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Review Article

The Pathway from Radiation to Fibrosis: LET Dependence

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ABSTRACT

It is well-known that Radiation-induced fibrosis (RIF) is a late event occurring months to years after the initial radiation exposure. Fibrotic lesions have been shown to manifest in many tissues including the skin, heart, lung, liver and kidney. Fibrosis occurs due to abnormal accumulation of extracellular matrix (ECM) proteins that result in loss of normal tissue and organ function. The cell type involved in RIF is myofibroblasts, which do not undergo apoptosis after healing but instead continue to accumulate, producing excessive amounts of ECM proteins, thereby damaging the tissues and organs. Reactive oxygen species, generated in response to radiation, is one signal that helps maintain the myofibroblast phenotype. In this review, we discuss molecular mechanisms leading to this late radiation event, known biomarkers for prediction, preclinical animal models of radiation-induced toxicity and current clinical trials designed for mitigation and treatment of radiation-induced fibrosis. We also discuss other physical properties such as linear energy transfer (LET) than the ones used in the clinics today which may have the potential to change our understanding on this inevitable pathway from radiation treatment to organ fibrosis.

Introduction

Radiation-induced Fibrosis (RIF) develops several months or years following radiotherapy [1-3]. Also, RIF occurs due to differentiation of normal fibroblasts into myofibroblasts, which in turn deposit aberrant amounts of extracellular matrix protein instead of undergoing apoptosis. Persistence of active myofibroblasts occurs through autocrine and paracrine signals and influx of inflammatory cells [4, 5]. In the context of radiation exposure, a major trigger towards development of RIF is reactive oxygen species (ROS), generated immediately after radiation exposure. Radiation-induced bystander effects (RIBE), where unirradiated cells exhibit effects of irradiated cells due to signals received from nearby irradiated cells, contribute to amplifying the ROS levels [6-10]. Generation of ROS is believed to induce epigenetic changes that cause differentiation of fibroblasts to myofibroblasts [11, 12]. Ionizing radiation sources used in cancer therapy include gamma rays and X-rays, which possess sufficient energy to displace electrons from atoms. When these energy waves interact with water molecules, it leads to excitation and ionization of water molecules to form free radicals and ROS. Generation of ROS takes place in three cellular compartments: mitochondria, endoplasmic reticulum (ER) and cytosol. The ROS generating processes at all three locations, mutually affects each other while also being influenced by the exogenous ROS, thereby amplifying the production of ROS from initial levels. Maintenance of homeostasis is achieved by biochemical mechanisms in place in the normal physiological setting, to counteract the damaging effects of free radical damage by ROS. These include action of enzymes such as super oxide dismutase (SOD) and DNA methyltransferases, p53 interplay and its regulation to restore the cell to its normal redox state. Ionizing radiation induced oxidative stress depends on radiation dose, dose rate, and linear energy transfer (LET) [13]. High LET radiation also causes persistent ROS, DNA damage, genomic instability in long term effect. Failure to maintain the ROS levels at physiological concentrations help maintain the myofibroblasts phenotype leading to radiation-induced fibrosis. While RIF is an unnecessary complication that occurs months to years following radiation, advances in understanding the molecular mechanisms have identified biomarkers that can predict patients at increased or decreased risk of treatment-related injury, with a goal toward improving the therapeutic ratio in order to enable physicians to optimize & individualize therapy for patients.

The TGF-β cytokine that stimulates fibroblast proliferation while upregulating ECM production has now been identified as a serum marker

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of developing RIF [14, 15]. Studies from pre-clinical animal models indicate Pravastatin to play a role in inhibition of rho kinase, the modulator of the Rho/ CCN2/extracellular matrix cascade and thereby improving radiation-induced fibrosis [16-19]. Clinical trials with Pravastatin have shown some reversal in radiation-induced fibrosis [20]. Charged particle radiation such as proton and helium, and heavy ion carbon radiotherapy with its unique properties to minimize scatter as particulate beams pass through the tissue, depositing energy at precise depths shows promise for reducing the molecular events leading to late radiation-induced fibrotic tissue damage. In this review, we summarize and discuss this pathway from initial radiotherapy to the tissue fibrotic endpoint with an aim to shed light into the importance of careful planning and evaluation on an individualized basis in order to reduce unwanted complications of radiation with severe consequences.

I Molecular Signaling in Radiation-Induced Fibrosis

i TGF-β Signaling Through SMAD Proteins

Transforming growth factor-β has a central role in the radiation-induced pathway to fibrosis [21]. Upon activation, either by irradiation *via* reactive oxygen species or other mechanisms, signaling is initiated by ligand binding to Type I and Type II TGF-β receptor complexes on the cell surface, inducing trans-phosphorylation of the glycine and serine residue-rich region (GS) in type I receptor by type II receptor kinases [21, 22-24]. The activated type I receptors phosphorylate SMADs 2 and 3 at the C terminal of the receptor. SMAD 2 and SMAD3 form a complex with a common SMAD 4. The activated SMAD trimer then enters the nucleus to activate gene transcription and promote cell growth and survival.

ii Smad3 Predominance in Radiation Induced Fibrosis

Given the predominant role of Smad3 in controlling downstream gene expression the TGF-β/Smad3 pathway is implicated in specific TGF-β associated pathologic fibrosis, since it controls the synthesis of proteins of the extracellular matrix such as Collagens 1, 5 and 6 and its role in epithelial to mesenchymal cell transition [21, 25, 26, 27,28]. Clinical data shows enhanced expression of TGF-β years after initial exposure [29].

iii Role of NF-κB in Radiation-Induced Response

NF-κB is a transcription factor that has an active role in the radiation-induced adaptive response [30]. The onset of radiation activates a group of NF-κB regulated cytokines including TNF-α, which contributes to enhanced sensitivity of a cell to radiation [31, 32]. TNF-α activates NF-κB via receptor activation and regulates the expression of several genes involved in the immune and inflammatory response TNF-α activated protein kinases activate I κB kinase and c-jun N-terminal kinase,that are involved in the NF-κB mediated radiation response [33-39].

iv Reactive Oxygen Species (ROS)

Radiation interacts with molecules of water in cells, causing them to ionize and produce ROS which includes superoxide (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), all of which result in progressive damage to cells and consequently cell death [40]. The majority of radiation-induced cell injury is a consequence of the hydroxyl (OH^-) radical [41, 42]. Increased production of ROS lead to toxicity of cells of parenchymal origin, which initiate a cascade, altering the mileu of cytokines in the microenvironment, leading to peroxidation of lipids, oxidation of DNA and protein and activation of proinflammatory cytokines both *in vitro* and *in vivo* [43-46]. Lipid peroxidation products activate fibrogenic cytokines that function as chemoattractants, mitogens and smooth muscle cell differentiation inducers in the blood vessel wall [47]. A summary of effectors of fibrosis and the biochemical mechanisms involved are illustrated in (Table 1).

Table 1: Modulators of radiation-induced fibrosis.

Modulator	Biochemical signaling pathway	Fibrotic manifestation
TGFβ	Smad dependent pathways	a SMA collagen
Cytokines	Smad, NFκB	EMT profibrotic responses
TNFα	NFκB	profibrotic responses
ROS/RNS	DNA oxidation, secretion of MMPs	ECM

II Effects of High LET and low LET Radiation on Fibrosis

High LET radiation is known to result in lower survival of the cell per absorbed dose, as compared to low LET radiation. Pro-fibrotic gene, plasminogen activator inhibitor I (PAI-1) is involved in radiation-induced tissue remodeling via p53, TGF-β and Smad pathway. Although high LET radiation induced more apoptosis than low LET induction of the pro-fibrotic gene, plasminogen activator inhibitor I (PAI-1) was similar with high LET and low LET [48]. Since expression of PAI-1 is regulated by p53, specifically by phosphorylation of the latter at serine 315, a correlation of phosphorylation at serine 315 of p53 and levels of PAI-1 protein expression were observed on Western blot and luciferase functional assays with high and low LET [48]. Functional assays did not show a correlation between phosphorylation of p53 at serine 37 and PAI-1 induction. There is a direct correlation between Serine 37 phosphorylation and apoptosis however, with higher apoptosis observed after high LET radiation [48]. Since overexpression of post-mitotic fibroblasts is the hallmark cellular phenomenon leading to fibrosis, another group of investigators studied the differentiation pattern of human fibroblasts along with quantifying the production of extracellular matrix components after induction of high and low LET radiation. Similar differentiation was demonstrated for 195MeVu $^{-1}$carbon ions with low LET compared with X-rays. Low energy carbon ions were observed to be more efficient than X-rays, versus nickel ions, which showed a lesser effect on induction of fibroblast differentiation [49]. For ECM protein production, a similar pattern of LET dependence was observed as was seen for differentiation [49].

III Dose Effects

A variety of factors affect radiation-induced fibrosis. Among these, total dose and dose per fraction, treated volume, and time course of radiation delivery are the primary factors known to have a significant impact on development of fibrosis in treated tissue [50-54]. However, the NTD_{50}

values vary among different studies depending on variables besides radiation, and the effect of dose rate on the development of fibrosis may become arguable [50]. The dose effect also depends on radiation quality. Carbon ion radiation induces persistent lung injury and inflammation in a mouse model at high doses (over 2 Gy) [55].

IV Biomarkers for Prediction of RIF

i Biomarkers in Blood

TGF-β1 levels in the serum serve as an early biomarker for development of moderate to severe RIF after IR [56-58]. Since, radiation induced damage is the consequence of an early activation of an inflammatory response leading to significant expression in the bloodstream of a cascade of cytokines besides TGF-β1, such as IL-6, KL-6, surfactant proteins and IL-1ra, the latter have potential to be used clinically as early response markers for radiation induced damage [59-61].

ii Genetic Biomarkers

Studies of molecular mechanisms of radiation sensitivity show associations between common variants in DNA damage and repair genes and development of adverse reactions to radiotherapy. Polymorphisms in the *XRCC1, ATM, hHR21 and TGFβ1* correlate to an increased risk of developing an adverse tissue reaction to radiotherapy, whilst one variant of the *ATM* gene has been reported to be radioprotective [62-70].

V Preclinical Animal Models for Treatment of RIF

A TGF-β/Smad Pathway Inhibitors

Since the TGF-β/Smad pathway is a major player in the development of fibrosis, its inhibition has been implicated as a possible therapeutic intervention. SM16, an inhibitor of TGF- βR1, has been demonstrated to be effective in reducing radiation-related lung damage in an animal model [71]. LY2109761, inhibitor of TGF- βI serine/threonine kinase, known to reduce p-Smad 2 and p-Smad I expression, has been shown to suppress and reduce pulmonary fibrosis [72]. Neutralizing antibodies against TGF- β inhibited both proliferation of rat lung fibroblasts and terminal differentiation of progenitor fibroblasts to post-mitotic fibrocytes [73]. SB203580 and WP631 are inhibitors of the Smad signal transduction pathway, have been shown to abrogate the excessive proliferation and reduced expression of p21 (WAF1/CIP1) and PAI-1 (a TGF- β/Smad-responsive profibrotic gene induced by gamma rays and TGF-β1 [74, 75]. MyD88 (an intracellular adaptor for TLR signaling) regulates innate immunity and NF- κB activated responses, attenuates long-term radiation-induced lung injury and protects against fibrosis by alleviating chronic lung injury [76]. Fluorofenidone (1-(3-fluorophenyl)-5-methyl-2-(1H)-pyridone, AKF-PD), a novel pyridine antifibrotic agent, reduced cardiac and kidney fibrosis by inhibiting CTGF (connective tissue growth factor) expression [77, 78].

B Targeting Chronic ROS/RNS Production

Targeting chronic generation of ROS/RNS post-radiation with long-term administration of free-radical scavengers such as superoxide dismutase (SOD) and/or catalytic manganese (Mn) porphyrin-based superoxide dismutase (SOD) mimetics alleviates oxidative stress, organ hypoxia, production of cytokines and injury to heart & lung. The recombinant enzyme SOD-TAT combats radiation-induced lung injury in mice [79]. SOD has also been used in a porcine animal model to successfully treat RIF [80]. Molecular hydrogen is an antioxidant that diffuses through cell membranes, reduces levels of ROS and decreases oxidative stress-induced injury in several organs [81]. The radioprotective drug Amifostine is used clinically for its properties of scavenging free radicals, DNA protection, and acceleration of repair and this drug has potential as a therapeutic for treatment of radiation-induced lung damage [82, 83].

C Anti-TNF Antibodies

TNF-α is known to play a major role in the pathogenesis of post-radiation tissue injury, causing cachexia in addition to tissue and organ damage and shock effects that are irreverible. It is therefore implied that Fibrosis could be prevented with antibodies to TNF. TNF-α receptors I and II in soluble form have potential for their application as inhibitors of radiation-induced tissue injury [84].

D Restoring Immunological Balance

Glucocorticosteroids have value to restore immunological balance in several affected organs and tissues [85]. Sivelestat, a neutrophil elastase inhibitor, significantly decreased deposition of collagen and accumulation of neutrophils in the lung parenchymal cells and showed improvement in static lung compliance of radiation-treated lung [86].

VI Clinical Trials

Since preclinical studies showed the possibility of the anti-oxidant SOD as a good candidate for clinical studies, a clinical trial using Lipsod (a Cu/Zn SOD in liposomal form) was used in a clinical study at the Necker Hospital in Paris in 1984. 34 patients manifesting RIF in skin and underlying tissues were treated from May 1984 to January 1986 and followed for a time frame of 5 years. All patients exhibited clinical regression of fibrosis at varying degrees [87]. In another study, involving 24 women previously treated with radiation for breast cancer, a 6-month treatment with a combination of pentoxifylline and alpha-tocopherol was shown to successfully treat superficial radiation-induced fibrosis [88, 89].

VII Future Directions

i Safer Radiation Protocols

Proton beam therapy (PBT) with unique properties to deliver proton beams with minimal dose deposition beyond the treatment target has the potential to minimize exposure to the heart, lungs, muscle and bone [90]. Historically, a relative biological effectiveness of protons in clinical practice has been established as 1.1, based upon *in vitro* and *in vivo* data of the biological effect of protons in the middle of the therapeutic radiation distribution or SOBP [91]. However, data also exists that the RBE of protons is greater at the distal end of the range. If not accounted for, this could result in a greater biologic effect and as a toxicity beyond the target at the ribs, lungs and heart. Modeling and accounting for the

optimal physical and biological parameters at the end of the treatment range remains as an important area for laboratory and clinical investigation. Close clinical follow-up and adopting a 'data pooling culture' will enable better correlation of individual treatments with long-term outcome of a large number of patients, so as to better understand the impact of variations in radiation treatment protocols [92].

ii Close Monitoring for Timely Intervention

Cardiac damage occurs in more than 50% of patients treated with radiation therapy. Close monitoring of post-radiation effects and clinical intervention in the ischemic stage of RICAD might be valuable since this is a reversible and treatable, thereby suppressing the subsequent progression of damage to irreversible events.

iii Therapeutic Invention Towards the TGF-β Inhibition

Since TGF-β1 is considered the central player in the switch towards fibrosis, future therapeutics should be focused towards inhibition of TGF β1 receptor activity [93]. Promising results have been achieved using small-molecule inhibitors of the TGF-β receptor I kinase in preclinical models to treat radiation-induced lung fibrosis [94]. Inhibition of integrin receptors which have an important role in cell-matrix interactions, serve as another promising new approach for antifibrosis therapy [95-97].

Conclusion

Fibrosis is a consequence of various converging paths including inflammation, oxidative stress and chronic alterations in gene expression. The complexity of the interplay between these pathways may present a varied number of therapeutic targets for combating fibrosis. On the other hand, since some of these targets have pleiotrophic effects, caution should be exerted to ensure knocking down one fibrotic pathway does not lead to toxicity from another interconnected pathway and thereby reduce the overall clinical benefit. This communication attempts to discuss the end-point of current radiation protocols (heart and lung damage), an inevitable consequence of radiation, clinically manifesting as hardening of usually soft organs such as heart and lung by deposition of extracellular matrix components which should have undergone apoptosis in the normal setting. This phenomenon is referred to as Radiation-induced Fibrosis (RIF). RIF has been traditionally considered an irreversible process.

Although there is no known treatment for this inevitable and undesirable side-effect of radiation, contemporary research studies indicate that regression of fibrosis can occur using the anti-oxidant Superoxide dismutase (SOD) and a combination of pentoxifylline and Vitamin E which work synergistically with each other to cause clinical regression and successfully treat fibrosis at different levels [87-90]. Even though, the end-point of Fibrosis is not the scope of our lab, as a social responsibility to the community at large, we have discussed this, since it points to the fact that an even higher regression can be achieved with the safer radiation beams. It opens up future research possibilities with proton beams and charged particles (known to reduce non-targeted effects to normal cells and tissue) and points to the fact radiation-induced fibrosis may be treatable with a combination of safer forms of radiation as well as mild drugs. Our recent studies explore the possibilities to understand and mitigate the radiation related fibrosis [98-100].

Conflicts of Interest

The authors whose names are listed below certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject material discussed in this manuscript.

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