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Cellular Inflammatory Infiltrate in Pneumonitis Induced by a Single Moderate Dose of Thoracic X Radiation in Rats

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Abstract

The goal of these studies was to characterize the infiltrating inflammatory cells during pneumonitis caused by moderate doses of radiation. Two groups of male rats (WAG/RijCmcr, 8 weeks old) were treated with single 10- or 15-Gy doses of thoracic X radiation; a third group of age-matched animals served as controls. Only 25% rats survived the 15-Gy dose. Bronchoalveolar lavage fluid and whole lung mounts were subjected to cytological and histological evaluation after 8 weeks for distribution of resident macrophages, neutrophils, lymphocytes and mast cells. There was a modest increase in airway and airspace-associated neutrophils in lungs from rats receiving 15 Gy. Mast cells (detected by immunohistochemistry for tryptase) increased over 70% with 10 Gy and over 13-fold after 15 Gy, with considerable leakage of tryptase into blood vessels and airways. Circulating levels of eight inflammatory cytokines were not altered after 10 Gy but appeared to decrease after 15 Gy. In summary, there were only modest increases in cellular inflammatory infiltrate during pneumonitis after a non-lethal dose of 10 Gy, but there was a dramatic rise in mast cell infiltration after 15 Gy, suggesting that circulating levels of mast cell products may be useful markers of severe pneumonitis.

INTRODUCTION

We previously described a model of structural and functional alterations and vascular injuries in the lungs of female Wistar rats (WAG/RijCmcr) after whole thoracic irradiation (1,2). The single doses used for those studies, 5 and 10 Gy, were non-lethal and were expected to demonstrate pulmonary pathologies that may occur in survivors of a radiological attack. We conducted multiple assays at six times between 3 days and 1 year after irradiation in these models. Some of the assays were non-invasive (e.g. breathing rate and arterial oxygen saturation). Histological examination of hematoxylin and eosin (H&E)-stained sections as well as planar angiograms and hemodynamic studies of whole lungs showed perivascular edema, inflammation and structural injuries to the pulmonary arterial tree. Most of the end points examined demonstrated derangements and dysfunctions between 7–8 weeks after 10 Gy but not after 5 Gy (1,2). These injuries were partially or completely reversed by 1 year (compared to age-matched control animals). While histopathology revealed mild fibrosis in the 10-Gy cohort at 1 year after irradiation, it was not debilitating, and it failed to alter lung function. In

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summary, while 10 Gy caused radiation pneumonitis at 2 months in our experiments, we did not observe appreciable fibrosis, as has commonly been reported as a second phase of lung injury (3–5).

A feature of radiation pneumonitis is recruitment of inflammatory cells into the lung (3,6,7). Our previous studies included counts of total cells in the bronchoalveolar lavage fluid in irradiated and control rats, which were not increased significantly at 3 days to 1 year. We reported a rise in protein content in the bronchoalveolar lavage fluid at 1 month after 10 Gy, coinciding with an increase in perivascular edema observed by histology. However, we did not evaluate the associated interstitial inflammatory infiltrate.

Recruited circulating inflammatory cells have the potential to generate significant pneumonitis in combination with resident cells. Macrophages, monocytes, neutrophils, mast cells and lymphocytes enter the irradiated lung parenchyma and alveolar spaces, presumably attracted by locally secreted proinflammatory cytokines and chemokines. A study of rats irradiated with 25 Gy to the lower lungs found different results in the lung tissue compared to the bronchoalveolar lavage fluid. There was a pronounced increase of mast cells and mononuclear cells in the lung, while the bronchoalveolar lavage fluid was richer in neutrophils and lymphocytes (8). Bronchoalveolar lavage fluid from patients undergoing radiotherapy for localized breast cancer contained an increased number of lymphocytes and mast cells, which paralleled changes in extracellular matrix proteins and connective tissue in the lung (9,10). Male Wistar rats given whole-lung irradiation at 15 Gy showed an increase in macrophages, lymphocytes, polymorphs and protein in the bronchoalveolar lavage fluid from 4–8 weeks (11). Mast cells were increased in the perivascular and peribronchial areas, and there was a temporal and spatial relationship between the distribution of mast cells, with collagen deposition in the lungs at 20 weeks. However, not much is known about the cellular infiltrate in rodents at a dose of 10 Gy, which gave rise to vascular injuries and pneumonitis in our model (1,2).

Changes in cytokine profiles in the lung and circulation after irradiation have been studied to identify surrogate markers that may predict inflammation. Circulating cytokines were measured in patients receiving thoracic radiotherapy (12). A panel for interleukin 1 alpha (IL-1 α), interleukin 6 (IL-6), monocyte chemotactic protein (MCP-1), E-selectin, L-selectin, transforming growth factor beta1 (TGF- β 1) and basic fibroblast growth factor (bFGF) were tested 12 weeks after irradiation. Only IL-1 α and IL-6 levels were significantly elevated in patients who developed pneumonitis, though these values were also higher before radiotherapy, making them less likely to have been caused by the radiation. Partial-volume irradiation of rat lungs with 10 Gy caused activation of macrophages by 1 h, and the levels of cytokine mRNAs and activated macrophages fluctuated in waves that were similar both in- and out-of-field (13).

While gastrointestinal (GI) injury and bone marrow failure could be very important at whole-body doses in the range of 10–15 Gy, so could lung injury. With the development of supportive therapy for GI and bone marrow loss, pulmonary injury is a potential survival-limiting factor. In addition, lung injury occurred in radiation accidents (14), and there are many scenarios (e.g., inhalation exposure, upper hemibody exposure, combined injury) where lung tolerance could be exceeded without fatal GI or bone marrow injury. The study of radiation lung injury using survivable doses is therefore relevant to the terrorism scenario. To characterize inflammatory mediators in a model of lung injury after moderate doses of radiation that may be survivable in a radiological terrorist attack, we examined rats given thoracic doses of 10 Gy. We evaluated changes in cellular inflammatory infiltrate and circulating cytokines both in the bronchoalveolar lavage fluid and within the lung parenchyma. Rats receiving a higher dose of 15 Gy that resulted in considerable morbidity were also examined. Characterization of the

cellular infiltrate is critical for identifying the pathophysiological basis of lung injury by survivable doses of radiation and for developing mitigators and treatments for this injury.

MATERIALS AND METHODS

Animals

The study was approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. Specific-pathogen-free male rats from the MCW colony of WAG/RijCmc were used; WAG/RijCmc is the ILAR strain designation for the strain previously referred to as WAG/Rij/MCW. The study was conducted with three groups of rats; eight were irradiated with 10 Gy and 12 with 15 Gy, and eight were controls (0 Gy). All rodents were housed in a moderate-security barrier and were allowed sterilized food and water *ad libitum*. Rats were checked daily, and those showing continuing distress or significant signs of morbidity such as poor grooming along with hunching, lack of eating or drinking, or severe respiratory problems were euthanized.

Injury Model

Unanesthetized 8-week-old male rats were placed in a Plexiglas holding jig and given 10 or 15 Gy of radiation limited to the thorax. An orthovoltage system (X-RAD from Precision X-Ray, North Branford, CT) was used with a 320 kVp beam, a half-value layer of 1.4 mm copper, and a midline dose rate of 1.43 Gy/min. The dose was measured using a calibrated Farmer-type ionization chamber. The radiation dose was delivered by two equally weighted lateral beams to improve the dose uniformity. An $8 \times 7.5\text{-cm}^2$ collimator was used to define a radiation field that encompassed the whole thorax, including the lungs, the heart and a small subphrenic portion of the liver. Irradiated rats and their age-matched controls were studied for various markers of inflammation at 51–61 days after irradiation (around 16 weeks of age).

Lung Lavage and Histology

Rats from each cohort were anesthetized intraperitoneally with isoflurane or pentobarbital (50 mg/kg), a tracheostomy was performed, and the chest was opened. Blood was sampled via the right ventricle and an 18 G cannula with a flexible tip was then inserted into the trachea. The lungs were infused with a total volume of 5 ml of phosphate-buffered saline and withdrawn. This was repeated three times. Cytospins were prepared from the bronchoalveolar lavage fluid using a standard clinical protocol. Differential counts were performed on Wright's-stained cytospin by a pathologist who was blinded to the identity of each sample.

For histopathological examination, the heart and lungs were removed en bloc; the left lung was dissected, inflated by 10% neutral buffered formalin, and fixed for 24 to 48 h. Tissue sections were cut through the hilum, including the apex and base of the lung, and processed on a Sakura Tissue Tek VIP5 processor (Sakura Finetek, Torrance, CA) according to standard protocols for formalin-fixed, paraffin-embedded tissue. Sections (4 μm thick) were stained with H&E and also with trichrome. Digital images of the sections were captured at appropriate magnifications with an Axiovision camera mounted onto a Nikon microscope with image acquisition software. H&E-stained sections were scored in a blinded manner according to a semiquantitative scale (15) for acute and chronic parenchymal changes and alterations to airways and vascular compartments as in our previous study (1). Trichrome-stained sections were also analyzed using a score between 0 to 100. These scores were assigned for relative intensities and areas that stained blue compared to the counterstains (red) for each field of the lungs by two independent investigators who were blinded to the experimental treatments of each sample. In addition, Image J analysis software (version 1.43d; NIH) was used to quantify intensities of red and blue signals after Masson's trichrome staining. The algorithms in Image J program use a color deconvolution method to separate contributing colors. Using the red,

green and blue (RGB) optical density vectors as determined by the software, each color was quantified individually for every image. The ratio of blue (collagen) and red (non-collagen) signal intensities were calculated to compare normalized collagen content. Average ratios for each treatment group (0, 10 and 15 Gy) were calculated as an indicator of fibrosis.

Cytokines

Whole blood was obtained from the heart as described above and collected in tubes containing buffered sodium citrate in a ratio of 1 part anticoagulant and 9 parts blood. The tubes were then centrifuged at 1000g at 4 °C for 10 min, and the supernatant was collected and filtered through a sterile 0.22-µm filter. The plasma samples were assayed immediately for the levels of nine cytokines using the Bio-Plex rat cytokine 9-plex panel kit (catalog numbers-171K11070, 171-305008; Bio-Rad, Hercules, CA). We selected this panel of “inflammatory cytokines” that were developed commercially to detect multiple markers in rats. All samples were run as triplicates in a single plate, and the levels of nine cytokines were read simultaneously in each well of a 96-well filter plate with the Luminex 100 xMap system (Bio-Plex High Throughput Fluidics, Bio-Rad) and quantified using five-parameter logistic regression with the Bio-Plex manager software.

Immunohistochemistry

Formalin-fixed, paraffin-embedded longitudinal sections of the whole-mount left lung were deparaffinized followed by antigen retrieval in citrate buffer. Endogenous peroxide activity was quenched by incubating the samples in 3% H₂O₂. The sections were incubated with the optimized dilutions of primary antibodies for tryptase, myeloperoxidase and CD 45 for identification of mast cells, neutrophils, lymphocytes and macrophages (Catalog numbers: IMG 80250, Imgenex, San Diego, CA; 550566, BD Pharmingen, San Diego CA; ab 15484 Abcam, Cambridge MA). For detection horseradish peroxidase (HRP)-conjugated secondary antibodies and a peroxidase-based system (labeled streptavidin biotin method, LSAB) were used for signal amplification. Mayer's hematoxylin was used as the counterstain. Appropriate positive and negative controls of relevant histological tissue sections were included for each stain to ensure specificity of the signal. Immunohistochemical results were analyzed in a blinded fashion, and the frequency of each cell type was quantified from a minimum of three and usually over 10 fields per lung section.

Statistical Methods

Data for animal mortality were represented graphically as Kaplan-Meier survival plots. Significance was assessed by an extension of the Kruskal-Wallis test as implemented by Lee and Desu (16). For other studies mean values with standard deviations (as detailed in the figure legends or text) were plotted. Values from the three test groups were compared using one-way ANOVA with all pairwise multiple comparisons computed by the Holm Sidak method. Differences between groups were considered significant if the *P* value was ≤ 0.05.

RESULTS

Survival

No morbidity was observed in the unirradiated or 10-Gy male rats up to 8 weeks after irradiation (Fig. 1). However, 8/12 males of the 15-Gy group met the defined criteria for euthanasia after 35–39 days and one rat at 45 days. Statistical analysis determined that survival after 15 Gy was different from that after 0 or 10 Gy (*P* < 0.005).

Analysis of Bronchoalveolar Lavage Fluid

The cellularity in the bronchoalveolar lavage fluid was generally low in unirradiated animals and moderate in the 10- and 15-Gy cohorts. One of eight samples from the 10-Gy group and one of three from the 15-Gy group demonstrated marked increases in cellularity. No difference in red blood cells was seen. Differential counts from 200–500 cells per rat (Fig. 2) indicated that segmented neutrophils ranged from 0.5–4.4% and lymphocytes from 0–4.4%. The majority of the cells were macrophages (from 95–99.5%; data not shown). The differential cell counts were not significantly different between groups. Bronchoalveolar lavage fluid from the 15-Gy group contained occasional to moderate phagocytes and occasional iron-laden macrophages, which were observed only rarely in the control or 10-Gy groups.

Histology of Lungs

Hematoxylin and eosin-stained sections were scored by two clinicians for injuries to the alveolar spaces, airways, vessels and interstitium. Although multifocal minor alveolar reactive changes (plump reactive alveolar lining, minimal edema and minimal to mild increase of cellularity in alveolar walls) were seen after 10 Gy, without fibrosis, this was more accentuated and frequent after 15 Gy (Fig. 3A–C). However, no significant differences between groups in scores were observed when these injuries were evaluated according to Canzian *et al.* (15) under low power by light microscopy.

Immunohistochemistry

1. Mast cell distribution—Increasing numbers of mast cells (stained with anti-tryptase antibody) were observed in lung sections from rats receiving 10 and 15 Gy compared to unirradiated controls (Fig. 4A–D). In animals receiving 10 Gy, distinct perivascular mast cell distribution was seen with a multifocal pattern and a 70% increase in density over baseline in affected fields (Fig. 4A, B). In animals receiving 15 Gy, the inflammation extended more peripherally, with diffuse prominent perivascular confluence of mast cells (Fig. 4C) and associated interstitial edema.

Peribronchial mast cell clusters were rare to focally prominent in unirradiated lungs; after 10 and 15 Gy they were consistently increased (overall about twofold), with similar densities per affected individual field in these locations (around larger airways). At 15 Gy, associated moderate to marked interstitial edema was also observed. In more peripheral airways and airspaces (alveolar sacs and alveolar walls), the density of mast cells per affected field increased by over 50% after 10 Gy. After 15 Gy, there was involvement of the entire peripheral lung parenchyma with an average 13-fold increase in mast cell density in the affected fields (Fig. 4A and C).

At 15 Gy, in addition to the marked increase of mast cells and associated interstitial edema, there was evidence of local/interstitial degranulation of mast cells and leakage of tryptase into intravascular spaces, airways and airspaces (Fig. 5A–D). This was seen on tryptase immunohistochemical stains as light but distinct membranous stain on the ciliary bronchial/bronchiolar epithelial surfaces (solid arrow in Fig. 5B), alveolar lining and intraluminal/intra-alveolar macrophages. Similar membranous staining was present on vascular endothelial surfaces and on some circulating red cells (Fig. 5D). These findings were not present in control animals or after 10 Gy (Fig. 5A and C). Regional marked bronchial epithelial vacuolation was seen in one rat after 15 Gy (not shown).

Subpleural distribution of mast cells appeared to be largely independent of radiation, with a nonsignificant increase after a dose of 10 Gy and non-distinct patterns blending into the diffuse marked peripheral alveolar distribution patterns after a dose of 15 Gy (not shown).

Overall the above patterns revealed a primary central distribution of mast cells in the periairway and perivascular interstitium after irradiation of the lung with 10 Gy. After 15 Gy, there was a marked peripheral parenchymal extension as well as histological features consistent with consequences of mast cell degranulation (edema, vascular leakage, and leakage into airways and airspaces).

2. Distribution of lymphocytes, macrophages and neutrophils—Lung sections were also stained with CD 45 and anti-myeloperoxidase antibodies to detect lymphocytes, macrophages and neutrophils. CD 45 stained larger macrophages (solid arrows in Fig. 6) and smaller lymphocytes (dotted arrows in Fig. 6) in a characteristic light membranous manner. There was no difference in lymphocytes between groups (Fig. 6D). However, after 15 Gy, significant lymphocytic endotheliitis and subendotheliitis (Fig. 7) were observed in two rats, which included long dense stretches of lymphocytes lined up subendothelially and also included focal vasculitis, subendothelial fibrin deposition, and, in one animal, fibrointimal hyperplasia. Intra-alveolar macrophages increased after irradiation from rare scattered in the control group to mild to moderate after a dose of 10 Gy and moderate after 15 Gy (Fig. 6A–C).

The overall counts of neutrophils in association with airways and airspaces was decreased in lungs from unirradiated or 10-Gy rats compared to rats receiving 15 Gy (Fig. 8A–D).

Fibrosis

Trichrome staining was performed to confirm the predicted absence of fibrosis at this stage of pneumonitis. Characteristic blue stain (marked by arrows in Fig. 9A–C) was observed in a normal periairway and perivascular pattern, with no difference in the parenchyma of any specimens. Relative scores for the three groups, obtained in a blinded manner for each whole mount, were not significantly different. In addition, we performed a color deconvolution analysis of the trichrome-stained images as described in the Materials and Methods section. We observed no difference between groups in the ratio of blue to red components of the images (0 Gy, $n = 8$, 1.24 ± 0.04 ; 10 Gy, $n = 8$, 1.26 ± 0.06 ; 15 Gy, $n = 3$, 1.24 ± 0.06). The relative intensities of red signal also were not different between groups (0 Gy, $n = 8$, 288 ± 9 ; 10 Gy, $n = 8$, 288 ± 13 ; 15 Gy, $n = 3$, 305 ± 11).

Circulating Cytokines

Levels of interleukin-1a (IL-1a), IL-1b, IL-2, IL-4, IL-6, IL-10, interferon gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α) were evaluated in animals irradiated with 10 and 15 Gy and in age-matched controls (Fig. 10). After irradiation with 10 Gy, the levels of all the cytokines were not different from those in age-matched controls. With 15 Gy, however, each of the cytokines except TNF- α showed a decreasing trend compared to controls, but none of these differences were significant.

DISCUSSION

Our goal was to examine inflammatory markers and infiltrating cells during radiation pneumonitis that results directly or secondarily from radiation exposure (17,18). Our results further characterized a model of lung injury using a moderate dose of X radiation (10 Gy) that may be survivable after a radiological attack. We also examined rats receiving a higher dose of 15 Gy. Though 15 Gy to the thorax of rats has been reported not to result in mortality for up to 30 weeks (11), others have found a dose of 14 Gy to be lethal by 7 weeks (19). We observed considerable morbidity at 35–39 days after 15 Gy, and only 3/12 rats survived to 51 days. Morbidity in our 15-Gy rats coincided with the onset of radiation pneumonitis, making it an important limiting factor for survival. We therefore used survival as well as inflammatory

cell migration as end points for our study. Doses at or lower than 10 Gy to the thorax did not result in sufficient lung injury to cause death in an otherwise healthy rat, while 15 Gy caused death by 7–8 weeks. Therefore, analysis at 7–8 weeks after 10–15 Gy spans a relevant time (of pneumonitis) for both doses as well as a relevant dose range for mitigation studies.

The bronchoalveolar lavage fluid from irradiated rats showed little change in the differential counts for neutrophils, lymphocytes and macrophages. However, total cellularity may have been increased in the irradiated rats, though this is difficult to quantify accurately due to variable recovery of bronchoalveolar lavage fluid and the lack of a reliable marker of airspace volume sampled. We did not observe an increase in cell numbers in the bronchoalveolar lavage fluid at 8 weeks after 10 Gy in a previous study (1), though that study was conducted with female rats compared to males in the current work. In this study, there were no appreciable differences in infiltrating lymphocytes in the peripheral parenchyma after 10 or 15 Gy, but neutrophils were modestly increased after 15 Gy. The most dramatic differences between the three groups of rats were the numbers of infiltrating mast cells, which increased by 70% after 10 Gy and by over 13-fold after 15 Gy. Tryptase leakage was detected on membranes of the alveolar lining, bronchial epithelial cilia, vascular endothelia and red blood cells in rats receiving 15 Gy. This suggests that levels of tryptase, serotonin or histamine (released by mature mast cells of the lung) may provide circulating markers for radiation pneumonitis. Some of the rats given 15 Gy developed considerable vasculitis and subendotheliitis, which would have contributed to the morbidity and mortality we observed.

We did not observe a difference in circulating cytokines in the 10-Gy cohort. The higher dose of 15 Gy tended to decrease cytokines other than TNF- α . For this characterization of radiation pneumonitis, we did not study the temporal progression of circulating cytokine levels. Though molecular changes in cytokine mRNA are believed to precede histopathological or clinical changes in the lung, circulating cytokine profiles have not been reliable indicators of radiation injury in humans (12,20,21).

Repair and remodeling after radiation injury are regulated by a multitude of factors including inflammatory cells, their secretagogues, cytokines and possibly mast cells. We observed that the number of inflammatory cells during pneumonitis does not appear to be correlated with the dose of thoracic radiation. There is a modest rise in neutrophil counts only after exposure to 15 Gy, a dose that can result in lethal pneumonitis. It is possible that cellular infiltration had occurred before pneumonitis and subsided by the time we identified pulmonary dysfunction at 6–8 weeks (1,2). The distribution of inflammatory infiltrates (as suggested by Figs. 6 and 7) and/or inflammatory cell function may be altered during pneumonitis causing pulmonary vasculitis and subendotheliitis. Others reported that inflammation did not adequately resolve after irradiation and that there was a chronic overproduction of cytokines and secreted factors that maintain aberrant matrix and vascular remodeling and that maintain the progression of injury (22). We observed considerable tissue restitution by 5 months after 10 Gy, suggesting a balance between accumulating injury and tissue repair.

The role of mast cells in radiation pneumonitis in rats has been explored widely at doses above 15 Gy (7,11,23,24). Mast cells were found to be predominantly protective for radiation-induced heart disease in rats 6 months after exposure to 18 Gy; rats deficient in these cells exhibited more severe postirradiation changes than their mast cell-competent litter mates (25). Mast cells typically begin development in the bone marrow and mature after they migrate to remote sites such as the lung, skin, connective tissue compartments and small intestine (26). Factors controlling movement to the lungs remain unknown, though migration to the small intestine is controlled by the integrin $\alpha 4 \beta 7$ (26). Thoracic irradiation of male Wistar rats with 15 Gy increased mast cell density in perivascular and peribronchial areas at 7 weeks (11). This is similar to our observations after 10 Gy (Fig. 4). Administration of steroids after 15 Gy

suppressed mast cell numbers but did not attenuate chronic fibrosis (3). Mast cells increased in density around fibrosing alveoli after the third week after 30 Gy to the lower third of the lungs (22). No mast cells were found in the lumen of the alveoli or in the epithelium in that study. The most pronounced increase (200-fold) was seen in the subpleural regions, though the radiation field was localized to the lower third of both lungs, suggesting that the infiltration was concentrated in the radiation field (23). Irradiation with 30 Gy induced an increase in mast cells over the alveolar surface area that was decreased by 40% in rats treated with 50 mg/kg/day of captopril (24). Captopril also reduced hydroxyproline associated with fibrosis in this model, implying that the mast cells may be involved in fibrosis (24). However, A/J mice developed alveolitis after irradiation that correlated with increased levels of pulmonary mast cells, while C57BL/6J mice responded differently by developing fibrosis with fewer inflammatory cells that instead correlated with the presence of neutrophils in the lavage (27). Studies estimating cell numbers in the lung after 30 Gy to one lung reported a 540-fold increase in mast cells but no increase in macrophages (7). However, there was a marked decrease in the epithelial and capillary surface area in that study.

Similar to our current studies, Johnston *et al.* (6) reported that there were no differences in cell numbers or differentials in the bronchoalveolar lavage fluid of mice 8 weeks after a single dose of 15 Gy to the thorax. However, at 16 weeks, infiltration of macrophages and lymphocytes was observed; this response was driven by the lung parenchyma and not bone marrow-derived inflammatory cells. Mast cells were not examined in that study.

Numerous models of radiation pneumonitis and fibrosis have been described, though a well-accepted consensus that clearly links cause and effect leading to pulmonary dysfunction has not emerged (3,5–8,11,^{19,21},23,24,27–32). Without identifying events that lead to pneumonitis and fibrosis, it may be difficult to identify specific therapeutic targets. We reported pneumonitis in rats after 10 Gy to the thorax (1,2) that resolved spontaneously without debilitating fibrosis. In the current study, we found that though mast cell infiltration was increased by 10 Gy, it was much less severe than that observed at the higher radiation doses that are later associated with fibrosis. These results along with those of Ward *et al.* (24) describing the use of captopril to decrease mast cell accumulation and fibrosis support a role for mast cells in fibrosis. Suppression of mast infiltration with steroids has been reported to reduce pneumonitis (3,30). However, there are also numerous reports that mast cells may *not* be responsible for radiation-induced fibrosis (7,11,25,27).

It is not clear how mast cells infiltrate the lungs. There is a well-documented increase in edema and vascular permeability 2–4 weeks after lung irradiation (1,11,30). Graham *et al.* (30) tested 13 drugs against early radiation-induced vascular permeability and concluded that inhibitors targeting prostaglandins, leukotrienes and histamines, products released by macrophages and mast cells, were effective mitigators. Complement activation, proteolytic enzymes and neutrophil migration did not appear to play a major role in increasing vascular permeability (30).

In previous studies, we documented a drop in the density and reactivity of pulmonary arteries/arterioles during pneumonitis induced by 10 Gy (1,2). Endothelial injury in lung vasculature was described by others (7,29), though with doses higher than 10 Gy. Taken with our current observations of only a marginal increase in cellular infiltration after 10 Gy, we suggest that structural and functional derangements of the vasculature may play a more significant pathophysiological role than cellular infiltrates present during pneumonitis. Endothelial injury first increase vascular permeability and also promote infiltration of inflammatory cells, but later, the secondary loss of pulmonary vessels and vascular reactivity may decrease perfusion and induce the respiratory dysfunction observed at pneumonitis. We were surprised by our results demonstrating a relative lack of inflammatory cells (excluding mast cells) after 15 Gy

in rats that were close to death and exhibited from vasculitis and subendotheliitis. In summary, pulmonary dysfunction and even death may occur in the absence of accompanying elevations of the numbers of lymphocytes, macrophages or even neutrophils in the lung.

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FIG. 1. Actuarial survival curves for control rats and rats receiving 10 and 15 Gy thoracic radiation. Numbers of animals: 10 Gy: 8, 15 Gy: 12. Numbers in parentheses show the numbers of animals still at risk at various times.

FIG. 2.

Differential counts of cells recovered in bronchoalveolar lavage fluid. Bars are means $\pm$ SD for differential counts of neutrophils (open bars), lymphocytes (hatched bars) and macrophages (shaded bars). The number of rats for each group is shown within each bar. There were no significant differences between groups.

FIG. 3.

Lung sections stained with hematoxylin and eosin. Panel A: Typical section from a control rat showing normal alveoli. Panel B: Rat lung after 10 Gy demonstrating focal minor reactive alveolar change (solid arrow). Panel C: Rat lung after 15 Gy showing focal mild increase in perivascular cellularity with inflammation (between arrows) and reactive alveolar changes.

FIG. 4.

Rat lung stained with anti-tryptase antibody to show mast cells in brown. Panel A: Control lung showing only rare perivascular mast cells, with no notable peripheral parenchymal mast cells. Panel B: Lung irradiated with 10 Gy showing distinct perivascular mast cell infiltration, with very few peripheral parenchymal mast cells. Panel C: Lung irradiated with 15 Gy showing diffuse infiltration of the parenchyma (including alveolar walls) by mast cell. Panel D: Cell counts from sections stained with anti-tryptase antibody. Cells were counted in five randomly selected fields from each lung. Bars are means $\pm$ SD; the number of rats is shown within each bar.

FIG. 5.

Rat lungs stained with anti-tryptase antibody for mast cells. Panel A: 10 Gy, interstitial mast cell. There is no tryptase leakage onto the alveolar lining (dotted arrow). Panel B: 15 Gy, interstitial mast cell in alveolar walls, with tryptase (post degranulation) adsorbed onto the alveolar lining (solid arrow). Panel C: 10 Gy, endothelial lining free of stain from leaked tryptase (dotted arrows). Panel D: 15 Gy, membranous endothelial stain (solid arrow) and red cell stain (double-lined arrow) secondary to mast cell degranulation and tryptase leakage.

FIG. 6.

Rat lung stained with anti-CD 45 antibody to show lymphocytes and larger macrophages that stain with light membranous positivity. Panel A: Control rat lung demonstrating scattered small lymphocytes (dotted arrow) and larger macrophages (solid arrow) in the interstitium as well as within the alveoli (red arrows). Panel B: Rat lung irradiated with 10 Gy demonstrating perivascular accentuation of lymphocytes (dotted arrow). The solid arrow points to a macrophage. Panel C: Rat lung irradiated with 15 Gy showing lymphocytic vasculitis (example of most severe lesion), with numerous lymphocytes infiltrating through vascular wall and a few into the subendothelial space (dotted arrows), and macrophages (solid arrows) showing membranous stain in perivascular parenchyma. The grouped arrows encompass a subendothelial fibrinoid cushion in the artery. Panel D: Counts of anti-CD45-positive cells. Bars are mean numbers of cells $\pm$ SD for five randomly selected fields. The number of rats is shown within each bar.

FIG. 7.

Rat lung stained with anti-CD 45 antibody demonstrating vasculitis and endotheliitis at 15 Gy. Panel A: Vascular wall of unirradiator lung with lymphocytes rolling on the endothelial surface, but not infiltrating. Solid arrow points to intact endothelial cell. Panel B: Vascular wall of lung after 10 Gy showing early endotheliitis/subendotheliitis in the endothelial layer. Solid arrow points to an endothelial cell. Panel C: Rat lung irradiated with 15 Gy showing marked lymphocytic subendotheliitis. Numerous lymphocytes (dotted arrows) traversing and underlying the endothelium (solid arrows), which is partially lifted.

FIG. 8.

Rat lungs stained with anti-myeloperoxidase antibody to show neutrophils in brown. Panel A: Control rat. Panel B: Rat irradiated with 10 Gy. Panel C: Rat irradiated with 15 Gy. Panel D: Cell counts from sections stained with anti-myeloperoxidase showing an increase in neutrophils after 15 Gy. Bars show number of cells for five randomly selected from each lung. The number of rats is given within each bar.

FIG. 9.

Rat lung sections treated with trichrome stain to demonstrate fibrosis (blue) at 8 weeks after irradiation. Panel A: Control rat. Panel B: Rat irradiated with 10 Gy. Panel C: Rat irradiated with 15 Gy. Panel D: Scores for relative areas of blue stain (marked by arrows) compared to counterstain (red). Bars show mean scores $\pm$ SD for each group. The number of rats/group is indicated within each bar.

FIG. 10.

Levels of eight rat cytokines measured in serum from rats at 8 weeks after irradiation of the whole thorax with 10 Gy (solid bars) or 15 Gy of X rays (hatched bars) and age-matched controls (open bars). Values are the percentage of the control $\pm$ SD for each cytokine.