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ARTICLE

Radiation Induced Endothelial Cell Retraction *in vitro*: Correlation with Acute Pulmonary Edema

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We determined the effects of low dose radiation (<200 cGy) on the cell-cell integrity of confluent monolayers of pulmonary microvascular endothelial cells (PMEC). We observed dose- and time-dependent reversible radiation induced injuries to PMEC monolayers characterized by retraction (loss of cell-cell contact) mediated by cytoskeletal F-actin reorganization. Radiation induced reorganization of F-actin microfilament stress fibers was observed ≥30 minutes post irradiation and correlated positively with loss of cell-cell integrity. Cells of irradiated monolayers recovered to form contact inhibited monolayers ≥24 hours post irradiation; concomitantly, the depolymerized microfilaments organized to their pre-irradiated state as microfilament stress fibers arrayed parallel to the boundaries of adjacent contact-inhibited cells. Previous studies by other investigators have measured slight but significant increases in mouse lung wet weight >1 day post thoracic or whole body radiation (≥500 cGy). Little or no data is available concerning time intervals <1 day post irradiation, possibly because of the presumption that edema is mediated, at least in part, by endothelial cell death or irreversible loss of barrier permeability functions which may only arise ≥1 day post irradiation. However, our *in vitro* data suggest that loss of endothelial barrier function may occur rapidly and at low dose levels (≤200 cGy). Therefore, we determined radiation effects on lung wet weight and observed significant increases in wet weight (standardized per dry weight or per mouse weight) in ≤5 hours post thoracic exposure to 50–200 cGy x-radiation. We suggest that a single fraction of radiation even at low dose levels used in radiotherapy, may induce pulmonary edema by a reversible loss of endothelial cell-cell integrity and permeability barrier function. (Pathology Oncology Research Vol 5, No 1, 49–55, 1999)

Key words: radiation, lung, edema

Introduction

We are in general agreement with those investigators that have described a role for radiation-induced alterations in the pulmonary endothelial structure and function as the critical mediator of the pathogenesis of lung injury. It is known that the severity of radiation induced injuries to the endothelium is largely dependent on the volume of lung included in the radiation treatment field.[1,2] These injuries are thought to be largely a result of the lethal irradiation of the endothelium and are manifest by loss of endothelial monolayer integrity and denudation of the microvascular lumen.[3,4] However, most studies directed towards analysis of edema in animals exposed to single fractions of high dose (>500 cGy) radiation, as opposed to the lower dose levels (<200 cGy) used clinically.[5,6]

The radiation-induced loss of endothelial cell integrity leads to the exposure of basal lamina which results in the leakage of plasma from vessels into the interstitium.[7,8] This radiation-induced vascular permeability is an essential element, if not an initiator, for the development of the acute and late radiation injuries of edema and fibrosis.[9-11] The time course between lethal exposure and denudation or loss of vascular integrity has usually been found to be

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approximately several days to several weeks post irradiation.[5,8] In contrast the immediate and reversible effects of non-lethal levels of radiation on endothelial morphological integrity have gone relatively unstudied. Moreover, non-lethal radiation may be a contributing cause or even the initiator of acute post radiation lung injuries. Because edema should be detected at time intervals (<1 day) post irradiation that are much earlier than the observed denudation of vascular structures, we felt it important to examine the effects of non-lethal radiation at short time intervals post radiation. In addition, the dose levels used in the studies reported here are significant in that they are within the range that lung microvasculature in the target volume would receive during a single exposure of fractionated radiotherapy, or that the lung microvasculature in the treatment field might receive during the cumulative course of multifraction therapy.[12,13]

Reports by other investigators suggest that the rapid and reversible endothelial retraction could be a result of F-actin reorganization.[14,15] For example, increased permeability of the pulmonary microvasculature has been shown to follow the disruption of the microfilament apparatus and the disruption (loss of cell-cell contact) between apparently healthy endothelial cells. It was demonstrated that retraction of the cell cytoplasm and the disruption of the microfilament bundles occurred upon the exposure of pulmonary artery endothelial cell monolayers to cytoskeletal disrupting agents (e.g., hormone, oxidants) and were presumably responsible for the increased permeability.[14,16,17] Therefore, the initial focus of studies presented here are the effects of low dose radiation on the morphology and microfilaments organization of confluent, contact-inhibited pulmonary microvascular endothelial cell monolayers. We examined the rate and extent of endothelial retraction by phase contrast microscopy as well as the rate and extent of F-actin depolymerization by indirect immunofluorescence.

Acute radiation damage to the endothelium results in metabolic dysfunction of eicosanoid metabolism and increased permeability.[18] Similar observations have been made *in vivo* and *in vitro* in normal host tissue and in tumors.[19] It is apparent that the amount of radiation (i.e., 300–5000 cGy) as well as the time interval between radiation and determination of response (i.e., 1–14 days), are critical variables affecting the perceived response(s) of endothelial cells to radiation.[18-20] Recent studies have demonstrated that significant levels of lipoxygenase products are released from irradiated bovine aortic endothelial cells.[21] Moreover, we have observed (data not reported) that radiation stimulates endothelial cell (PMEC) biosynthesis of several eicosanoid metabolites including the lipoxygenase products 11-, 12-, and 15-HETE and unresolved leukotrienes. Products of both the COX and the LOX pathways have been implicated in several aspects of radiation damage, including the loss of barrier function.[22,23] Thus, we sought to test, indirectly, whether radiation-induced lipoxygenase metabolites play a role in acute edema by pretreatment with the non-specific lipoxygenase inhibitor, NDGA.

Materials and Methods

Pulmonary microvascular endothelial cells (PMEC)

Lungs were aseptically removed from C57Bl6J mice and immersed in Ca^{++} and Mg^{++} free Hank's Balanced Salt Solution (HBSS). Following this initial wash, the pleural lining of the lung was fixed gently by applying 70% ethanol over the lung surface. This procedure eliminated mesothelial cell contamination in the developing culture preparation. After rinsing in HBSS, small tissue pinches (1–2 mm), using fine forceps, were obtained close to the lung periphery to avoid obvious large vessels. Tissue explants were then treated with 0.1% collagenase (Type II, Worthington, Malvern, Pa) in $Ca^{++,}$ Mg^{++} free HBSS for 20 min at 37°C. The collagenase treated tissue was carefully removed and plated as explants into 100 mm tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS). Isolated colonies of endothelial cells devoid of spindle cells were selectively trypsinized using colony penicylinders. Three separate endothelial cell clones were isolated, grown to confluency and recloned. One surviving clone was established and routinely subcultured at split ratios 1:2 every 7–10 days and was designated PMEC (pulmonary mouse endothelial cell). Endothelial cell characteristics were verified by growth behavior pattern, presence of factor VIII and prostanoid production according to previously published procedures. For use in the studies presented here, PMEC (between passages 14–20) were cultured in sterile DMEM +10% FBS. They were maintained at 37°C, 95% room air, 5% CO_2 in a water saturated incubator. Medium was changed every three days and cultures were passaged [2.5 mM EDTA (Sigma Chemical Co, St Louis, MO) and 0.25% trypsin (Worthington Biochemical Corporation, Freehold, NJ)] when a contact-inhibited monolayer was achieved (approximately once a week). Cultures used for experimental purposes were seeded in T-75 culture flasks and used when confluent.

Endothelial cell retraction studies

PMEC were grown to confluency in alternate rows of 16 mm 24-well plates (Falcon 3047, flat-bottom). Cells were plated at the appropriate density ($1.5x10^5$ cells/well) so that a confluent monolayer was formed in less than 48 hours. Monolayers were irradiated using a Picker X-ray unit (dose rate of 205 cGy/ min, 280 kV, 20 mA, 1.3 mm

Cu HVL). Following X-irradiation, samples were incubated 37°C 5% CO_2) for various time intervals (0, 2, 4, 8, 24, 48 hour). After the appropriate incubation period, culture medium was aspirated from the wells and 1 ml of fixative (1% paraformaldehyde, 2% glutaraldehyde in HBSS, pH 7,4) was added. Cells were fixed for 10 minutes at room temperature. They were than washed once and stored under 0.75 ml of HBSS. After fixation samples were immediately observed under phase-contrast and photographed at a magnification of 400x using Kodak Panchromatic T-Max 400 ASA film.

Immunofluorescent staining of cytoskeletal elements

Rhodamine-labeled phalloidin (Molecular Probes Inc., Eugene, Oregon) was used for identification of F-actin filaments. Sterile 18 mm^2 coverslips were transferred to 35 mm 6-well flat bottom plates (Corning, NY). PMEC ($2.5x10^5$ cells/ml) were added to coverslips in DMEM + 10% FBS to form contact-inhibited monolayers in 48 hours. Monolayers were irradiated (12.5-200 cGy) and then were terminated at appropriate time intervals post irradiation. Lysis squirting was used to access the cytoskeleton of PMEC. This technique uses osmotic swelling and cell lysis to remove the dorsal cell surface to expose the cytoskeletal elements. Cells were rinsed with HEPES buffer (10 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$ 3 mM EGTA, pH 7.0) and then incubated (10 min) in a 20% dilution of HEPES buffer to induce osmotic swelling and cell lysis visualized by visual inspection using phase-contrast microscopy. After cell lysis samples were rinsed rigorously (to remove membrane fragments), and fixed with 4% paraformaldehyde in HEPES buffer (room temperature (30 min). Samples were then washed 3X with HEPES buffer. Labeling of F-actin (rhodamine phalloidin) was accomplished by a single step staininq using 75 µl of 1:50 rhodamine phalloidin. After labeling, samples were rinsed (5x, HEPES) and mounted in a medium containing 1:2 glycerol:HBSS supplemented with 0.1 g Cytifluor (Amersham, Arlington Heights, IL) and 0.2 g of Mowiol 4–88 (Calbiochem Corporation, La Jolla, CA) to prevent rapid quenching under fluorescent excitement. Samples were analyzed at 600X magnification under oil using a Nikon Optiphot Microscope. Micrographs were recorded on Kodak T-MAX 400 Panchromatic film.

In vivo thoracic radiation

Unanesthetized C57Bl6J male mice, Jackson Laboratories, Bar Harbor ME (6–7 wk old) were exposed to 0.5–2.0 Gy of radiation [X-ray source (Picker unit, 280 Kev) that delivered 275 cGy/min.]. Mice were held in a plastic cylinder which is blocked with lead and calibrated to ensure that the target volume was restricted to the thoracic region (between the mediastinum and head). In all experiments, mice were randomly chosen front stock cages for each treatment group. Mice were pretreated, simultaneously treated and/or post-treated with specific lipoxygenase inhibitors using specific schedules and vehicles. [Note: Animal care was in accordance with institutional guidelines.]

Determination of lung wet weight

Immediately after sacrifice, the lungs were dissected and rinsed by saline spray under a dissecting microscope to be cleaned of all other tissue. Care was taken that the saline did not enter the trachea by inverting the lungs and gently shaking off the fluid. After cleaning, the lungs were placed on absorbent tissue, gently blotted five times and transferred into pre-weighed polycarbonate weigh boats which were covered and weighed. Dry weights were also determined. Lungs were dried for about 40 hours in an oven at 60°C and reweighed. The weight of each lungs was determined and correlated with dry weight and mouse weight. The average weight of <7 week old mice is approx. 12 grams. Mice were irradiated on same day (Wednesday) of each week at the same time of day (9:00 AM) to standardize for possible chronological effects on weight etc. [Control and irradiated C57Bl6J mice were sacrificed by three methods, CO_2 gassing, sodium pentobarbitol anesthesia and direct cervical dislocation. No significant differences in lung weights among groups were observed.]

Results

Radiation therapy of the thorax for treatment of breast and lung cancer and Hodgkin's disease is often associated with pulmonary edema and fibrosis resulting in compromised lung function.[1,2] At the clinical level the manifestation of these pathological states can only be detected a posteriori when the patient is symptomatic for these syndromes. We believe that an understanding of the mechanism(s) which mediate radiation induced edema and fibrosis may lead to the development of adjuvant therapies and the use of specific inhibitors of eicosanoid metabolism to greatly reduce or inhibit the development of these injuries.

We used phase contrast photomicroscopy to record that radiation (50–200 cGy) initiates retraction of contact inhibited PMEC monolayers. We found retraction was time-dependent and dose-dependent[24] in that the degree of retraction increased as the time interval between radiation and observation increased. *Figure 1a–d,* is representative of our studies. *Figure 1a* is control PMEC monolayer demonstrating the characteristic morphology contact-inhibited capillary endothelial cells. *Figure 1b* demonstrates the F-actin cytoskeleton prior to irradiation, Note the prominent stress fiber spanning large areas of the cell body. Retraction of cells of the PMEC monolayer was

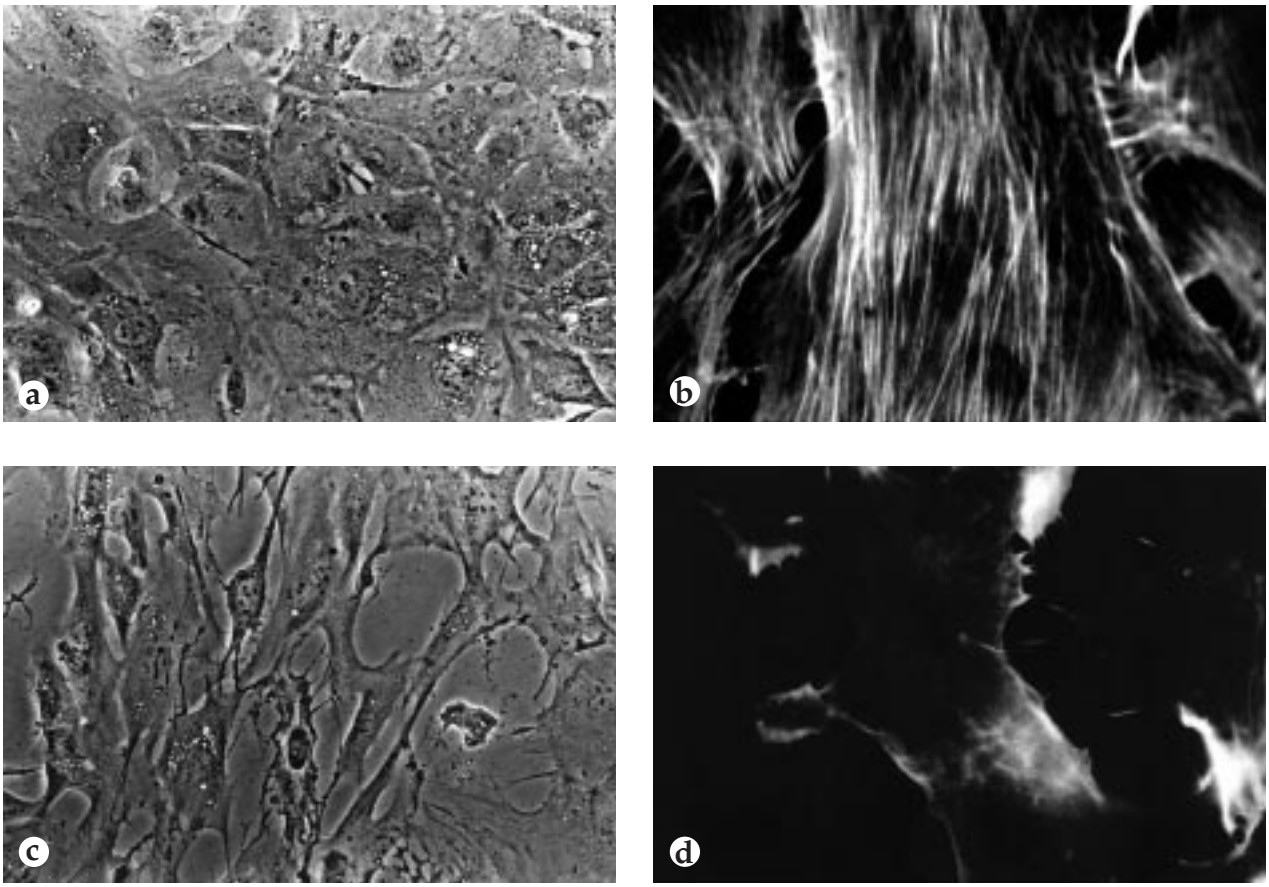

Figure 1. *Effect of x-irradiation (50 cGy) on the morphology of pulmonary microvascular endothelial cell monolayers (PMEC). PMEC were seeded on fibronectin 24 hours to form confluent monolayers. After irradiation, PMEC were incubated for various time intervals before fixation. This figure is representative of the studies.* ***1a*** *represents PMEC monolayers prior to radiation exposure. The characteristic morphology of contact-inhibited capillary endothelial cells is clearly evident.* ***1b*** *presents the F-actin cytoskeleton prior to irradiation, Prominent stress fiber spanning large areas of the cell body are readily visible.* ***1c*** *represents the retracted cells induced by a single fraction of 0.5 Gy. Concomitant analysis of F-actin demonstrated the loss of cytoskeletal organization, although some fibers are still visible at the retracted cell periphery* ***(1d)****.*

induced by a single fraction of 0.5 Gy. Significant retraction was recorded 4–8 hours post exposure *(Figure 1c)*. Analysis of F-actin demonstrated the loss of cytoskeletal organization. The F-actin stress fibers have resolved, although some fibers are still visible at the retracted cell periphery *(Figure 1d)*. Retraction was reversible, and the PMEC cells resumed their appearance as contact inhibited monolayers within 24 to 30 hrs post irradiation, which corresponded to the repolymerization of F-actin fibers (not shown). The ability of the cells to regain their monolayer appearance and develop cell-cell contacts indicates that energy-dependent cytoskeletal reorganization was not impaired by 0.5 Gy radiation and suggests that levels of radiation below that dose do not significantly impair normal PMEC metabolic activity. There appears to be a threshold for radiation initiated PMEC retraction, and once crossed, further increases in radiation dose level fail to increase the rate or extent of retraction or decrease the lag time for the induction of retraction or cytoskeletal reorganization (data not shown)

Our working hypothesis predicts that radiation promotes endothelial retraction via stimulation of lipoxygenase metabolism. Accordingly, a general lipoxygenase inhibitor should inhibit radiation induced retraction. We treated confluent PMEC monolayers with the lipoxygenase inhibitor NDGA (10 μM) 15 minutes prior to and during 50 cGy radiation. Retraction was complete inhibited, whereas pretreatment with the cyclooxygenase inhibitor indomethacin failed to inhibit retraction (data not shown).

Previous studies by other investigators have measured slight but significant increases in mouse lung wet weight >1 day post thoracic or whole body radiation (>500 cGy).[22,32] Little or no data is available concerning time intervals <1 day post irradiation, possibly because of the presumption that edema is mediated, at least in part, by endothelial cell death or irreversible loss of barrier permeability functions

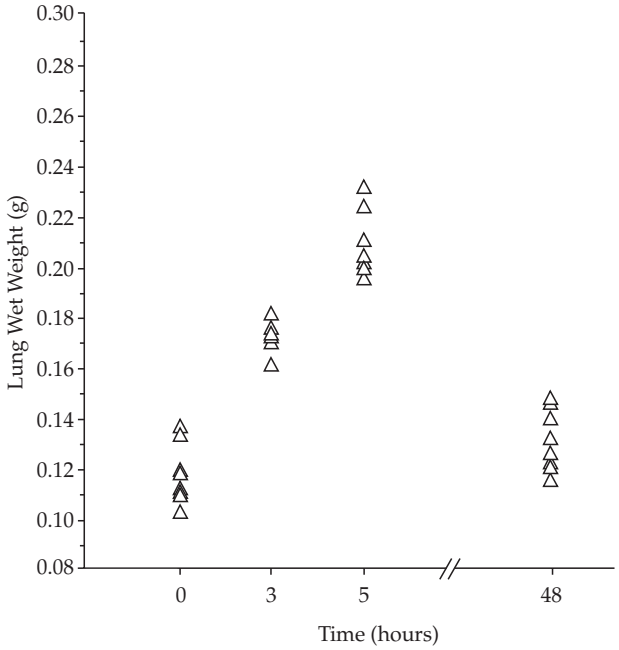

Figure 2. *C57Bl6J mice were randomly selected for sham or exposure to 200 cGy radiation. Data are for individual total lung weight from each mouse used in the study. The weight of lungs from mice sacrificed 5 hours post radiation exposure were significantly different (p<0.01 by students t test) when compared to the weight of lungs from mice in the sham irradiated group or from mice sacrificed 48 hours post radiation exposure.*

which may only arise >1 day post irradiation. However, our working hypothesis predicts that low dose radiation at levels traditionally employed for radiotherapy should induce acute edema in the pulmonary microvasculature. This edema would be mediated by loss of endothelial cell-cell integrity induced by the direct effects of radiation on microvascular endothelial morphology as well the impetus supplied by adherent and migrating neutrophils and monocytes as they passage from the lumen to the subendothelial matrix and to the interstitium. Our *in vitro* data clearly demonstrated that loss of endothelial barrier function occurs rapidly (≤4 hours) and at low dose levels (≤200 cGy). Therefore, we performed a series of studies to verify a correlation between the time and dose effects for radiation-induced loss of endothelial cell-cell integrity *in vitro* and radiation induced acute edema (as determined by effects on lung wet weight) *in vivo*. We first demonstrated a time course for radiation-induced edema. Mice were exposed to thoracic radiation of 200 cGy. We observed significant increases in lung wet weight (standardized per dry weight or per mouse weight) for time points 3 and 5 hours post irradiation *(Figure 2)*. By 48 hours post-irradiation, there was no statistically significant increase in lung weights, suggesting a recovery from acute edema. We also observed a dose-response effect, with increased radiation exposure resulting in increased edema. There was a significant increase in lung wet weight at all dose levels five hours post irradiation *(Figure 3)*. At three hours post irradiation, 100 and 200 cGy, but not 50 cGy exposure resulted in significantly increased lung weights (data not shown). Finally, we observed protective effect by pretreatment of animals with the lipoxygenase inhibitor NDGA, which quite effectively blocked acute edema *(Figure 4)*. NDGA was administered i.p. 15 minutes prior to radiation exposure.

Discussion

In the treatment of pulmonary neoplasms, breast carcinoma, esophageal carcinoma or Hodgkin's disease, the risk of complications to the normal pulmonary tissues is a major limitation in the prescription of the therapeutic dose.[1-3] The treatment of neoplasia by radiation requires part or the whole of the thorax to be in the radiation field. Radiation induced edema, pneumonitis and fibrosis are well-documented complications in patients receiving such treatments.[25,26] Early reactions develop within days or weeks, whereas late reactions require months or years.[25-27] The clinical presentation of radiation-induced lung damage principally depends on the lung volume irradiated, the radiation dose and the pre-existing lung disease.[28,29] Mah et al have established a distinct dose-response relationship between

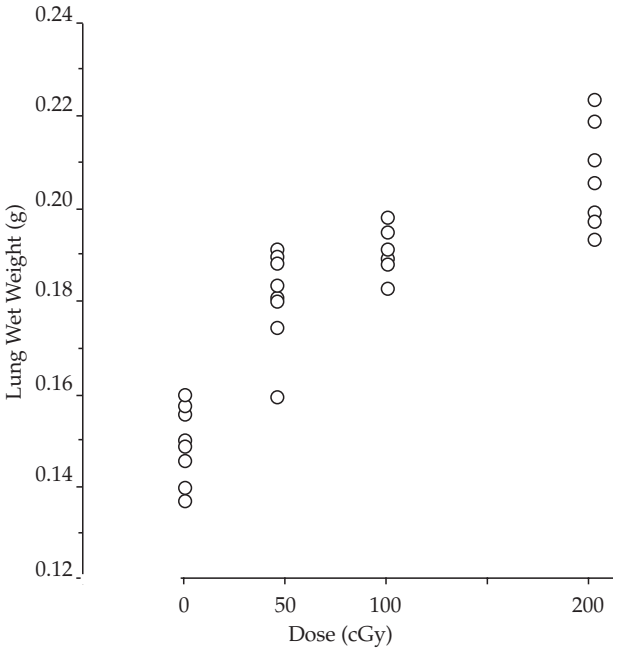

Figure 3. *C57Bl6J mice were randomly selected for sham or exposure to 50, 100, or 200 cGy radiation. Data are for individual total lung weight from each mouse used in the study. The weight of lungs from mice exposed to 100 or 200 cGy were significantly different (p<0.01 by student's t test) when compared to the weight of lungs from mice in the sham irradiated group.*

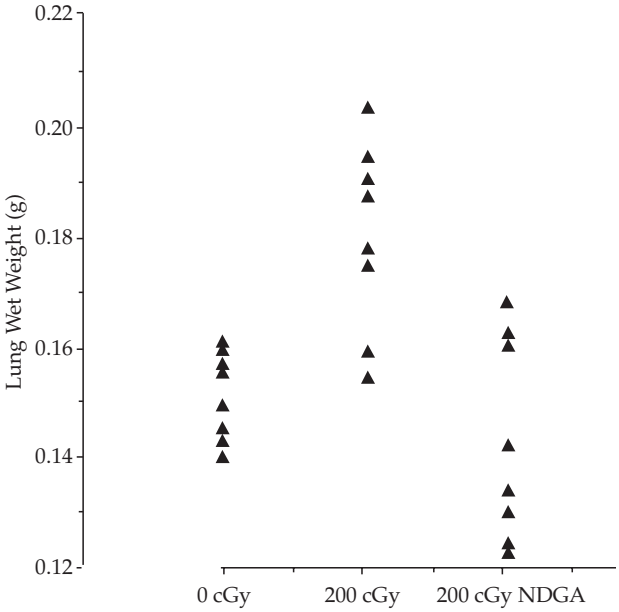

Figure 4. *C57Bl6J mice were randomly selected for sham or exposure to 200 cGy radiation. Sham mice were randomly treated with NDGA (25 mM, ip injection, 15 minutes prior to procedure) or vehicle. Mice exposed to 200 cGy radiation were similarly randomized to vehicle and NDGA treated groups. Data are for individual total lung weight from each mouse used in the study. The weight of lungs from sham exposed mice treated with vehicle or NDGA were not significantly different and were pooled. The weight of lungs from mice treated with vehicle and exposed to 200 cGy were significantly different ($p<0.05$) from control mice. In contrast, the weight of lungs from mice treated with NDGA prior to radiation exposure were similar to the control group.*

the incidence of acute radiation-induced pulmonary damage for human pulmonary tissues to fractionated radiotherapy using average lung dose in the high dose region.[30]

Control of radiation lung damage has been attempted using many procedures which have centered on fractionated doses and low dose rates.[31] Lung correction and shielding are routinely employed in radiotherapeutic practice to reduce adverse lung injury. Unfortunately in the treatment of pulmonary and thoracic neoplasms it is inevitable that a certain part of normal lung tissue will fall within the treatment volume. Adjuvant therapy using corticosteroids which are potent inhibitors of inflammatory edema[32] are used to prevent radiation injuries but the precise cellular/intracellular target sites of action are unknown.[9,10] An understanding of the basic biochemical mechanisms underlying the events leading to edema, pneumonitis and fibrosis would facilitate the identification or specific inhibitors capable of blocking both the acute/early and late injuries.

We report here that low dose radiation (50–200 cGy) produces significant changes in the morphology and microfilament organization of pulmonary microvascular endothelial cells characterized by retraction and the resulting loss of close contact between individual cells within the monolayer. By phase-contrast microscopy, one observes an apparent retraction and loss of contact between cells resulting in the formation of gaps (between the cells) The radiation-induced cellular retraction was time and dose-dependent. Retraction was first observed at >1 hour post radiation and the extent of retraction increased with time. At the earliest stage of retraction, the cells usually demonstrated a loss of association with the adjacent cells but only in limited areas of the cell periphery, not around the entire cell margin. The extent of loss of contact between cells increased with time, and at maximum retraction (>4 hours), there was a complete loss of contact between adjacent retracted cells and large regions of the monolayer had resolved into isolated cells that were completely separated from adjacent cells. We also observed that the extent of retraction was dose dependent and that the time interval to reach maximum retraction decreased with increased dose level.

Because a role for microfilaments, but not for microtubules, has been previously demonstrated in transient hormone-induced cellular retraction and respreading, we examined the effects of radiation on microfilament organization. We observed that the centrally located stress fiber bundles "disappear" in response to radiation, and it is this radiation induced depolymerization of the microfilaments that comprise the centrally located stress fiber bundles that appears to be causal for the morphological change of retraction. We observed an absolute and positive correlation between radiation-induced F-actin depolymerization and the dose- and time-dependent radiation-induced retraction. The time course for changes in microfilament organization (i.e., F-actin depolymerization) were perfectly coincident with the time course for morphological changes. For example, profound F-actin depolymerization was seen at 2 hours post radiation at 50 and 100 cGy, which coincides with the retraction seen at these two doses at 2 hours. Conversely, lower dose levels (12.5, 25 cGy) failed to initiate F-actin depolymerization at 2 hours and no retraction was observed at this time.

We also demonstrated (indirectly) a role for lipoxygenase products in radiation-induced endothelial cell retraction. Pretreatment with a variety of lipoxygenase inhibitors (e.g., NDGA) blocked radiation-induced retraction. Inhibition was both dose- and time-dependent, and application of NDGA after irradiation failed to block retraction. In contrast, pretreatment with the cyclooxygenase inhibitor, indomethacin, failed to block retraction.

We used the radiation dose levels and time course for PMEC retraction *in vitro* to design studies to determine radiation-induced acute edema *in vivo*. We demonstrated that low dose thoracic radiation induces pulmonary edema as characterized by increased lung wet weight. The inci-

dence of increased weight was radiation dose-dependent to 2.0 Gy and was coincident with the time course for radiation-induced endothelial retraction in vitro. Finally, we determined that pretreatment of animals with 25 μM NDGA 15 minutes prior to radiation exposure inhibited radiation-induced edema. These observations were also in perfect agreement with our *in vitro* studies.

We suggest that our PMEC model system may prove useful for the screening of compounds that may prove clinically useful for the prevention of acute and late radiation injuries to the lungs and other normal tissues. The studies presented here demonstrate an initial step in identifying agents (e.g., NDGA) which block radiation injuries *in vitro* and *in vivo*.

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