Endothelial Injury and Repair in Radiation-Induced Pulmonary Fibrosis

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Cytokinetic relationships between endothelial cells and fibroblasts during lung injury and repair in mice have been studied in a morphologic, autoradiographic, and biochemical study following whole body irradiation. After 650 rads, endothelial injury accompanied by interstitial edema was seen between weeks 1 and 2. The cell labeling curve had two components: predominant endothelial labeling to 3 weeks, then a smaller rise in DNA synthesis in interstitial cells. There was focal fibrosis but little change in total hydroxyproline to 20 weeks. After 1000 rads, cell injury, still confined to the endothelium, was more severe and lasted up to 6 weeks. Increased DNA synthesis occurred in the endothelium between Weeks 2 and 8 and in interstitial cells from Week 3 to 16, when total hydroxyproline was significantly elevated and many fibrotic areas were seen in the lung. The results indicate that acute endothelial injury may be rapidly repaired with little fibroblastic stimulation, whereas severe or prolonged injury with delayed regeneration disturbs endothelial–mesenchymal relationships. This may be a key factor in promoting fibroblast proliferation and the deposition of collagen. (Am J Pathol 1983, 112:224–230)

STUDIES of cellular injury and repair in the lung have indicated that disturbed epithelial–fibroblastic cell relationships are important in the induction of pulmonary fibrosis.[1-3] Severe epithelial injury with delayed or modified regeneration results in loss of fibroblastic control, a process analogous to wound healing in the skin. The lung also has a very large endothelial surface, and it is not known whether disruption of endothelial–fibroblastic cell interactions is equally important in pulmonary injury and repair. Although acute endothelial damage may be rapidly repaired with no fibrosis,[4] it has been suggested that more severe injury with delayed regeneration may result in fibrocellular lesions in the vascular wall.[5]

In the present experiments whole-body irradiation is used as a model of pulmonary endothelial cell injury. Patterns of DNA synthesis by endothelial and interstitial cells in repair are correlated with collagen synthesis to identify relationships between endothelial injury, repair, and fibroblastic control.

Materials and Methods

Swiss–Webster mice (25-g males) were divided into groups of 100 and received chlortetracycline (2 g/l) in drinking water 2 weeks before irradiation and continuously afterwards. One group received 650 rads of whole-body irradiation, and the second group received 1000 rads. The animals were held in individual sections of a plastic box equidistant from a ^{60}Co source. Thirty nonirradiated animals served as controls and were killed monthly in groups of 4. Irradiated mice were killed in groups of 4 by intraperitoneal sodium pentobarbital at frequent intervals to 2 weeks then at Weeks 3, 4, 6, 8, 10, 12, 16, and 20. One hour before death, each animal received 2 μCi/g tritiated thymidine. Blood was taken from the heart, and a leukocyte count was made with the use of an automated cell counter after lysis of red cells. A tracheotomy was performed, and the bronchus leading to the right lung was clamped. This lung was removed, weighed, and frozen for biochemical determinations. The left lung was inflated with 2% glutaraldehyde and removed. After 1 hour the tissue was

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sliced, and most tissue was postfixed in formalin and embedded in glycol methacrylate for light-microscopic study. The remaining tissue was postfixed in osmic acid and prepared for electron-microscopic study.

Autoradiography

Plastic sections 0.75 μ thick were prepared for autoradiography by exposure to Kodak NTB2 emulsion for 2 weeks, development, and staining with basic fuchsin. The percentages of labeled cells (at least 4 silver grains per nucleus) in the lung were determined at each time after irradiation by counting 3000 lung cells (excluding bronchial epithelium) per animal. Differential counts of pulmonary cell types were made on 300 labeled cells per animal at each time. Cells were designated as epithelial, endothelial, interstitial, intravascular, or alveolar macrophages on the basis of morphologic features and the precise anatomic location.[4] Means and standard deviations were calculated on a per-animal basis. The radiographic index for each cell type was also calculated. This is the product of the total cell labeling index and the differential percentage for a particular cell type, e g, the overall percentage of labeled cells multiplied

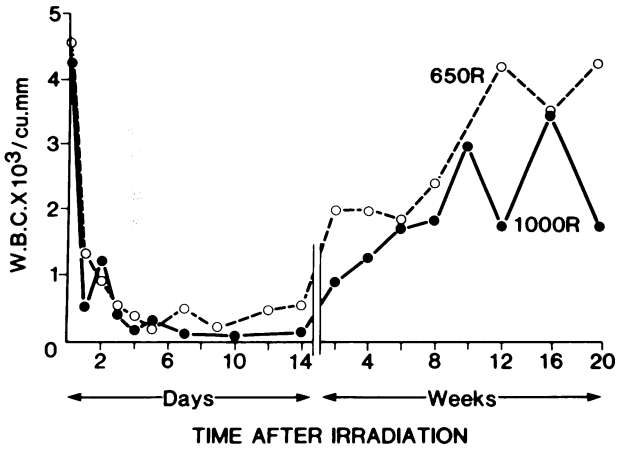

Figure 1 — Mean leukocyte counts of the 4 mice per group at various times after each dose of whole-body irradiation.

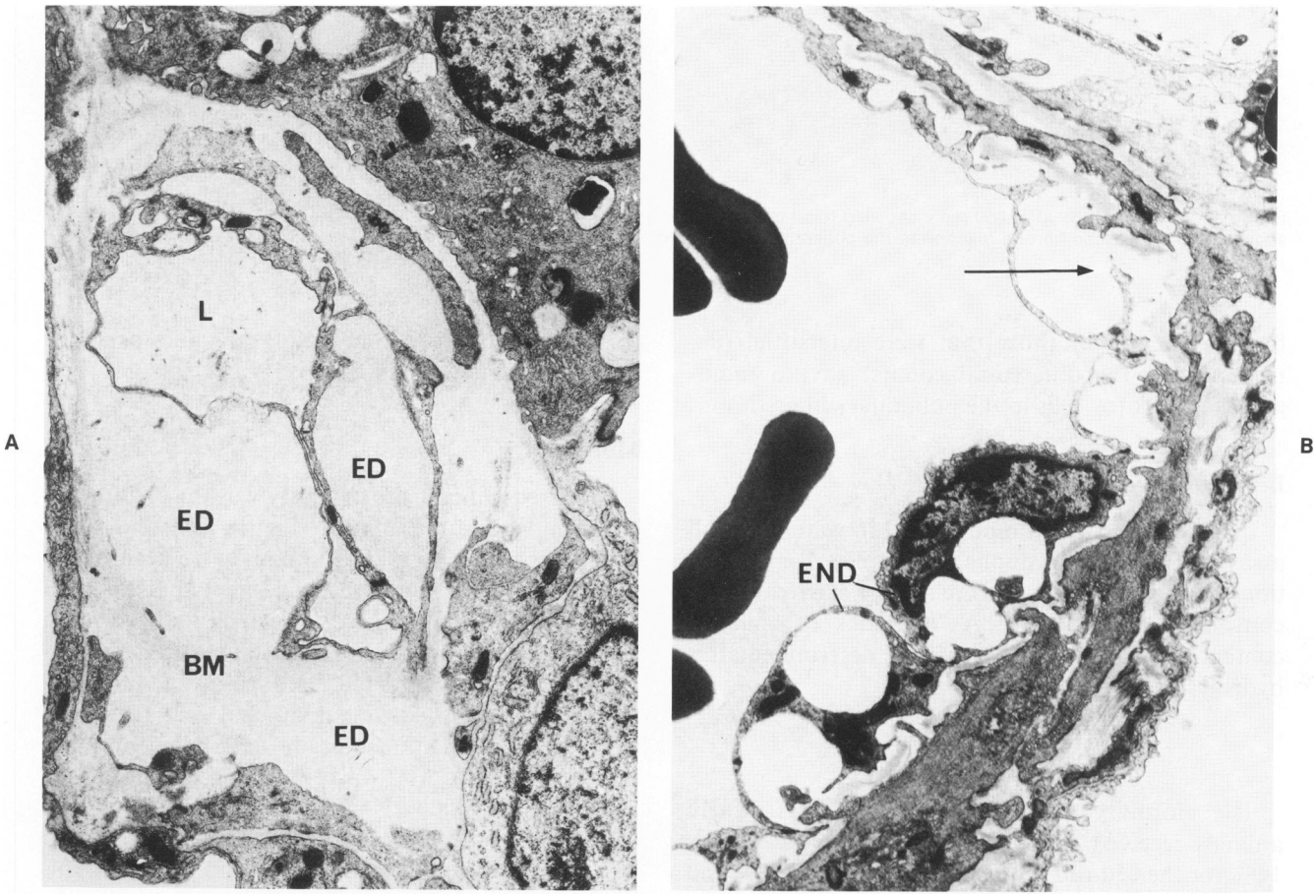

Figure 2 — Electron micrographs 4 weeks after 1000 rads. **A** — Endothelium, separated from basement membrane (*BM*) by interstitial edema (*ED*), bulges into the capillary lumen (*L*). Normal Type I and Type II epithelial cells are seen. (×8000) **B** — Endothelial cells (*END*) of arteriole are vacuolated and separated from basement membrane (*arrow*). (×3500)

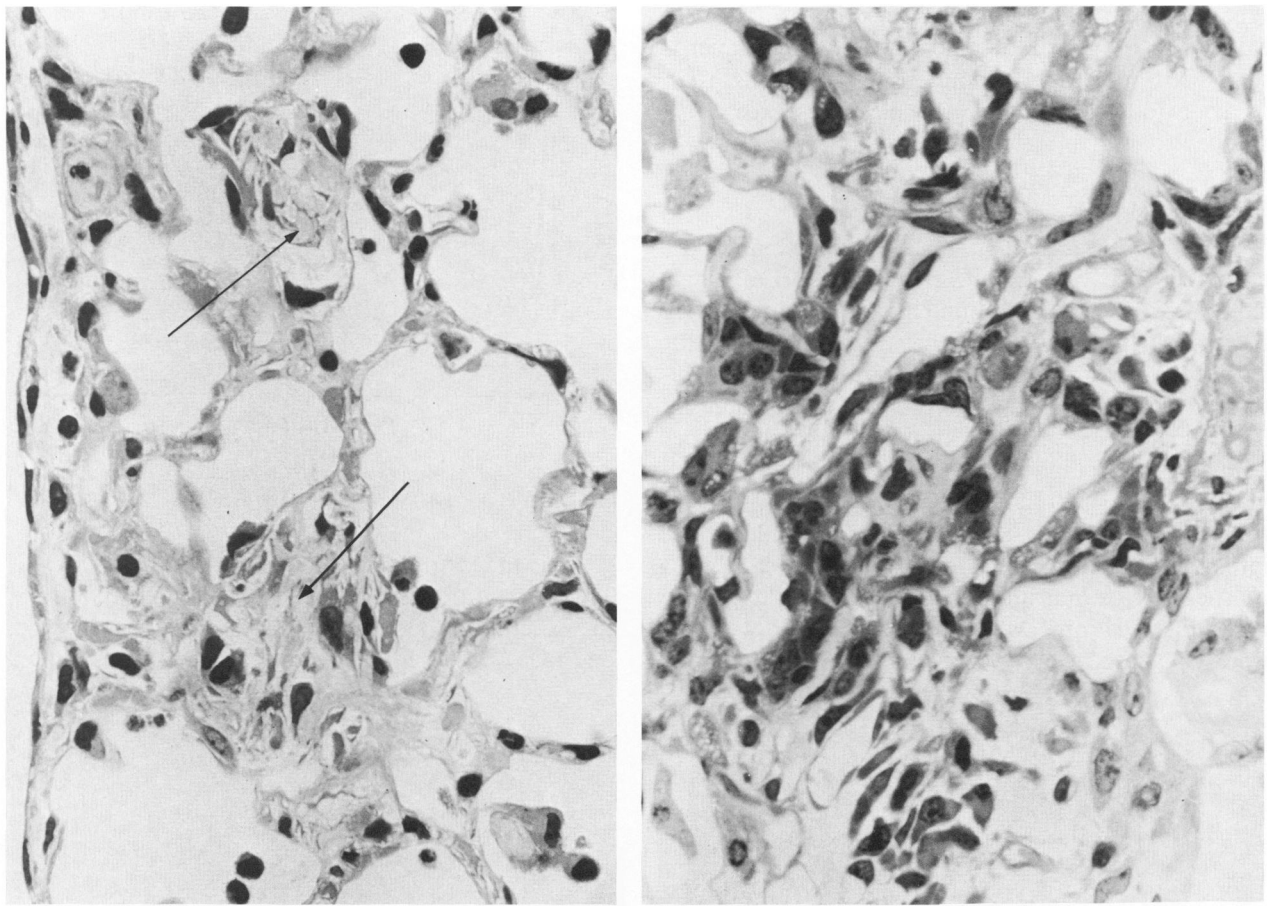

Figure 3 **A**—Four months after 650 rads, showing focal areas of interstitial fibrosis (*arrows*). (×400) **B**—Four months after 1000 rads, showing a more diffuse fibrotic reaction in the pulmonary interstitium. (H&E ×500)

by the fraction of those that were interstitial (determined by the differential counts) gave a radiographic index for cells in the pulmonary interstitium.

Biochemistry

Lung samples were homogenized in water, and all assays carried out on duplicate samples. Determinations of DNA and total protein were carried out by conventional methods.[6,7] As an index of collagen content, hydroxyproline levels were determined after hydrolysis with hydrochloric acid.[8]

Results

The mortality rate in these experiments, where animals received antibiotics in drinking water, was 14% for the 650-rad group and 34% for the 1000-rad group. The white blood cell count dropped rapidly after each dose and was very low for about 2 weeks (Figure 1). Recovery to near normal values occurred faster after the 650-rad dose.

Morphology

The predominant site of injury was the endothelial cells lining both capillaries (Figure 2A) and small blood vessels (Figure 2B). Lesions were seen in the first 2 weeks after 650-rad treatment and up to 6 weeks following the high dose, which induced more severe and extensive endothelial damage. Cell cytoplasm became vacuolated, and areas of subendothelial edema caused the endothelial cells to detach from the basement membrane and bulge into the lumen (Figure 2). At 2–4 weeks after 650 rads and 6–8 weeks after 1000 rads, a few focal areas of swollen cytoplasm in alveolar Type I cells were observed. These changes appeared minimal and were not followed by any obvious proliferation of Type II cells.

Subsequently, fibrotic changes developed in the

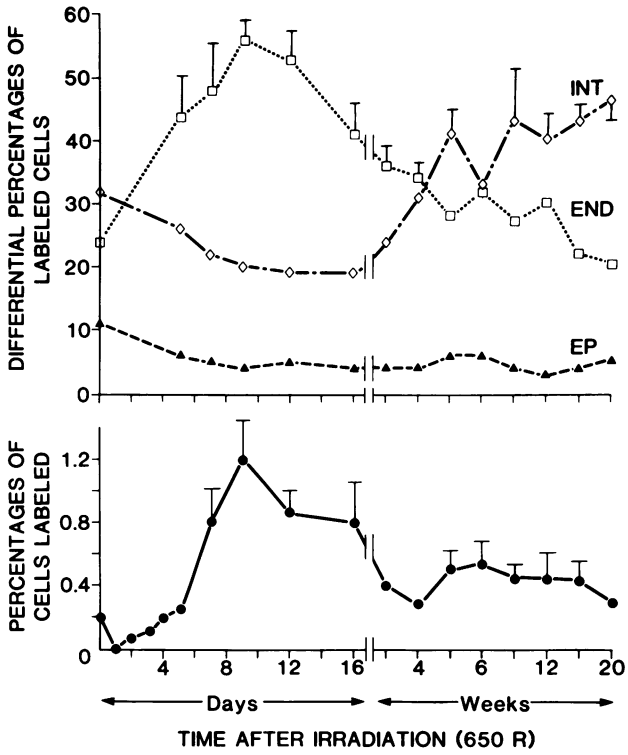

Figure 4 A—Differential counts of labeled cells: *EP*, epithelial; *END*, endothelial; *INT*, interstitial. Bars indicate mean ± SD when significantly greater than normal. $P < 0.05$ **B**—Percentages of labeled lung cells at intervals after 650 rads.

lungs, particularly in the high-dose group. A thickened air-blood barrier was seen by electron microscopy, and later fibrosis was obvious by light microscopy. In the 650-rad group, these changes, first observed at 6 weeks, were multifocal (Figure 3A), whereas in the 1000-rad group, fibrosis, first seen at 3 weeks, became progressively more severe and widespread with time (Figure 3B).

Autoradiography

The percentages of cells labeled in DNA synthesis after 650 rads is shown in Figure 4. Control values stayed constant at about 0.3%, whereas the graph of irradiated mice showed a biphasic increase, the first phase from 5 days to 3 weeks, and the second from 4 weeks onward. Differential counts of labeled cells were made, and the results are shown in Figure 4. The percentage of labeled endothelial cells rose from 23% in control mice to 55% at 10 days after irradiation. DNA synthesis in the second phase of repair was due to increased proliferation of interstitial cells. No increase in labeling was seen in epithelial cells, whose differential percentage of the total decreased as total labeling increased.

Cell proliferation was delayed in mice that received 1000 rads, and the graph again appeared biphasic (Figure 5). The first phase, 10 days to 8 weeks, reached a peak of 1.7%, with most activity between 3 and 6 weeks. Differential counts showed a prolonged increase of labeled endothelial cells in the first phase (Figures 5 and 6A) and a later large increase in labeled interstitial cells (Figures 5 and 6B). Again, there was no increase in DNA synthesis in alveolar epithelial cells.

In order to demonstrate the temporal relationships between endothelial and interstitial cell division, the radiographic index for each cell type was calculated (Figure 7). At the lower dose there was a rapid large increase in endothelial labeling accompanied by a smaller rise in interstitial cell division. From 4 weeks, however, a small steady increase in interstitial labeling was maintained. After the higher dose, DNA syn-

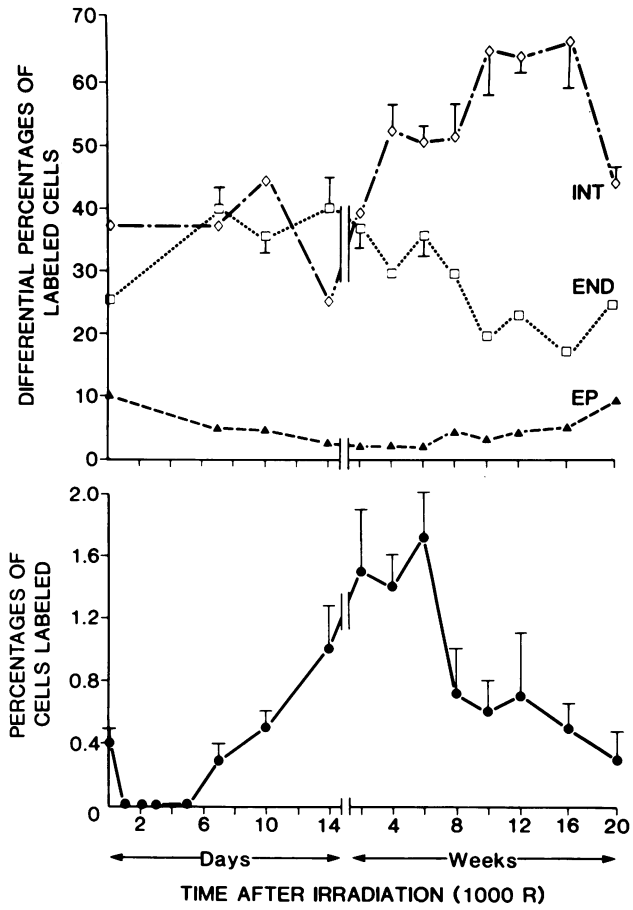

Figure 5 A—Differential counts of labeled cells. Bars indicate mean ± SD when significantly greater than control. **B**—Percentages of labeled lung cells (mean ± SD) at intervals after 1000 rads.

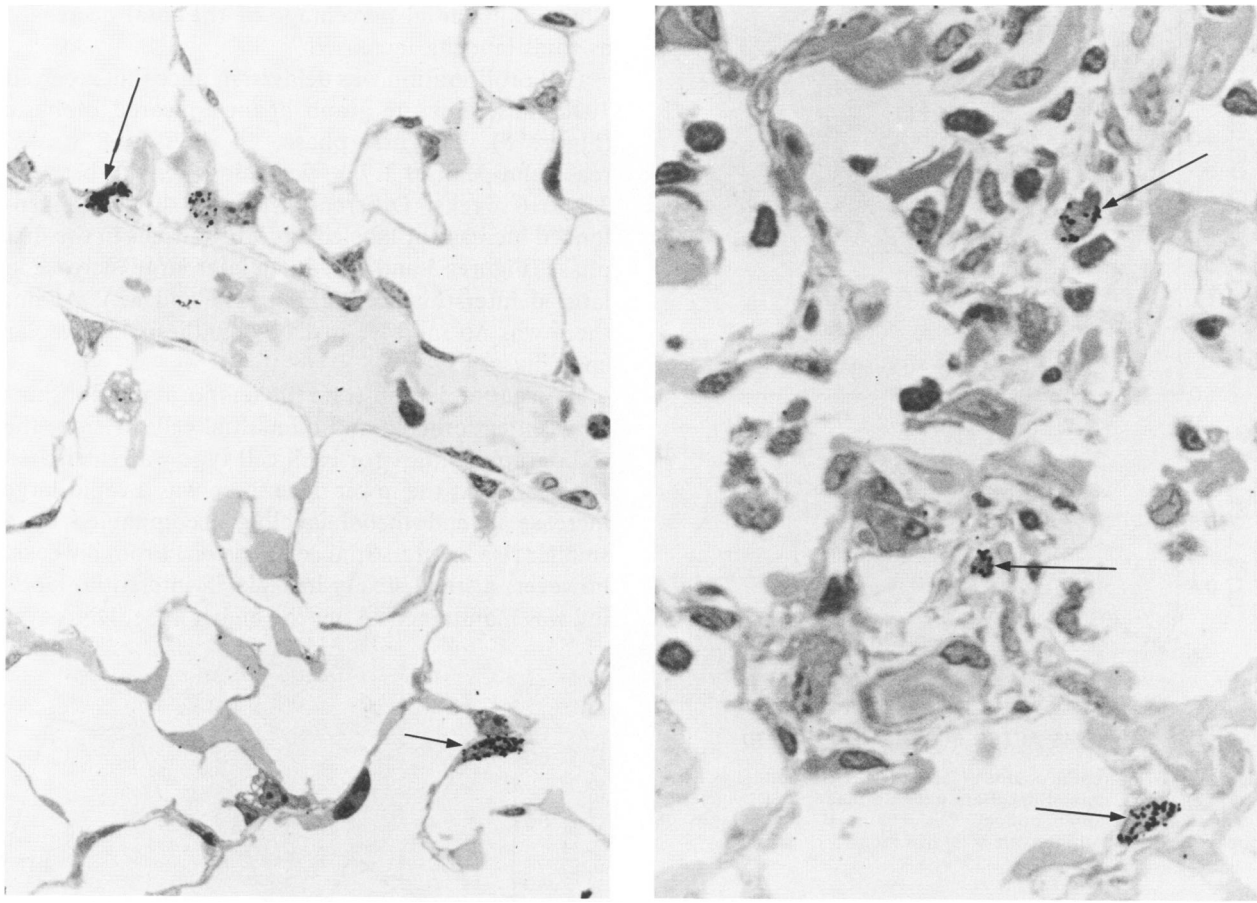

Figure 6A — Autoradiograph 2 weeks after 1000 rads, showing ^{3}H-thymidine incorporation into endothelial cells of capillary and venule (*arrows*). **B** — Autoradiograph 8 weeks after 1000 rads, showing labeled fibroblasts (*arrows*). (Fuchsin stain, ×500)

thesis was delayed in endothelial cells, and between 2 and 8 weeks both endothelial and interstitial cells proliferated; after 8 weeks there was continued interstitial cell division at a much higher level than that seen after 650 rads (Figure 7). The radiographic index for epithelial cells remained constant at control levels throughout the study.

Biochemistry

Determination of hydroxyproline (HYP) was correlated with protein and DNA levels in the lung. Control values increased gradually over the 20 weeks of the experiments, though there was a lower increment in the HYP/DNA ratio (Figure 8). Little change was seen in any of the experimental values in the 650-rad experiment, but significant changes were seen after 1000 rads (Figure 8). The total HYP rose after 4 weeks and was 50% higher than controls at 20 weeks. An increase in the ratio of HYP to total protein was seen after 3 weeks and again increased to about 50% higher than controls at 20 weeks. The ratio of HYP to DNA rose earlier, probably because of decreased DNA from destroyed endothelial cells and leukocytes in the lung. However, at the time of recovery for these cells (4–6 weeks) onward, as DNA synthesis slowed down, the ratio of HYP to DNA became 100% higher than controls and correlated well with the morphologic observation of fibrosis.

Discussion

The lung is particularly sensitive to cell injury induced by irradiation. Its effects are seen after whole-body exposure and, more markedly, after a larger local dose to the chest. Radiation pneumonitis and pulmonary fibrosis ensue and often result in death.[9] Early mortality is usually due to infections when leukocyte counts are low; but in the present experiments, antibiotic protection allowed most animals to survive the leukopenic period. Whole-body irradiation is a simple method of inducing

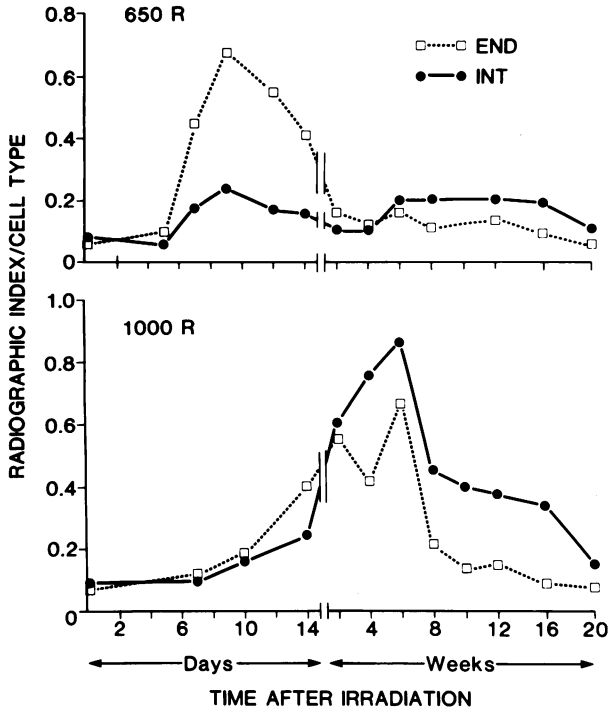

Figure 7—Radiographic indices for endothelial (*END*) and interstitial (*INT*) cells at intervals after each dose.

varying degrees of endothelial cell injury in order to study relationships between cellular regeneration and the induction of fibrosis.

There are a number of descriptive studies of radiation-induced injury in the lung utilizing a variety of exposure doses and conditions.[9] The results suggest that cell injury and subsequent fibrosis increase with the dose administered. There is some dispute, however, about the cells involved and their roles in the pathogenesis of fibrosis. Vascular injury, with resultant interstitial edema, has been described[10-12]; and the present study confirms these observations. The role of the epithelium in the pathologic process is not so clear. Changes in Type II cell secretion and reduced surfactant function have been reported,[13,14] but Gross maintains that changes in surface tension are secondary to leaked plasma proteins inactivating surfactant.[9] Other investigators have demonstrated focal cytoplasmic changes in Type I cells.[11,12] In the present study, we found no change in DNA synthesis in the epithelial population, indicating that alveolar epithelial injury and repair is not a major factor in the generation of fibrosis. Similarly, when rats received 3000 rads to the lungs, no increase in the number of Type II cells was observed subsequently.[15]

Endothelial cells have been identified as the predominant site of cell injury induced by irradiation. Damage is also implied from the appearance of interstitial edema and by other functional changes such as impaired amino acid transport.[16] In a study of oxygen toxicity, we have shown that the pattern of endothelial regeneration is a good marker of the anatomic location of cells most susceptible to injury.[4] With the use of a similar technique in the present study, endothelial injury, after 650 rads, was followed by rapid repair, with little stimulation of fibroblasts; whereas at the higher dose, injury was followed by delayed and prolonged endothelial cell proliferation. This enhanced proliferative response indicates greater vascular damage and correlates well with increased fibroblast proliferation and collagen secretion, as demonstrated morphologically and biochemically.

Altered relationships between epithelial and mesenchymal cells have been postulated as factors in the generation of pulmonary fibrosis. After oxygen, rapid epithelial repair restores the lung to normal, whereas severe injury and delayed proliferation is associated with fibroblastic stimulation.[1] Altered epithelial-interstitial interactions have also been seen

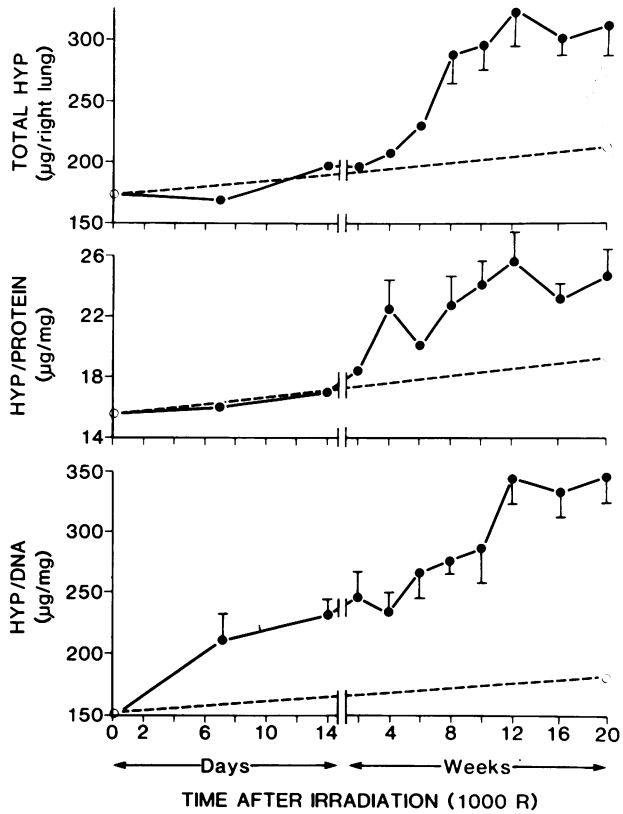

Figure 8—Mean values of total hydroxyproline, the hydroxyproline/protein ratio, and the hydroxyproline/DNA ratio at intervals after 1000 rads (*solid circles*). Standard deviation is shown when values are significantly above control (*broken line*) ($P < 0.05$).

in fibrotic animal lungs after bleomycin,[2] butylated hydroxytoluene,[3] and in human disease.[3] In the same way that loss of epithelial surface promotes fibroblast growth, we now propose a similar mechanism operates when there is loss of endothelial surface in the lung. When endothelial injury is followed by rapid cell proliferation, the vascular surface may be restored with little loss of fibroblast control. Delayed repair following severe injury to the endothelium, however, may cause an extended period of surface denudation with concomitant loss of fibroblastic control, resulting in cell proliferation and collagen deposition. It is postulated that prolonged disruption of the normal endothelium–fibroblast control system is the crucial event in radiation-induced pulmonary fibrosis.

Relationships between endothelial and interstitial cells have been demonstrated in other systems. Following damage to the rat aorta, injured intimal areas that are rapidly covered by regenerating endothelium are protected from the development of fibrocellular intimal lesions.[5] It appears that the extent of the injury is crucial in determining repair patterns, because a period of denuded surface is necessary for the initiation of a smooth-muscle reaction, whereas minimal injury may be repaired with no lesion.[17] Endothelial growth has been maintained *in vitro* by conditioned medium from other endothelial cells, a preparation that also contains a factor that stimulates the growth of smooth-muscle cells.[18] However, other investigators have shown that medium conditioned by endothelial cells inhibits the growth of smooth-muscle cells.[19] The importance of the substrate to cell division has also been stressed, because endothelial cells proliferate on Type I or III collagen but migrate preferentially on Type IV or V collagen.[20,21] It may be speculated that severe injury destroys endothelium and basement membrane, thereby releasing control of the interstitial fibroblast, which proliferates and secretes Types III and I collagen. This new substrate could then promote further endothelial proliferation to assist the reparative process.

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