil infiltration, and alveolar wall thickening typical of the radiation and burn injury group. Images are at 400x magnification. N = 4–5 mice per group.

Figure 2. Pulmonary congestion analysis

The area of 10 high power fields (400x) per mouse covered by alveolar tissue on H&E stained lung sections was measured and quantified using ImageJ software. The alveolar thickness is reported as a percentage of the total field of view. N = 4 mice per group. *p<0.05 compared to sham within time point. #p<0.05 compared to 5 Gy only within time point.

Figure 3. Neutrophil infiltration in the lung

Neutrophils were counted and scored by a pathologist in a blinded manner from 24 and 48 hour post-injury H&E stained lung sections. Each section was assessed for presence and count of neutrophils per alveolus. The score was based on the predominant pattern seen per 10 high power fields (400x magnification). A score of 0 was given if no neutrophils were present, a score of 1 if 1–2 neutrophils were found within alveolar walls, a score of 2 if 2–3 neutrophils were seen in alveolar walls, and a score of 3 was given if 3 or more neutrophils were seen in the alveolar walls and septum. N = 4–5 mice per group. * $p < 0.01$ compared to 48 hour 5 Gy.

Figure 4. Pulmonary myeloperoxidase (MPO) content

MPO content was measured and normalized against total protein for each sample. Data are shown as average Units MPO/mg protein $\pm$ sem. N = 4–6 mice per group. * $p < 0.01$ compared to all other 48 hour groups, + $p < 0.05$ compared to sham and 5 Gy 24 hour, # $p < 0.05$ compared to 5 Gy 24 hour.

Figure 5. Pulmonary KC (CXCL1)

Lung tissue was homogenized and analyzed by ELISA (R&D Systems), then normalized against total protein in each sample.

Data are represented as KC pg/mg protein $\pm$ sem. N = 4–6 mice per group. * $p < 0.05$ compared to 48 hour sham and 5 Gy.

Figure 6. Pulmonary MCP-1 (CCL2)

Lung tissue was homogenized and analyzed by multiplex bead array according to manufacturer's instructions (Bio-Rad), then normalized against the total protein content of each sample. Data are represented as MCP-1 pg/mg protein $\pm$ sem. N = 4–6 mice per group. N = 4–6 mice per group. *p<0.01 compared to all other 48 hour groups.