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Radiation-Induced Fibrosis

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Abbreviations

AP-1: activator protein-1.
CAT: catalase
DNA: deoxyribonucleic acid
ECM: extra cellular matrix
eV : electron volt.
Gpx: glutathione peroxidase
Gy : gray units.
IL-1: interleukin 1.
PDGF: platelet derived growth factor.
PMF: post mitotic fibroblast
MF:mitotic fibroblast.
mRNA: messenger ribonucleic acid.
NFkB: nuclear factor kappa B
RIF: radiation induced fibrosis.
ROS: reactive oxygen species.
SOD: super oxide dismutase
TGF: transforming growth factor
TNF: tumor necrosis factor.

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Abstract

Radiotherapy, the use of X- rays and other ionization radiation to treat cancer, involves a deadly compromise. Bombarding a tumor with cell-killing beams inevitably affects the healthy tissue in the tumor's vicinity. One of the most common complications is radiogenic fibrosis, the hardening of tissue. Fibrosis in cancer patients receiving radiotherapy results from an overproduction of extracellular matrix (ECM). In fibrosis, tissues gradually lose their elasticity as ECM fills the space between cells. Over the recent years, studies have shown that the induction and progression of fibrosis could be caused by a radiation induced shift in the differentiation pattern of fibroblast / fibrocyte cell system. One of the key modulators of this cellular response is the cytokine TGF-β1 (transforming growth factor -β1), which triggers the fibrotic response through autocrine and paracrine mechanisms. TGF-β1 has been very well studied as a cell-signaling molecule for its role in wound healing. TGF-β1 links to fibrosis relates to its role in regulating the production and remodeling of the extracellular matrix. Fibrosis in some ways is like wound healing gone haywire, with TGF-β1 converting healthy tissue to tough tissue similar to that seen in scars. The particular attention to the gene expression of fibrogenic cytokines has shown some link to the role of oxidative stress in fibrogenesis. Studies have also shown that antioxidants have a protective role in radiation-induced injury like fibrosis. The goal of this review is to emphasize the role of free radicals and oxidative stress in causing fibrosis following radiation therapy and the possible interventional strategies to minimize the radiogenic fibrosis.

Introduction

Radiotherapy has become more firmly established as a curative-organ conserving therapy, used alone or in combination with surgery, as the main local treatment of cancer. The success of

radiotherapy (RT) has led to longer patient survival. Unfortunately, this carries with the penalty of providing a greater opportunity for late adverse effects to appear. Late complications can occur in the form of edema, nerve damage or fibrosis. Studies shows that the incidence and prevalence of fibrosis is more common when compared to other radiation induced morbidities [1]. Accidental overexposures to radiation can also lead to fibrosis. Fibrosis is a complex tissue response whose predominant characteristics are massive deposition of extracellular matrix and excessive fibroblast proliferation. It is a dynamic process that involves constant tissue remodeling and long-term fibroblast activation. It has been described in many tissues, including skin [2], lung [3], heart [4] and liver [5]. The dangers of fibrosis depend on the site of fibrosis. In the breast, it can cause loss of mobility; in lungs, it can limit breathing and the ability to cough up infectious material; in the kidney, it can restrict fluid flow and lead to infection.

The origin of fibroblast activation in fibrosis has now become a major issue in this field of research. In normal wound healing, fibroblasts are transiently activated into myofibroblasts to proliferate and deposit the collagen matrix. Fibrosis can be considered as a wound where continuous signals for tissue repair are emitted. These continuous signals can lead to abnormal production of cytokines and growth factors, resulting in chronic, sustained long-term myofibroblast activation leading to fibrosis. Among the various growth factors TGF-β1 is considered as a master switch for this fibrotic mechanism [6].

The evolution of radiation injury

Ionizing radiations are forms of energy that can induce tissue damage. The biological lesion is induced by discrete random depositions of radiation energy in discrete microscopic subcellular critical volumes. The radiation energy may be in the form of kinetic energy in the case of particle

radiation (electrons, neurons, protons, alpha particles, pi-mesons and stripped heavy nuclei) or as discrete energy units (referred to as a photon or quantum) as in case of X rays or gamma rays. Between the initial energy deposition and the final expression of biological damage (in the form of a specific clinical early or late radiation effect), there is an intervening series of events, each evolving into the other with a different time scale. The free radical events following radiation can be described as follows: Incident X-ray photon → fast electron (e^-) → ion radical → free radical → chemical changes from breakage of bonds → biological effects.

Pathogenesis of radiation induced fibrosis

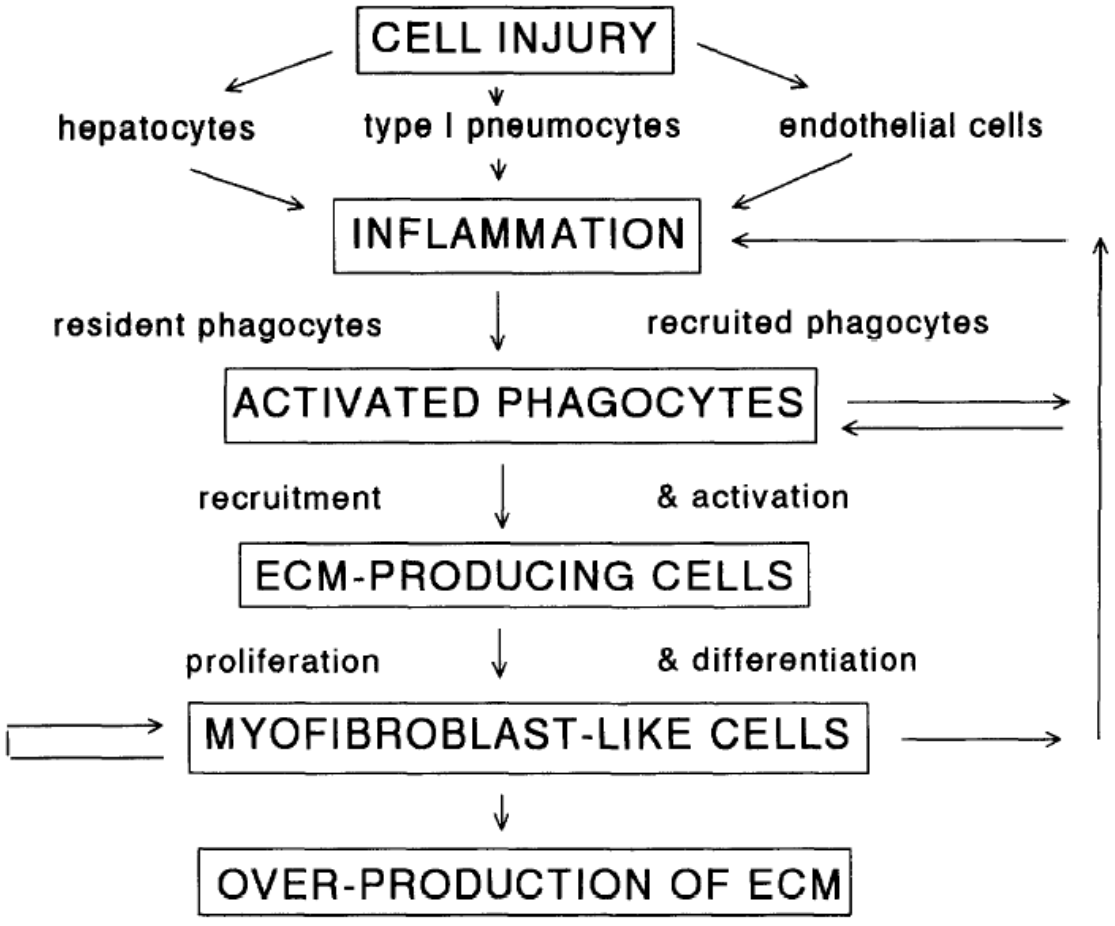

Figure 1. Shows the mechanisms of fibrosis common to liver fibrosis, lung fibrosis and vascular fibrosis [7].

The underlying mechanisms of the radiation-induced fibrosis still remain to be resolved. Figure 1. show the stereotyped mechanisms of fibrogenesis, independent of the initial site of injury [7]. It is of significance to note that in all these sites, an essential role is played by macrophages and by extra-cellular matrix producing cells (hepatic stellate cells in liver fibrosis and myofibroblast in pulmonary fibrosis). The inflammatory response evoked by the radiation injury results in a stimulative response of tissue cells like fibroblasts leading to an increased proliferation and differentiation as well as collagen secretion. We will try to understand how changes in proliferation and differentiation pattern of fibroblasts, the multicellular interactions of epithelial cells, endothelial cells, macrophages and fibroblasts, mediated by various fibrogenic cytokines following irradiation injury, will result in the overall fibrotic reaction. Recent studies to understand the fibroblast cell system of the connective tissue of skin, lung, kidney of various mammalian species has established the differentiation sequence resulting in functioning fibrocytes. On the basis of these investigations it can be concluded that in the fibroblast stem cell system three stem cells develop in the stem cell compartment along with the lineage S-1, S-2, S-3, three mitotically active progenitor fibroblasts differentiate along the sequence MF-1, MF-II, MF-III and three post-mitotic fibrocytes proceed in the fibroblast maturing compartment along the sequence PMF IV, PMF-V, PMF VI [8].

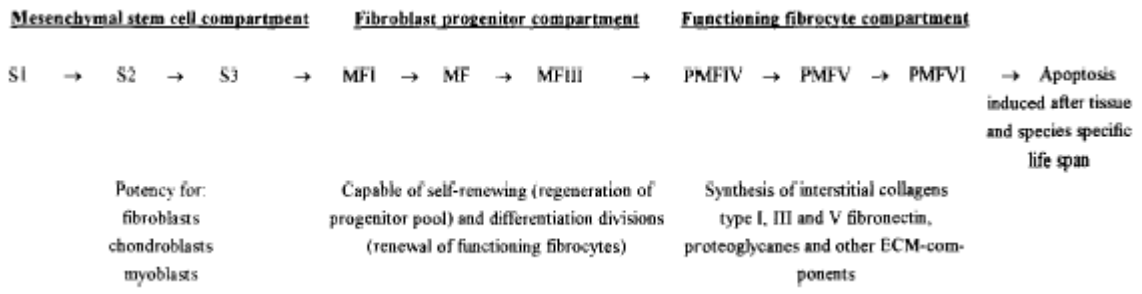

Figure 2. The mesenchymal stem cell system: differentiation sequence resulting in functioning fibrocytes [8].

The cell type PMF VI is the terminally differentiated end cell of the fibroblast stem cell system. PMF VI is characterized by a specific capacity for the synthesis of interstitial collagens types I, III, and V, proteoglycans and other extracellular matrix components [8]. Experimental results have shown that a cell type ratio of 2:1 between progenitor fibroblasts to post mitotic fibrocytes is essential for an ordered tissue function [8]. Disturbances in the differentiation processes of progenitor fibroblasts due to exogenous factors like radiation could be the basis for connective or interstitial tissue alterations seen in fibrosis.

Ionization radiation has been shown to induce premature terminal differentiation in the fibroblast / fibrocyte cell system, presumably by a significant depletion of mitotically active progenitor fibroblasts due to an induced transition into post mitotic fibrocytes. For the radiation induced post mitotic fibrocytes, it could be demonstrated that synthesis of interstitial collagens type I, III, and V is enhanced by a factor of 5 - 8 as compared to progenitor fibroblast populations [8]. These studies suggest that radiation is able to induce severe changes in tissue homeostasis resulting in fibrotic phenotype of various tissues.

Recent studies also suggest that radiation-induced fibrosis is a complex process involving with initiation and sustaining of fibrogenic process through an intercommunication of different cell types *via* the production of specific cytokines and growth factors. As described by various authors for different cell systems *in vivo* and *in vitro,* an immediate and transient expression of a number of proto oncogenes, eg., c-*fos* and c-*jun,* c-*myc* as well as growth factors, like platelet derived growth factor (PDGF), IL- 1, TGF-β and TNF-α occurs within hours of irradiation. This suggests that genetic expression of radiation injury probably occurs immediately, within hours and/or days as there are genetic events, which control and regulate the release of cytokines and receptors. These early modulation of gene expression may have profound effects on the

pathophysiology of the late radiation effect. This altered expression of growth factors and cytokines may result in the modulation of the cellular interactions of cell types involved in fibrotic reactions.

It can be assumed that the so called latent period of weeks or months before expression of radiation reactions can be replaced by appreciation of autocrine, paracrine and endocrine signals being synthesized immediately after the injurious exposure to radiation by a variety of cells, especially epithelial cells, endothelial cells, macrophages and fibroblasts. A perpetual cascade of cytokines has been identified post irradiation in lung tissues, beginning immediately at the time of irradiation and persisting up to the appearance of the late injury [9]. Identification of this continuing cytokine cascade and gene expression has shown that there is no latent period in a biological sense in the development of late radiation damage, although both clinically and using light microscopy, damage may not be seen in lethally irradiated tissue for weeks to months post irradiation.

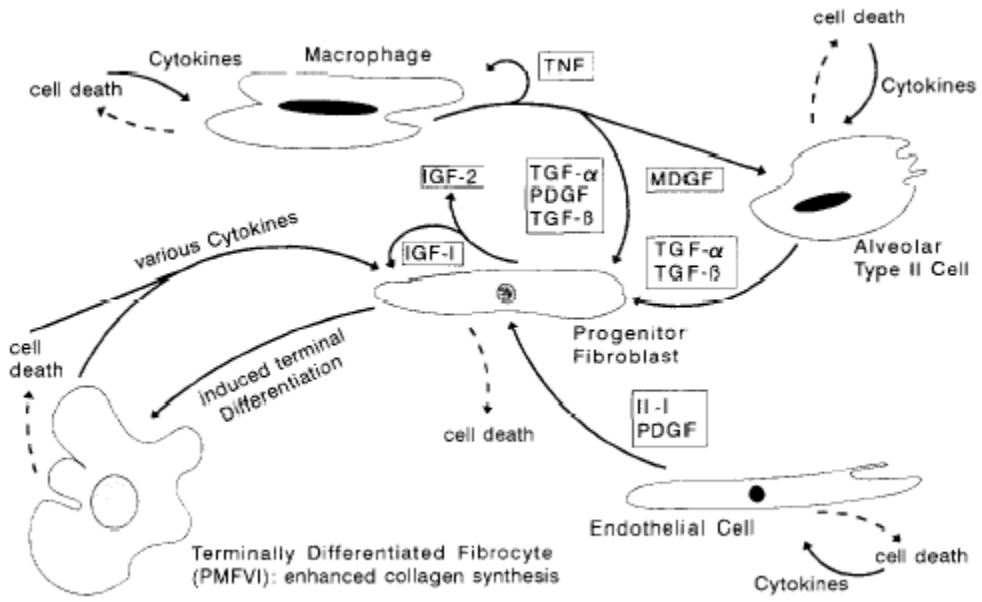

Figure 3 : shows the cell-cell interaction and control of gene expression by growth factors in lung injury [8].

The above schema (figure3) suggests that following irradiation injury of lung, alveolar macrophages and alveolar type II pneumocytes, maybe stimulated to produce altered amount of growth factors, notably interferon-α (INF-α), IL-1, PDGF, TGF-α, and TGF-β which then can alter the overall pattern of proliferation and differentiation and/or matrix gene expression of lung parenchymal fibroblasts [8].

A"afferent-efferent" intercellular radiation response paradigm of early molecular radiation responses has been presented to offer a possible explanation of late injury.

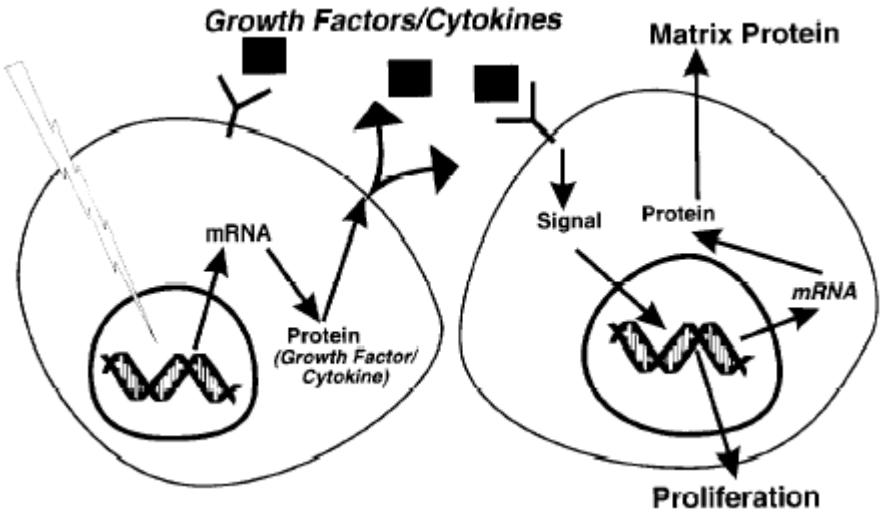

Figure 4. Shows the paracrine radiation effect. Afferent versus efferent cell cytokine response to explain the late injury following irradiation [9].

In the afferent limb (macrophage or type II pneumocyte), there is an immediate cellular damage following irradiation, which can result in either membrane and intracellular injury or DNA single and double strand breaks. The cellular injury leads to altered genetic expression or and the prompt release of growth factors such as TGFα and TGFβ. In the efferent limb or at the target cell (fibroblast) growth factor receptors are activated, leading to signal transduction. The stimulated fibroblast responds by turning on collagen genes, leading to the production of

extracellular matrix proteins [9]. It has been postulated that these early alterations, identified within days to weeks of the irradiation, persist and play a major role in the subsequent development of chronic fibrosis 5 to 6 months later. Complex scenarios of intercellular communication have been proposed. However, the exact mechanism of how these cells and cytokines interact has not been elucidated. Which cytokine play a dominant role in which process?

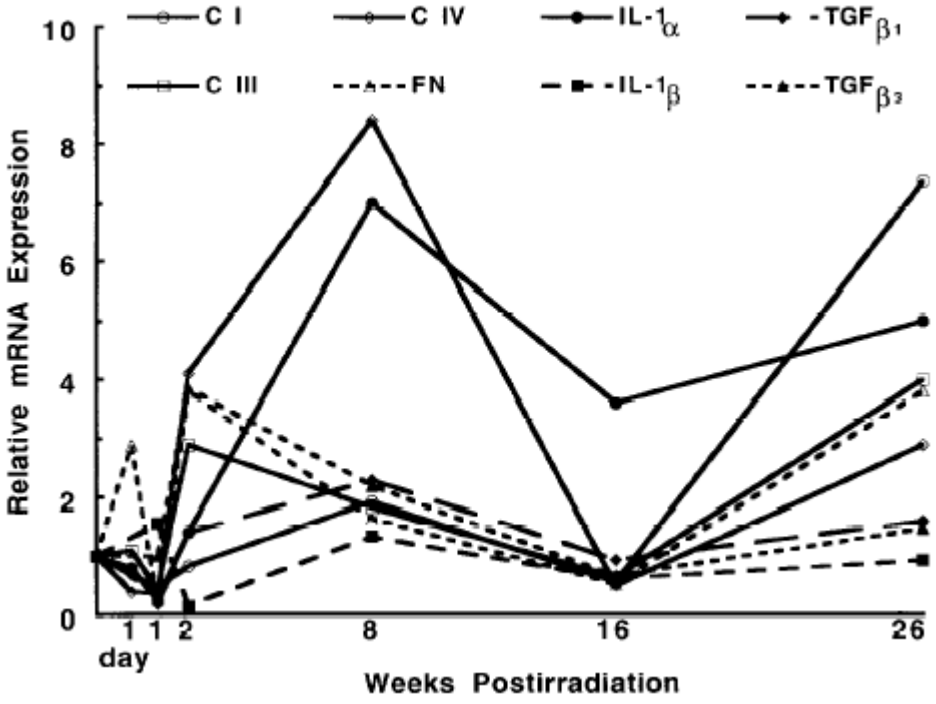

Figure 5 : (a) Shows the superimposition of cytokine and collagen gene expression following 12.5 Gy illustrating the cascade effect [9].

When all the cytokines and collagen gene expression were superimposed on each other, an apparent cascade was observed as shown in figure 5.

Pathological findings in irradiated tissues:

Extensive studies have been made to characterize the histopathologic and molecular changes following radiation injury. The maximum dose of radiation to tumor is often limited by the

radiation tolerance of normal tissues. A higher risk of radiation injury is expected for patients treated with combined radiation and chemotherapy, higher total dose, larger fraction size and larger volume of irradiated tissue. The pathology of radiation induced injury of skin, lung, heart and liver has been studied. Distinct histopathological changes have been seen in post-irradiated lung tissues, distinguished by difference in time of expression after irradiation. The hours to days preceding the overt appearance of radiation pneumonitis are referred to as the "latent period" as no overt histopathologic, radiographic or clinical signs and symptoms of radiation damage can be observed. But recent electron microscopic studies have revealed ultra structural injuries involving endoplasmic reticulum, mitochondria and plasma membranes of type I and type II pneumocytes and endothelial cells during this "latent period" [10]. Radiation pneumonitis occurs 2 weeks upto several months after irradiation and is characterized by edema of the interstitium and exudation into air spaces, infiltration of inflammatory cells, alterations of capillaries, and thickening of the alveolar septa [10]. This diffuse alveolar damage is followed by late lung injury, characterized by progressive fibrosis of alveolar septa, which eventually causes widespread obliteration of the residual alveoli. Thus lung fibrosis was explained as a repair process that follows the classic radiation pneumonitis.

Studies involving the immunohistochemical staining of TGF-β in irradiated lung tissue demonstrates an acute and long lasting increase in the expression of TGF-β in lung tissue following irradiation. The immunohistochemicl studies done by Rube *et al* [10] gives a good insight to the significance of TGF-β as well as the histopathological changes associated following irradiation of lung tissue. Figure 6 shows the increased immunoreactivity in the lung parenchyma during the first hours following irradiation. This is largely due to the increased

number of staining alveolar macrophages, which is suggestive of an ongoing process that is initiated shortly after irradiation.

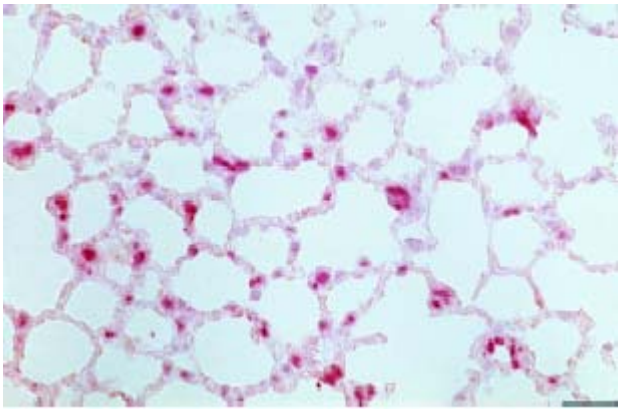

Figure 6: Immunohistochemical staining of TGF-β in irradiated lung tissue (12 Gy), 1 hour after irradiation. Staining related to alveolar macrophages and type II pneumocytes.Magnification x 250 [10].

The most striking increase in TGF-β immunoreactivity was seen at the beginning of the pneumonic phase (2 and 4 weeks postirradiation). Microscopic examination of the lungs revealed tissue inflammation with accumulation of inflammatory cells in the alveolar spaces and in the interstitium, particularly in perivascular and peribronchial areas (figure 7) but extending widely in the parenchyma. At the peak of TGF-β expression (2 and 4 weeks post irradiation), inflammatory cells constituted the majority of cells expressing TGF-β.

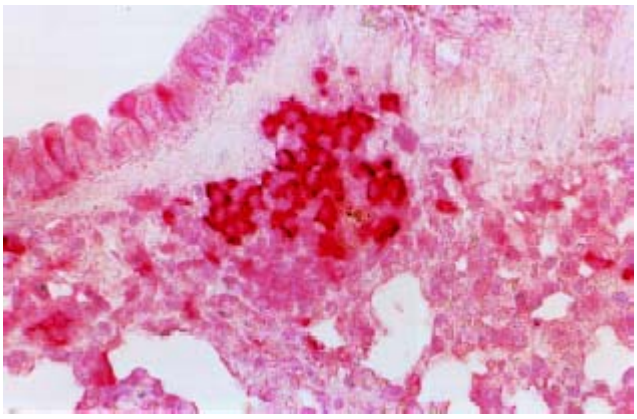

Figure 7: Immunohistochemical staining of TGF-β in irradiated lung tissue (12 Gy), 4 weeks after irradiation. Distinctive patch of positive pulmonary macrophages in peribronchial region. Magnification x 250 [10].

With advanced time, the fraction of positive staining type II pneumocytes is increased. During the later time points (8,16 and 24 weeks post irradiation) type II pneumocytes and fibroblasts served as an important source of TGF-β expression. TGF-β is a potent chemoattractant to

fibroblasts and triggers the expression of extracellular matrix components in pulmonary fibroblasts. This studies show that there is predominant localization of TGF-β in areas of inflammatory cell infiltrates and fibrosis suggests the involvement of this cytokine in the pathogenesis of consequential radiation induced fibrosis.

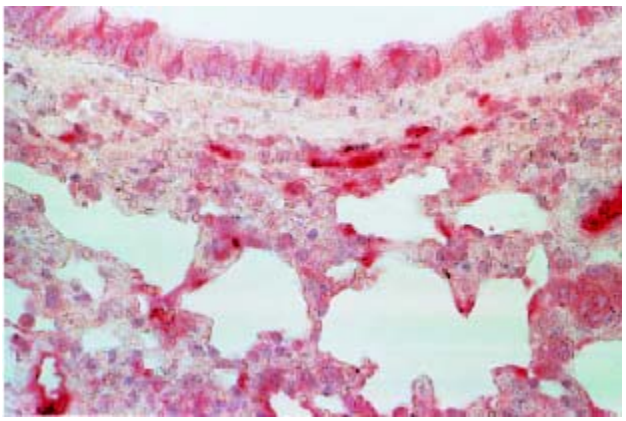

Figure 8 .Immunohistochemical staining of TGF-β in irradiated lung tissue (12 Gy), 24 weeks after irradiation. Distinctive positive fibroblasts in peribronchial region. Magnification x 250 [10].

Radiobiological effects of ionization radiation

Absorption of electromagnetic radiation by tissue is a function of the electronic excitability of its constituent molecules and it can lead to excitation or ionization of the biological material. Excitation is the phenomenon when photons or relatively low energy, such as those in visible or ultra violet frequency, are able to interact with electrons of organic molecules, and promote it to a higher energy level without the actual ejection of electrons. If the radiation has sufficient energy, such as γ-rays, to eject one or more orbital electrons from the atom or molecules, the process is called ionization. The important characterization of ionizing radiation is the localized release of large amounts of energy (33 eV / per ionizing event). The energy associated with a C=C bond is 4.9 eV [11]. So, this energy is more than enough to break a strong chemical bond. The biological effects of radiation result principally from damage of DNA, which is the critical target. When any form of radiation is absorbed in biological material, there is a possibility that it

will interact with directly with the critical targets in cells. The atoms of the target may itself be ionized or excited, consequently initiate the chain of events that leads to a biological change. This is called the direct action of radiation. The radiation may act with other atoms or molecules in the cells (particularly water) to produce free radicals that are able to diffuse far enough to reach and damage the critical targets. This is called the indirect action of radiation. The biological implications of the radicals generated are now considered important in causing the oxidative damage and hence fibrosis.

Factors influencing the magnitude of radiation induced injury

A higher risk of radiation injury is expected for patients treated with higher total dose, combined radiation and chemotherapy, larger fraction size and larger volume of irradiated tissue.

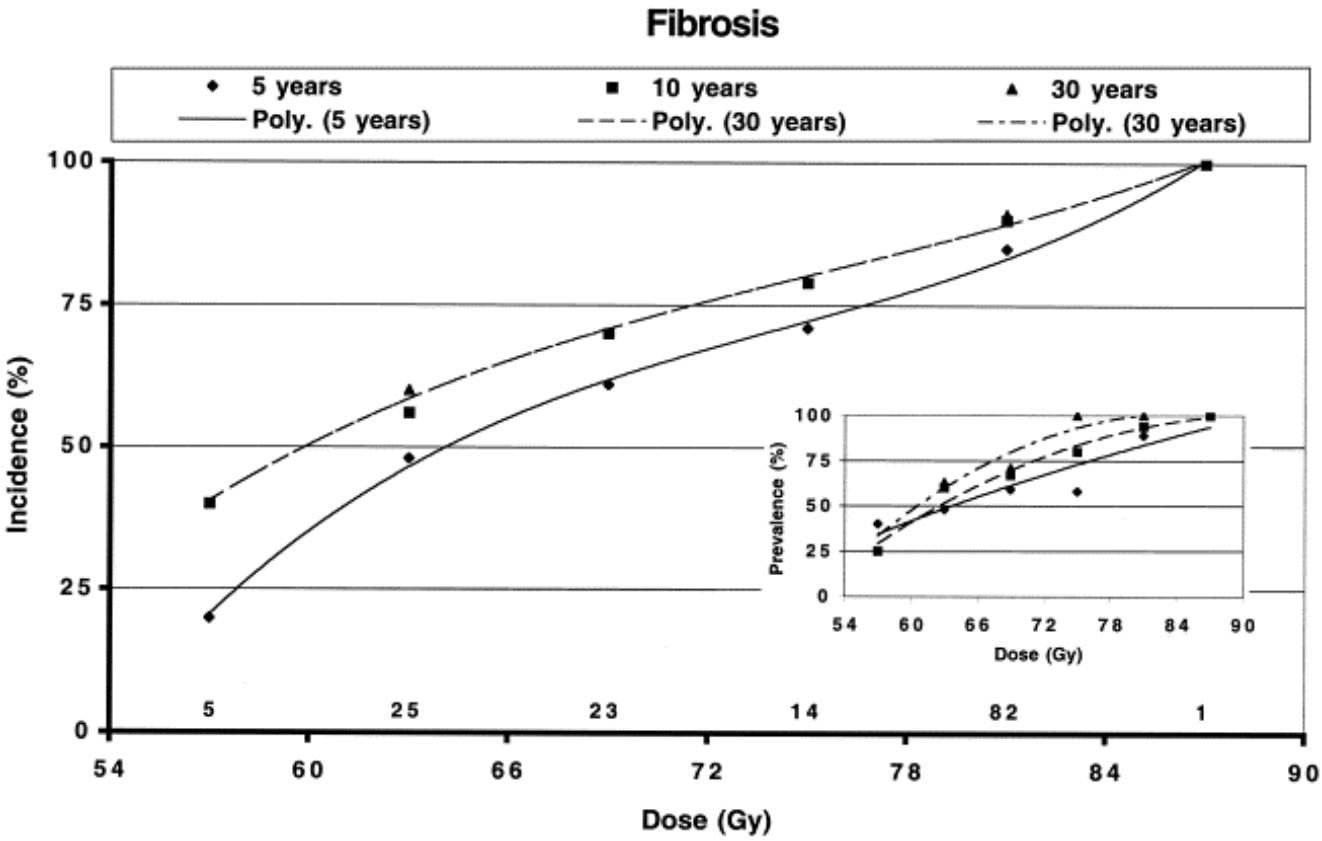

Figure 9. Dose response curve for fibrosis at 5, 10 and 30 years after radiation therapy. Doses expressed as 2-Gy fractions received by the brachial plexus. Patients are grouped in 6- Gy intervals. Numbers of patients within different dose windows are given. Prevalence (for comparison with incidence) of damage at 5, 10, and 30 years for the patients alive in different dose windows (Inset) [1].

Total dose of irradiation absorbed is an important factor contributing to radiation injury. Total radiotherapy dose has shown to be well correlating with late clinical changes [1]. Figure 9 shows data adopted from a retrospective study analyzing the records from a group of 150 patients with breast cancer treated with radiotherapy shows a clear dose dependence of fibrosis.

From the plot of the dose response curve (figure 9) it reveals that the dose dependence of fibrosis is apparent in all dose groups (shown in the figure 9) at 5 years but increases from 20 %(1 of 5) in the lowest dose group to 85 % (70 of 82) in the next highest dose group [12]. Prior irradiation to the tissue, is another risk factor that will enhance the toxicity of radiation injury.

Many chemotherapeutic agents may potentiate the damaging effects of radiation. The best-characterized drug enhancing radiation injury is bleomycin. The action and toxicity of these chemotherapeutics agents are very much linked to the production of ROS and redox cycling. So ROS may play a synergistic effect during the combined radiotherapy and chemotherapy regimens to potentiate the damaging effects. The fact that radiation and chemotherapy produce similar cytokine responses suggest that common molecular mechanisms may be the basis for enhanced late effect injury due to combined modality approaches.

Free radical production and oxidative stress:

Free radicals are chemical entities with one or more unpaired electrons and are usually formed by homolytic fission of some chemical bond from a proper precursor molecule. The selectivity of the respective bond splitting depends on the bond dissociation energy and on the source of energy. The unpaired electron state is associated with a high degree of chemical reactivity.

We know that 80% of the cell is composed of water molecules. These water molecules will get ionized, when it interacts with radiation energy induced by photons, X-rays, γ-rays or charged particle, such as electron or proton. The effects of radiation result from energy deposition in the irradiated material. The free radicals formed as a result of direct damage to the cellular molecules by ionization

$$2\,H_2O \rightarrow H_2O^{\bullet +} + e^- + H_2O^* \qquad (1)$$

where e^- represents an electron and H_2O^* , an excited state water molecule. Such excited molecules undergo hemolytic fission tin 10^{-14} to 10^{-13} seconds to give hydrogen atoms ($H^\bullet$) and hydroxyl radicals ($OH^\bullet$).

$$H_2O^* \rightarrow H^\bullet + OH^\bullet \qquad (2)$$

Within the same time scale $H_2O^{\bullet +}$ also reacts to form $OH^\bullet$

$$H_2O^{\bullet +} + H_2O \rightarrow H_3O^+ + OH^\bullet \qquad (3)$$

$$e^- + nH_2O \rightarrow e_{aq}^- \qquad (4)$$

The electrons become surrounded by clusters of water molecules and form hydrated electrons (e_{aq}^-).

$$H^\bullet + OH^\bullet \rightarrow H_2O \qquad (5)$$

$$e_{aq}^- + OH^\bullet \rightarrow OH^- \qquad (6)$$

$$e_{aq}^- + e_{aq}^- + 2\,H_2O \rightarrow H_2 + 2OH^- \qquad (7)$$

$$H^\bullet + H^\bullet \rightarrow H_2 \qquad (8)$$

$$OH^\bullet + OH^\bullet \rightarrow H_2O_2 \qquad (9)$$

In the presence of oxygen, superoxide radicals are formed.

$$e_{aq}^- + O_2 \rightarrow O_2^{\bullet -} \qquad (10)$$

$$H^{\bullet} + O_2 \rightarrow O_2^{\bullet -} + H^+ \quad (11)$$

The superoxide produced can form hydrogen peroxide as a secondary product by dismutation on a longer scale depending on pH.

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (12)$$

It is well known that, by the interaction of ionization radiation with living tissue, the production of free radicals plays a primary role in initiating the biological damage and the ensuing secondary inflammatory response. These radicals are responsible for a variety of adverse biological effects resulting in cell death and direct degradation of ECM.

ROS generation and fibrogenesis:

The increased steady state level of reactive oxygen species and how this will interfere with the activity of cells involved in inflammation and fibrosis has generated a wide interest. The tissue damage induces directional migration of PMNL (neutrophils) and macrophages into the extravascular parenchymal tissue and initiates fibroblast recruitment and proliferation. Extravascular neutrophils and macrophages are stimulated by contact with degraded collagen to release free radicals and proteases. This second wave of free radicals might play a central role in the formation and extention of fibrotic tissue in a self-maintained cycle.

A key pathological role for super oxide has been suggested in the development of radiation damage based on the observation that SOD inhibits radiation induced damages in a number of biological end points, including enzyme activity, membrane integrity, DNA damage, cell transformation and cell and organism survival. It is of interest to note that extracellular matrix is rich in dermatan sulaphte , hyaluronic acid and heparan sulfate. This is important as SOD preferentially binds to heparin like proteoglycans located in the endothelial cell surface and in the extracellular matrix. So supplying SOD supplements could directly or indirectly enhance the

cells natural antioxidant defenses and reduce oxidative stress at cellular and tissue levels and inhibit the process of fibrogenesis.

Studies show that ROS such as superoxide anion and hydrogen peroxide as well as other free radicals, pro-oxidants, and aldehyde end products of lipid peroxidation can modulate cell proliferation, possibly by operating along cell signaling pathways known to be activated by "conventional" growth factors [12]. Interestingly, most of these studies have been done on different lines of cultured fibroblast. The most important aspect of these studies is the modulation of gene expression by oxidant species. The fundamental observations were (1). Oxidative stress modulates the expression of genes encoding for inflammatory cytokines at the transcriptional level. (2). Lipid peroxidation up-regulates the synthesis of fibrogenic cytokines. (3) aldehyde end products of lipid peroxidation enhance type 1 collagen synthesis by fibroblasts.

It is of great interest to observe that transcription factors are sensitive to even modest variations of the intracellular oxido-reductive balance. In animal cells, ROS have been shown to induce the transcription of two families of transcription factors; nuclear factor kB (NF-kB) and activator protein1 (AP-1) [12]. The oxidation dependant activation of NF-kB and AP-1 leads to transcriptional upregulation, including genes encoding for inflammatory and/or fibrogenic cytokines. NF-kB binding sites are in the promoter region of GM-CSF, TNF β, IL-6 and growth factors relevant to inflammation, where as the gene activation of TGF β1, the most fibrogenic cytokine together with PDGF, occurs through binding to the AP-1 site present on its long terminal repeat [13].

TGF-β and the development of radiation fibrosis

TGF-β is a multifunctional cytokine that plays an important role in embryonal development, in immune responses and in regulating repair and regeneration following tissue injury [14].

The three mammalian isoforms TGF-β1, TGF-β2, TGF-β3 are structurally and functionally closely related to one another. The main biological function of TGF-β is in the regulation and general inhibition of cell growth, regulation of the deposition of extracellular matrix compounds, and some immunosuppressive activities.

Although the main effect *in vitro* of TGF-β on cell proliferation is inhibition, TGF-β1 can promote the proliferation of cultured mesenchymal cells such as fibroblasts and osteoblasts. The role of TGF-β to control the homeostasis of the extracellular matrix is crucial in fibrotic mechanisms. TGF-β1 causes the remodeling of extracellular matrix by simultaneously stimulating cells to increase the synthesis of most matrix proteins, decrease the synthesis of matrix degradation-proteases, increase the production of inhibitors of these proteases, and modulate the expression of integrins [15].

Experimental studies have shown an increasing causal relation between elevated production of TGF-β and tissue fibrosis. First, *in vivo* administration of TGF-β in healthy animals produced tissue fibrosis [16]. In newborn mice, subcutaneous injection of TGF-β caused the formation of granulation tissue within 2-3 days at dose levels of less than 1μgm [16] and in rats, intravenous injections of TGF-β for 2 weeks produced kidney and liver fibrosis [17]. In nude mice, generalized tissue fibrosis developed when TGF-β1 was given intraperitoneally at doses exceeding 2 μgm/day for 10 days [18]. The second line of evidence is by the generation of transgenic mice that TGF-β1 in specific organs brought definitive demonstration of the causative role of TGF-β in tissue fibrosis. Thus, transgenic mice that overexpressed TGF-β1 in the liver developed hepatic fibrosis as well as extrahepatic pathologies such as renal fibrosis, probably due to an increased level of circulating TGF-β in plasma [19]. In another transgenic model, a constitutively active human TGF-β1 gene was targeted to the liver, kidney and adipose tissue.

These mice developed severe fibrotic disease in these tissues and a severe reduction in body fat [20]. From these studies it shows that TGF-β1 is a key player in fibrosis.

Studies on pig models done by Martin *et al* suggested that TGF-β was detected in skin at 6 hours after γ -irradiation at both the protein and messenger RNA levels, for single doses of radiation ranging from 16 to 64 Gy [21]. Daburon and coworkers have shown the TGF-β1 was overexpressed in pig skin model of fibrosis through out all the phases of fibrosis development [21].

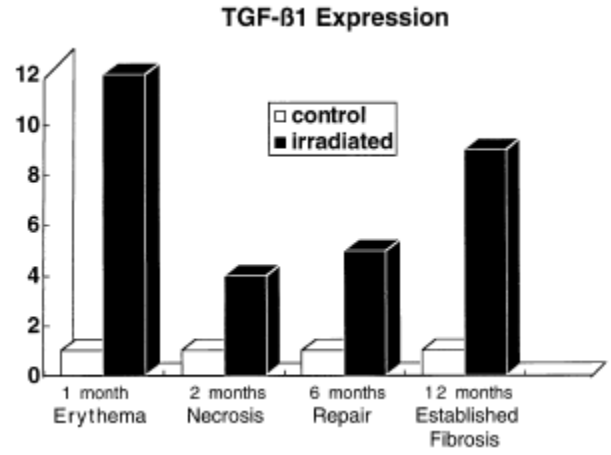

Figure 10: TGF-β1 mRNA was overexpressed at all stages of fibrosis development. Pig skin was removed at various times after irradiation. Five micrograms of mRNA was hybridized with the radiolabeled TGF-β1 probe. The quantification of the Northern blots is shown as TGF-β1 values corrected with the 18S values [21].

The TGF-β1 mRNA level was increased 12 fold in the irradiated skin during the early erythematous phase, which started 3 weeks after irradiation. During the later phases of fibrosis, from 6 to 12 months after irradiation, it remain 10 fold elevated over the basal level in the repaired skin [21].

Immunofluorescence staining of sections of irradiated skin revealed that fibroblasts, endothelial cells and epidermal cells secreted the TGF protein [21]. Further the study also shows that fos and jun proteins can regulate the TGF-β1 promoter region through the AP-1binding sites [21].

When comparing the general functions of TGF-β1 and its role in fibrosis, several points fit in. Its bimodal action on cell proliferation, with inhibition of epithelial cells and activation of

fibroblasts, can certainly favor fibrosis and scar development. Similiarly, the capacity of TGF-β to induce apoptosis in specific cell types can favor parenchymal damage and replacement by a fibrous tissue.

Although TGF-β is certainly a key cytokine, the fibrotic process cannot be explained by a single factor. It involves a complex network of interacting cytokines and growth factors, which include PDGF, IL-1, Insulin like growth factor-1 (IGF-1) and TNF-α.

Treatment and possible preventive strategies for radiation induced fibrosis.

Drugs of several categories have been tried in the management of fibroproliferative disorders, including anti-inflammatory agents (steroids, colchicines, D-penicillamine), drugs acting on blood flow (heparin, pentoxyphylline), and interferons. Corticosteroids are still the first line therapeutics. Understanding that TGF-β1 is a key factor in fibrogenesis offers a new possible target for developing therapeutic agents with potential antifibrotic effects. Biological modifiers targeting oxidative damage for radioprotection have been studies for many years. Since the discovery of SOD it has become clear that these enzymes provide an essential defense against the superoxide radical. It is believed that SOD may protect cells from radiation damage by removing free radicals produced by irradiation. In the presence of SOD, superoxide is dismutated to H_2O_2 and O_2. H_2O_2 is then subsequently eliminated by catalase and glutathione peroxidase to form water and oxygen [22].

$$O_2^{\bullet-} + O_2^{\bullet-} \rightarrow O_2 + H_2O_2 \qquad (13)$$

$$2\ H_2O_2 \rightarrow O_2 + 2\ H_2O \qquad (14)$$

$$ROOH + 2\ GSH \rightarrow ROH + H_2O + GSSG. \qquad (15)$$

The reactions 13, 14 and 15 are catalyzed by super oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) respectively.

The data obtained from studies done by Vujaskovic *et al* (2002) shows that the novel SOD mimetic(AEOL 10113) may be protective against radiation induced lung injury [23]. Recent investigation of SOD for radiation induced normal tissue injury have included a decrease in fibrosis by intramuscular injection of liposomal Cu / Zn –SOD in animal model and human clinical trial [23].

The development of standardized pig model of RIF provided an excellent tool to show the effects of SOD in fibrosis. The almost 75% decrease in the cutaneous surface area and volume of the fibrotic block were highly significant compared to the clinical and autopsy groups for the control animals are striking [24]. Another series of studies have demonstrated protection of lung and esophagus from radiation injury by Mn-SOD plasmid / liposome and Mn-SOD adenovirus gene therapy [25]. The possible mechanism of action could be a SOD induced downregulation of TGF-β secretion by myofibroblasts.

Future directions:

Radiation induced fibrosis is a common sequela to both accidental irradiation and cancer treatment by radiation therapy. Ionization radiations produce free radicals that cause the biological damage processes. These free radicals can modulate the gene expression of various cytokines like TGF-β and initiate fibrogenesis. If the radiation-induced fibrosis is mainly due to free radical reactions, then an increase in antioxidant defense should retard the radiation induced fibrogenic processes. Therefore further studies can be focused on understanding the mechanisms of antioxidant enzymes like SOD, CAT, GPx and many others in radiation-induced fibrosis.

The working paradigm for this proposal is that the premature terminal differentiation in the fibroblast plays a major role in the radiation induced fibrosis. A key event leading to fibrosis is the change in the fibroblast / fibrocyte phenotype. This premature terminal differentiation to fibrocyte leads to chronic upregulation of interstitial collagens type I, III, and V and ECM production. If the free radical processes is involved in initiating this premature termination, then antioxidant enzymes should decrease or delay the fibrotic processes.

The working hypothesis of this proposal is that in the presence of increased antioxidant enzymes, the production of radiation induced reactive oxygen species as well as the fibrogenic transforming growth factor (TGF-β) levels will be decreased. This can act in autocrine and paracrine manner to decrease the production of extracellular matrix gene products. I hypothesize that by using methods to increase or decrease the severity of the functional and morphological alternations seen in radiation fibrosis will be associated with a concomitant upregulation or downregulation of TGF-β and extracellular matrix gene products.

I will test this hypothesis by using the following specific aims:

A. *In vitro* studies.

Using adenoviral vectors containing appropriate antioxidants to transfect the *in vitro* cells. The increase or decrease in messenger RNA and /or protein levels of TGF-β and ECM gene products in transfected as well as non transfected cells, following irradiation will be studies. If the free radical mechanism is involved in the fibrogenic process, the expression of TGF-β as well as the ECM gene products will be low.

B. *In vivo* studies.

1). Using transgenic mice model of SOD to test the hypothesis that the increase of SOD in lungs can cause tolerance to radiation damage. After irradiating the mice, I will determine the increase or decreased survival, and the histopatholgical changes favouring increased or decreased fibrotic changes. Also the immunohistochemical staining will be done to see the TGF-β expression.

2). Using a gene knock out mice model of SOD, to test the hypothesis whether the decreased SOD levels in lung tissue can cause more exaggerated radiation induced fibrosis. After irradiation the increased or decreased survival of the SOD knock out mice will be determined. Also the increased or decreased morphological evidence of lung fibrosis will be determined.

Summary.

Radiation injury can be seen as a consequence of cascade of cytokine activities that ultimately begin with oxidative stress from radiolytic hydrolysis and formation of reactive oxygen species. Among the cytokines, TGF-β plays a key role. Responses of tissue to radiation is not only initiated by the production of radiation induced ROS during the primary ionizing events, but also may be sustained by the continuous production of ROS and their involvement in complex signaling and cytokine induction / activation. Based on the recent findings in molecular radiobiology and cellular biology, fibrosis can be seen as a multicellular process involving various interacting cell systems in the target organs resulting in the fibrotic phenotype of the fibroblast / fibrocyte cell system.

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