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NRF2 deficiency reduces life span of mice administered thoracic irradiation

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Abstract

Subsets of cancer survivors who have been subjected to thoracic irradiation face the prospect of developing pulmonary injury. Radiation-induced pulmonary fibrosis is an insidious injury that presents 6 to 24 months after irradiation and continues to progress over a period of years. TGF- β and reactive oxygen species contribute significantly to the pathogenesis of this injury. The transcription factor NRF2 controls antioxidant gene expression and therefore regulates the cellular oxidant burden. This work demonstrates an additional paradigm for NRF2: suppression of TGF- β -mediated signaling, assessed by measuring expression of a surrogate TGF- β 1 target gene (PAI-1) in lung fibroblasts. Thoracic irradiation of *Nfe2l2* ($-/-$) mice resulted in rapid expression of PAI-1 and FSP-1 compare to irradiated wild type mice. Examination of lung tissue 16 weeks after thoracic irradiation of *Nfe2l2* ($-/-$) mice revealed the presence of distended alveoli and decreased numbers of alveoli compared to wild type mice. Suppression of NRF2 expression shortened life span in mice administered 16 Gy to the thorax. *Nfe2l2* (+/-) and ($-/-$) mice exhibited a mean life span of 176 days compared to wild type mice that lived an average of 212 days. These novel results identify NRF2 as a susceptibility factor for development of late tissue injury.

Keywords

Radiation-induced fibrosis; late normal tissue injury; NRF2; TGF- β ; Nrf2 null mice; PAI-1; FSP-1

Introduction

‘Late normal tissue injury’ secondary to radiation therapy can limit prescribed dose [1] and can result in significant morbidity and mortality among cancer survivors. Radiation-induced pulmonary fibrosis (RiPF), characterized by the loss of parenchyma, the progressive accumulation of myofibroblast progenitors, the development of fibrosis, and the subsequent remodeling of lung interstitium [2–4], presents 6 to 24 months after irradiation, and continues to progress over a period of years [5].

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TGF- β and pathophysiological reactive oxygen species (ROS) are central to the pathogenic process of RiPF [6–10]. We and others have shown that mouse lung contains significant amounts of activated TGF- β 1 following thoracic irradiation [6, 11]. This is a consequence of ROS-mediated activation of latent TGF- β 1 [12]. TGF- β signaling, in turn, increases the generation of intracellular ROS [13, 14]. This ROS activates additional latent TGF- β , as well as produces further tissue injury [15]. ROS is also required for the differentiation of myofibroblasts [16].

The contribution of TGF- β signaling to fibrogenesis is multifactorial and involves both R-Smad-dependent and independent signaling. TGF- β /R-Smad signaling is initiated by ligand binding to a type II receptor, which in turn, binds to and phosphorylates type I receptor. The type I receptor phosphorylates key Ser residues in the C-terminus of Smad2 and 3, allowing R-Smads to complex with Smad4, translocate into the nucleus, undergo p300/CBP-mediated acetylation [17], and bind at Smad Binding Elements located in the proximal promoters of TGF- β target genes. TGF- β signaling promotes epithelial cell apoptosis, proliferation/transdifferentiation of myofibroblasts, induction of plasminogen activator inhibitor-1 (PAI-1), as well as collagen synthesis [18]. The role of TGF- β in the development of RiPF has been validated in experimental mouse models [2, 4, 6, 9, 10, 19]. Clinical studies have shown a correlation between *TGF β 1* SNPs and onset of RiPF [4, 20].

The use of well defined genetic mouse models has been instrumental in the development of a fibrogenesis paradigm. These models have shown that plasminogen activator (uPA/tPA) and plasmin activity are antifibrotic. Conversely, plasminogen activator inhibitor-I (PAI-1) activity has been shown to be profibrotic and life shortening [21–23]; PAI-1 null mice are resistant to bleomycin-induced fibrosis [22, 23]. PAI-1 inhibition of uPA/tPA prevents plasmin activity, which is required for fibrolysis, as well as for release and activation of hepatocyte growth factor (HGF) from the ECM [24]. The interaction of HGF with its receptor c-Met induces COX-2 activity, which generates bioactive prostaglandins. PGE2/EP2 receptor signaling is antifibrotic, due in part to inhibition of myofibroblast proliferation and synthesis of extracellular matrix (ECM) components [23]. An important caveat to the model described above is the knowledge that the PAI-1 experiments were conducted using bleomycin, which produces acute, not late lung fibrotic injury. Currently it is not known if pulmonary PAI-1 expression is induced by ionizing radiation.

Nuclear factor (erythroid-derived 2)-like 2 (NRF2) regulates Antioxidant Response Element (ARE)-dependent expression of Phase II/antioxidant genes. The current paradigm describing NRF2-mediated transcription involves NRF2 heterodimerization with transcription factors such as MAFs or CJUN. Heterodimerization licenses NRF2 binding to Antioxidant Response Elements (AREs) located in the proximal promoters of Phase II/antioxidant genes such as SOD2, CAT, GPX1, HO-1, and GCLC, which encodes the catalytic subunit for the enzyme glutamate cysteine ligase, the rate limiting enzyme for the synthesis of the antioxidant glutathione. The importance of antioxidant gene expression in RiPF can be appreciated by the knowledge that expression of SOD2, CAT, or HO-1 reduces the severity of pulmonary fibrosis in mouse models [7, 25–27].

We now report that in addition to regulating antioxidant gene expression NRF2 functions as a repressor of TGF- β /Smad-mediated signaling. Because NRF2 expression is depressed in normal lung tissue in subsets of patients [28–32], a consequence of aging, as well as the presence of functional SNPs in its promoter [33, 34], it was of interest to determine if Nrf2 deficiency would promote radiation induced pulmonary fibrosis. Life shortening in Nrf2 wild type C57BL mice administered thoracic irradiation is not associated with pneumonitis, rather it is associated with the presence of fibrotic lesions [35]. We found that thoracic

irradiation of *Nfe2l2* hemizygotic, and null mice shortened their life span compared to irradiated wild type mice.

Materials and Methods

Reagents

The following antibodies were used for immunohistochemistry and for immunoblotting: FSP-1 antibody was purchased from Millipore (#07-2271); NRF2 and PAI-1 antibodies were purchased from Santa Cruz (sc-13032- NRF2; sc-8979 -PAI-1); Smad3 antibody was from Cell Signaling (#9520).

Mice

These experiments were conducted using a well characterized colony of C57BL/6J mice that originated from crossing a 129/Sv-Nfe2l2tm mouse into a C57BL background. The F1 B6.129-Nfe2l2^{+/-} mice were backcrossed with C57BL/6J 10 times. Genotyping of the resulting B6.129-Nfe2l2tm (F₁+10) mice using 53 microsatellite markers indicated a B6 background of greater than 95% (data not shown). At the time of experimentation mice were between 7 and 10 months old. Mice were maintained under specific pathogen free conditions and all procedures performed on animals were approved by the Institutional Animal Care and Use Committee at Vanderbilt University and at the University of Texas MD Anderson Cancer Center, and complied with all state, federal, and NIH regulations.

Radiation Treatment

The procedure is described in ref [35]. In brief, unanaesthetized mice were immobilized in a specially constructed irradiation plastic device. The whole thorax of a mouse was irradiated to 16 Gy using a 300 kVp x-ray unit. The remainder of the body was shielded with 3 mm lead. Morbidity was assessed 3 to 4 times a week for the duration of the experiment. Any mouse exhibiting signs of distress, including lethargy, hunched back, ruffled fur, or increased breathing frequency was sacrificed.

Histology and Fibrosis Scoring (Phenotype)

Five micron sections were placed on charged slides, deparaffinized, and rehydrated. The sections were placed in heated Target Retrieval Solution (Dako, Carpinteria, CA). Endogenous peroxidase was neutralized with 0.03% hydrogen peroxide followed by a casein-based protein block (Dako, Carpinteria, CA) to minimize nonspecific staining. The sections were incubated with primary antibody and incubated for one hour at room temperature. Mouse lung samples served as positive controls. The Dako Envision+ HRP/DAB System (Dako) was used to produce localized, visible staining. The slides were lightly counterstained with Mayer's hematoxylin, dehydrated and coverslipped. Histological scoring of fibrosis was performed as described previously [36].

PAI-1 and FSP-1 immunohistochemical staining in lung was quantified as follows. Slides were inspected (10x objective) in their entirety to insure staining uniformity. Staining intensity of 10 random microscopic fields (20x objective) per slide was determined and was based on the following: 0 = negative; 1 = minimal staining; 2 = moderate staining; 3 = intense staining. Twenty to 30 fields were averaged together for statistical comparisons [37].

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described in [38].

Chromatin Immuno-Precipitation Assay (ChIP)

ChIP assays were performed as previously described [39]. The primers for CAGA PAI-1 were (208 bp): sense: 5'AGTCAACCTGGCAGGACATC3' and anti-sense: 5'ACACCTCCCTCTCTGGGACT3'.

Gel Mobility Shift Assay (GMSA)

These assays were performed as previously described [40]. Oligoes, 5'-TCG AGA GCC AGA CAA AAA GC-3' and 5'-GCT TTT TGT CTG GCT CTC GA-3', were synthesized by Invitrogen, Inc. (Carlsbad, CA) and annealed to obtain CAGA.

Reporter Assays

IMR-90 cells were co-transfected with the following vectors: a luciferase reporter under the control of a synthetic CAGA promoter [41]; β -galactosidase to correct for transfection efficiency; human wild type Nrf2 or dominant negative Nrf2 [42], plasmid expressing Smad3, insertless pcDNA3.1. Luciferase/ β -Gal activity was measured 72 hrs after transfection.

Results

NRF2 deficiency decreases life span after thoracic irradiation

We compared the radiation response of a well characterized inbred colony of C57BL/6J mice [36] to the response of congenic wild type B6.129-Nfe2l2 mice (Figure 1A). Life span was not significantly different between the two strains of mice, whether they were male or female ($p > 0.05$ Student's t test). Comparison of irradiated female to male mice yielded the expected gender susceptibility ($p < 0.001$, Student's t test). Sixteen Gy administered to the thorax resulted in an average life span of 160 days in female mice compared to 209 days in male mice (Figure 1A).

The data presented in Figure 1B and 1C clearly show that *Nfe2l2* nullizygosity decreased life span in female mice administered 16 Gy to thorax ($p = 0.0032$). Most interestingly, *Nfe2l2* hemizygosity ($n = 33$) decreased mean life span of male mice significantly compared to wild type mice ($n = 22$; $p < 0.0001$; Figure 1B). This point is illustrated graphically in Figure 1D. *Nfe2l2* nullizygosity decreased mean life span by an additional 2% in male mice compared to hemizygous mice ($n = 28$; $p = 0.027$). Thus, the major decrease in life span occurred when a single allele was lost.

Fibrotic Lesions

We quantified the size of fibrotic lesions in the left lung. H&E stained fixed lung sections were obtained from moribund mice and from all mice that survived until experimental termination. Representative images are shown in Figures 2.

The area(s) containing fibrotic lesions observed in H&E sections was quantified as described previously [43]. An average of 5.4 ± 0.7 (SEM) percent of the left lung from irradiated *Nfe2l2*^{+/+} male mice (mean life span of 212 days, $n = 19$) was fibrotic. The percent fibrosis for each individual mouse plotted as a function of survival time is shown in Supplement Figure 1. It is clear that the size of the lesion alone does not determine life span. Lungs from irradiated *Nfe2l2*^{+/-} male mice (mean life span of 179 days; $n = 27$) and null mice (mean life span 174 days; $n = 24$) exhibited similar degrees of fibrosis ($5.3 \pm 0.9\%$ (SEM) and $4.9 \pm 0.6\%$ (SEM), respectively).

We also performed trichrome staining of fixed lung sections and quantified them using the same approach as that used for the H&E stained sections. The analysis yielded results

similar to the H&E analysis (Data now shown). Representative sections are shown in Figure 4.

We characterized alveoli at a microscopic level. Alveoli were quantitated by examining H&E lung sections obtained from *Nfe2l2* wildtype (n=3) and null (n=3) mice using Image-Pro^R software (Figure 5A). One hundred and twelve days (16 weeks) after irradiation lung tissue obtained from *Nfe2l2*^{-/-} mice exhibited a 70% decrease in alveolar number (p = 0.002, Student's t test). Those alveoli present in *Nfe2l2* null lung were distended (Figure 5B) compared to age-match irradiated wild type mice. Thus, NRF2 deficiency produced marked alveolar distention, as well as a reduction in the number of alveoli.

Inflammatory Responses

Inflammatory responses represent an important process in acute models of pulmonary fibrosis such as that produced by bleomycin. An NRF2 deficiency exacerbates the response in these models [44]. However, inflammation does not appear to be central to the development of idiopathic pulmonary fibrosis [45] or in radiation-induced pulmonary fibrosis [35], which develops months after injury [5]. Conversely, radiation pneumonitis is considered an acute reaction that exhibits a self-limiting course [5]. We used a differential cell analysis of bronchoalveolar lavage (BAL) to determine if loss of NRF2 expression affected radiation-induced inflammatory responses in the BAL. As shown in Supplemental Figure 2, 16 Gy administered to the thorax resulted in a significant inflammatory response but this response was independent of NRF2.

Plasminogen Activator Inhibitor-1 (PAI-1) expression in irradiated lung

Alveolar distention has been linked to TGF- β -mediated signaling that promotes expression of matrix components, including PAI-1 [46], a critical enzyme for development of fibrosis following acute bleomycin injury [22]. However, we are unaware of any reports in the literature that indicate that PAI-1 expression is affected by pulmonary irradiation. Therefore experiments were undertaken to determine if PAI-1 expression was increased in response to injury produced by pulmonary irradiation. PAI-1 expression was greater in the lungs of irradiated *Nfe2l2* wildtype mice (n=3) compared to age matched unirradiated wildtype littermates (n=3) 24 hrs (p = 0.022, Student's t test), as well as 6 weeks (p < 0.001, Student's t test) after irradiation, as assessed by semiquantitation of immunohistochemical staining (Figure 6A). A similar analysis indicated that 24 hrs (p = 0.003, Student's t test) and 6 weeks (p < 0.001, Student's t test) after irradiation PAI-1 expression was greater in the lungs of irradiated *Nfe2l2*^{-/-} mice compared to age matched unirradiated *Nfe2l2*^{-/-} littermates (Figure 6A). Representative images of immunohistochemical staining are shown in Figure 6B. The analysis further indicated that PAI-1 immunohistochemical staining in irradiated lung obtained from *Nfe2l2*^{-/-} mice was greater than that obtained from *Nfe2l2*^{+/+} mice when analyzed 24 hrs or 6 weeks after irradiation (p < 0.001, Student's t test).

Expression of fibroblast specific protein-1 (FSP-1/S100A4) in irradiated lung

FSP-1 is a fibroblastic marker that can be used to detect pulmonary myofibrocytes following oxidant-induced injury [47, 48]. Wild type (n=3) and *Nfe2l2* null (n=3) mice were administered 16 Gy to the thorax. Immunohistochemical staining of paraffin embedded sections revealed low levels of FSP-1 in unirradiated lung obtained from wild type *Nfe2l2* mice. Staining increased significantly 24hrs after irradiation (p < 0.001, Student's t test), reaching maximum expression 6 weeks after irradiation (p < 0.001, Student's t test, Figure 7A). In contrast to the staining observed in mouse lung treated with bleomycin [47], FSP-1 staining remained elevated for 16 weeks after the initial insult. Representative images of immunohistochemical stained paraffin embedded sections is shown in Figure 7B. Surprisingly, the staining pattern observed in unirradiated 10 month old *Nfe2l2* null mice

was significantly higher than unirradiated lung from the *Nfe2l2* wild type mouse ($p < 0.001$, Student's *t* test), reminiscent of what is observed in human IPF disease [47]. FSP-1 staining was significantly more intense in the lung of irradiated *Nfe2l2* null animals compared to wild type, when assayed 24 hrs after irradiation ($p < 0.001$, Student's *t* test, Figure 7A). By 6 weeks the staining intensity of the two phenotypes was not significantly different but at this time interval there were twice as many fibroblastic foci presence in lung sections derived from *Nfe2l2* null mice compared to wild type. Thus, an NRF2 deficiency initiated an early onset of the FSP-1 fibrogenic marker.

NRF2 inhibits TGF- β /Smad-mediated signaling

We found that NRF2 and Smad3 form an endogenous nuclear complex in pulmonary fibroblasts. Nuclei from human IMR-90 lung fibroblasts were isolated and protein solubilized in RIPA buffer. Immunoprecipitation of either Smad3 (Figure 8A) or pSmad3 (pSer423/425; Supplemental Figure 3) and immunoblotting using an Nrf2 antibody revealed the presence of a single polypeptide in the precipitated complex that exhibited a Mr of approximately 70,000, indicating the presence of NRF2. We queried fibroblasts because these cells synthesize and secrete extracellular matrix components, as well as PAI-1 during fibrogenesis [49, 50]. In addition, fibroblastic foci are a hallmark of oxidant-induced fibrosis[51].

Use of a ChIP assay demonstrated that NRF2 could bind to a Smad Binding Element (SBE), as defined by [52], located in the proximal promoter of the TGF- β surrogate target gene PAI-1 (Figure 8B). As a positive control, an antibody to Smad3 was used to demonstrate the binding of Smad3 at this SBE. A GMSA was used to address the question of whether NRF2 binds to SBE elements. Cells were exposed to 2 ng/ml of TGF- β 1, which increased CAGA DNA binding activity (Figure 8C). A 50 fold excess of unlabeled CAGA double strand oligonucleotide abolished DNA binding activity. Nrf2 antibody also abolished CAGA DNA binding activity (Figure 8C). We have found that Smad3/NRF2 complexes can also bind ARE cis elements (manuscript in preparation). Therefore we added a 50 fold excess of unlabelled ARE double stranded oligonucleotide to nuclear lysate and found that the unlabeled ARE could compete for CAGA DNA activity (Figure 8C). However, mutated ARE, which is incapable of binding NRF2 [53], was unable to inhibit CAGA DNA binding activity. We interpret these results to indicate that the NRF2 forms a complex with Smad3 capable of binding CAGA sites, such as found in the PAI-1 promoter.

We found that co-transfection of NRF2, but not dominant negative NRF2[42], into IMR-90 fibroblasts along with a synthetic (CAGA) luciferase reporter [41] produced a statistically significant inhibition of reporter activity ($p < 0.05$ ANOVA; Figure 8D). The activity of the CAGA-luciferase reporter reflects the presence of endogenous TGF- β in the serum of the growth medium. A similar result was observed when cells were exposed to 2ng/ml of TGF- β 1 for 2 hrs. Taken all together, these results demonstrate that NRF2 and Smad3 form a nuclear complex at SBEs and can inhibit Smad-mediated transactivation.

Discussion

The American Cancer Society estimates that in 2010 approximately 450,000 people were diagnosed with esophagus, lung, or breast cancer (ACS Facts and Figures 2010). Approximately 50% of these patients will undergo radiation therapy in the course of their treatment [1, 54]. Technical improvements in this modality have contributed greatly to the increase in long-term cancer survivorship [5, 20]. Unfortunately, approximately 10% of patients [55] who have received pulmonary irradiation will present with progressive pulmonary fibrosis that can compromise quality of life, as well as result in respiratory failure [5].

Radiation-induced pulmonary fibrosis presents 6 to 12 months after a tissue injury. Initial events include vasodilation and leakage of plasma proteins into connective tissue, expression/activation of cytokines (eg, TGF- β 1), chemokines, and matrix metalloproteinases. This is followed by dysregulated epithelial-myofibroblast cross talk, formation of fibroblastic foci, and deposition of extracellular matrix [45, 56]. This pathogenic process is well modeled in C57BL/6J mice. Radiation induced fibrotic lesions formed in the lungs of irradiated C57 mice consist of atelectatic regions characterized by significant cellularity and superimposition of extracellular matrix components such as collagen [43]. Additionally there is alveolar damage: distension and fewer alveoli. There is a direct correlation between mice that are moribund and the presence of fibrotic lesions that encompass 3 to 5% of the lung. Decreasing the radiation dose to the lung lengthens the time until a mouse become moribund, while increasing the dose shortens this time interval. However, the extent of fibrosis remains the same – approximately 5%. Furthermore, there is excellent agreement between this extent of fibrosis and respiratory distress, as measured by breathing rate. Additionally, exposure to WR-2721, a scavenger of radiation-induced oxidant radicals, produces a statistically significant decrease in radiation-induced fibrosis, breathing rate, and lethality. The degree of protection afforded by WR-2721 is the same for each end point [35, 57, 58]. Thus, we interpret this body of literature to indicate that in the radiation-induced lung injury model fibrotic lesions that encompass 5% of the lung, coupled with alveolar damage result in respiratory failure and death. However, such an interpretation needs to be tempered with the knowledge that the data are correlative.

TGF- β /Smad-mediated signaling is a critical factor that promotes many facets of pulmonary fibrosis, including promoting ROS generation, activation of myofibroblasts and fibrocytes. Synthesis of extracellular matrix components such as collagen is TGF- β dependent. We and others have found that pulmonary irradiation results in activation of TGF- β 1 [6, 11, 59].

TGF- β cytokines are found primary in a latent form that requires activation (discussed in [60]). Activation can be accomplished by radiation-induced oxidation [59] or by enzymes such as plasmin [60]. Radiation-induced activation of latent TGF- β should be considered a rapid event. Absorption of ionizing energy occurs in less than a picosecond. Oxygen radicals are generated within a microsecond and these radical have a very short half life. Plasmin is a protease formed by cleavage of the zymogen plasminogen by tissue-type or urokinase-type plasminogen activator. Plasmin-mediated activation of latent TGF- β would occur at a much slower rate than radiation-mediated activation.

Although plasmin can activate latent TGF- β , it is considered an antifibrotic enzyme, discussed in [56]. While targeted disruption of PAI-1 or intranasal administration of PAI-1 siRNA attenuates fibrotic injury following acute bleomycin injury [22, 23], PAI-1 null mice exposed to plasmin inhibitor tranexamic acid develop pulmonary fibrosis [21]. Plasminogen null mice are susceptible to acute pulmonary fibrosis [61]. Conversely, infusion of plasminogen activator into lungs of bleomycin treated mice inhibits the development of fibrosis [62]. PAI-1 is an inhibitor of both tissue-type and urokinase-type plasminogen activator and it is postulated that this inhibition helps to explain the contribution of PAI-1 to expression of acute pulmonary fibrosis [56].

Although hyperexpression of PAI-1 is an important facet of TGF- β signaling observed in acute models of pulmonary fibrosis [63], its role in late injury produced by irradiation is not well characterized. It is of particular interest as Maasilta et al [64] found that plasmin and plasminogen activator could be induced in the bronchoalveolar lavage of patients following hemithorax irradiation for treatment of pleural mesothelioma or non-small cell lung cancer.

We found that PAI-1 expression was elevated 24hrs after irradiation and that an NRF2 deficiency significantly potentiated PAI-1 expression. These results are congruent with those of Zhao et al [65] who found that *in vitro* irradiation of rat mesangial cells induced PAI-1 expression and those of Brown et al [66] who observed a statistically significant increase in PAI-1 renal expression following *in vivo* irradiation. The observation that enhanced PAI-1 expression in *Nfe2l2* null mice was observed early after radiation injury while alveolar damage manifested itself many weeks later leads to the hypothesis that PAI-1 expression is an initiator of fibrogenesis in the radiation model. However, further experimentation is required to directly address this question.

Antioxidant/Phase II genes compose a canonical NRF2-responsive pathway, in which NRF2 heterodimerization with transcription factors such as MAF induces Antioxidant Response Element-directed transactivation of target genes such as GCLC, GCLM, SOD1, SOD2, and CAT. NRF2-dependent Phase II/antioxidant genes are of particular interest because expression of SOD2 or CAT in mouse models can reduce the severity of RiPF [7, 8, 25, 67]. Conversely, *Sod2* haploinsufficiency is sufficient to increase RiPF severity [68]. The interpretation of these outcomes is that SOD2 and CAT function by mitigating pathological ROS.

We now show that NRF2 can suppress TGF- β /SBE-directed gene expression, in addition to regulating canonical ARE-directed gene expression. We found that endogenous Smad3 and NRF2 form an immunoprecipitable nuclear complex. GMSAs showed that NRF2 can bind to SBEs. ChIP analysis demonstrated that NRF2 can be crosslinked to a CAGA site in the PAI-1 promoter, a TGF- β /Smad-responsive profibrotic gene [63]. These results support the hypothesis that NRF2/Smad interactions can suppress TGF- β -target gene expression.

An analysis of *mNRF2* expression in normal human lung tissue using the Oncomine data base revealed diminished *mNRF2* expression in 5 of 21 individuals ($p \leq 1 \times 10^{-5}$) [28–32]. An analysis of normal human breast tissue yielded a similar conclusion but with a lower percentage of individuals exhibiting a *mNRF2* deficiency (27–31). Suppression of *mNRF2* can be a consequence of the presence of functional polymorphisms in the *NFE2L2* promoter [33], as well as to aging [34]. An NRF2 deficiency has been shown to increase susceptibility to benzo[a]pyrene-induced carcinogenesis in murine models [69]. Wang et al [70] found that expression of NRF2/ARE-regulated genes was significantly lower in smokers who develop lung cancer (n=20) compared to smokers without lung cancer (n=24). Thus, we hypothesize that there are NRF2 deficient cancer survivors who have undergone radiation therapy to control their smoking-induced lung cancer.

We administered 16 Gy to the thorax of *Nfe2l2* null, heterozygotic and wild type mice in order to determine if an NRF2 deficiency would affect RiPF. We found that radiation-induced apoptosis, as measured by immunohistochemical staining for the presence of cleaved Caspase-3, was not enhanced by loss of NRF2. FSP-1 is a marker for the presence of myofibroblasts that accumulate following fibrotic-inducing injury [47, 71]. Furthermore, FSP-1 can form a complex with either phospho-Smad2 or phospho-Smad3 and potentiate TGF- β target gene transactivation [72] and thereby contribute to formation of extracellular matrix components. Unexpectedly, we found high levels of FSP-1 staining in unirradiated pulmonary tissue obtained from *Nfe2l2* null mice. As FSP-1 expressing cells are associated with the presence of activated TGF- β 1 [73], up regulation of FSP-1 in tissue from *Nfe2l2* null mice may reflect an inverse relationship between TGF β 1 and *mNrf2* levels reported by Choi et al [74]. We interpret these results to indicate that FSP-1 expressing cells present in unirradiated NRF2 deficient tissue contribute to fibrosis susceptibility that has been observed following acute lung injury in this model [75].

FSP-1 expressing cells were observed in the lung within 24 hrs after irradiation and FSP-1 staining was more intense in tissue from irradiated *Nfe2l2* null mice compared to wild type. Recently, van der Velden [76] found that TGF- β 1-mediated induction of FSP-1 requires phosphorylation of both the linker region and the C-terminus of Smad3. Thus, the observation that an NRF2 deficiency promotes FSP-1 expressing cells is consistent with the knowledge that activated TGF β 1 can be present in lung after irradiation [11] and that TGF- β /Smad3 signaling is empowered by loss of NRF2. Additionally, these data suggest the hypothesis that recruitment of FSP-1 expressing fibroblasts represents a very early event for a fibrogenesis that presents months later.

We found several differences between our results obtained in the late responding radiation-induced fibrosis model and previous reports that utilized acute response models initiated by bleomycin. Following treatment with bleomycin there is a linear relationship between the duration of survival and the percent of lung involved with a fibrotic lesion [77]. In contrast, our findings demonstrate that this relationship is not linear in a radiation model ($r^2 = 0.06$; Supplemental Figure 2). In the bleomycin model an NRF2 deficiency increases the percent of the lung involved with a fibrotic lesion [77]. This was not observed in the late injury model produced by x-irradiation. Loss of NRF2 enhances inflammatory responses in acute models of fibrosis [75] but not in late occurring radiation model. The reasons for these differences are not currently understood.

Taken all together, the data show that loss of NRF2 empowers TGF- β /Smad signaling. Following the oxidative stress produced by ionizing radiation there is rapid expression of the myofibroblast marker FSP-1 and of the profibrotic enzyme PAI-1. Irradiated lung in *Nfe2l2* null mice was characterized by a reduction in the number of alveoli, as well as alveolar distention. Loss of NRF2 expression shortened the life span of irradiated mice. Remarkably, *Nfe2l2* haploinsufficient mice exhibited the same degree of life shortening as the null mice. We interpret this to indicate that small changes in NRF2 expression are sufficient to impact life span following thoracic irradiation. Further, these studies suggest that NRF2 may represent a therapeutic target for managing RiPF. Screening of cancer survivors for NRF2 expression may identify subsets of patients that would benefit from inducing NRF2 expression. Compounds such as CDDO-im (1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl)imidazole) exhibit significant anticancer activity [78] and have significant potential for inducing NRF2 [79].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Loss of NRF2 shortens life span in mice administered 16 Gy to the thorax. A) Comparison of life span between irradiated inbred male and female C57BL/6J with life span of irradiated congenic wild type male and female mice derived from Het B6.129-Nfe2l2tm mice; B) Comparison of life span of congenic *Nfe2l2* ^{+/+}, *Nfe2l2* ^{+/-}, and *Nfe2l2* ^{-/-} male and female mice following exposure to 16 Gy to the thorax; C) Survival of *Nfe2l2*^{+/+} and *Nfe2l2*^{-/-} female mice administered 16 Gy to the thorax; D) Survival of *Nfe2l2*^{+/+}, *Nfe2l2*^{+/-} and *Nfe2l2*^{-/-} male mice administered 16 Gy to the thorax.

Figure 2.

Representative H & E staining of lung tissue obtained from *Nfe2l2*^{+/+} and *Nfe2l2*^{-/-} mice. Mice that had become moribund following administration of 16 Gy to the thorax and their age-matched sham control mice were sacrificed and lung tissue subjected to H&E staining. The number of days the irradiated animal survived is noted in the figure.

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Figure 4. Representative Trichrome staining of lung tissue obtained from *Nfe2l2*^{+/+} and *Nfe2l2*^{-/-} mice. Mice that had become moribund due to administration of 16 Gy to the thorax and their age-matched sham controls were sacrificed and lung tissue prepared for Trichrome staining. The number of days each irradiated animal survived is noted in the figure.

Figure 5.

Thoracic irradiation of NRF2 deficiency mice resulted in alveoli distention and a reduction in the number of alveoli. A) The relative number of alveoli per field were determined by examining H&E lung sections obtained from irradiated *Nfe2l2*^{+/+} (n=3) and *Nfe2l2*^{-/-} (n=3) mice using Image-Pro^R software. The mean number of alveoli per field determined from wild type mice 42 days after irradiation was assigned a relative value of 1.0; B) Representative H&E staining of lung tissue obtained from wild type and *Nfe2l2* null mice, 5X.

Figure 6.

NRF2 deficiency increases PAI-1 expression in irradiated lung. A) Quantification of PAI-1 immunohistochemical staining in fixed left lung tissue obtained from irradiated *Nfe2l2*^{+/+} (n=3) and *Nfe2l2*^{-/-} (n=3) mice and age-matched unirradiated mice (*Nfe2l2*^{+/+} (n=3) and *Nfe2l2*^{-/-} (n=3)). The histogram illustrates the percent area stained per field, as well as staining intensity per field; B) Representative images of immunohistochemical staining.

Figure 7. NRF2 deficiency increases FSP-1 expression in irradiated lung. A) Quantification of FSP-1 immunohistochemical staining in fixed left lung tissue obtained from irradiated *Nfe2l2*^{+/+} (n=3) and *Nfe2l2*^{-/-} (n=3) mice and age-matched unirradiated mice *Nfe2l2*^{+/+} (n=3) and *Nfe2l2*^{-/-} (n=3) mice. The histogram illustrates the percent area stained per field, as well as staining intensity per field; B) Representative images of immunohistochemical staining.

Figure 8.

NFR2 represses Smad3-dependent CAGA-directed transcription in human IMR-90 fibroblasts. A) Nuclei were isolated IMR-90 fibroblasts and solubilized in RIPA buffer. Endogenous Smad3 was immunoprecipitated and the immunoprecipitate immunoblotted for the presence of NRF2; B) Human IMR-90 cells were subjected to a ChIP assay using antibody to Smad3, NRF2 or control IgG antibody. PCR amplification of a 208 bp fragment from the PAI-1 promoter is shown; C) Nuclear lysate from IMR-90 cells exposed to 0 or 2 ng/ml TGF-β1 was subjected to a Gel Mobility Shift Assay (GMSA) using a double stranded ³²P labeled CAGA oligonucleotide. A 50 fold excess of unlabelled CAGA oligonucleotide, GCLC ARE4 [53], or a mutated GCLC ARE4 [53] was added to some reactions prior to electrophoresis. NRF2 antibody was added to one reaction prior to electrophoresis; D) IMR-90 cells were transiently co-transfected with a luciferase reporter under the control of a synthetic CAGA reporter [41], a vector expressing β-galactosidase, vectors expressing Smad3, wild-type human NRF2, dominant-negative NRF2, or insertless pcDNA3.1. The TGF-β1 exposure (2ng/ml) was for 2 hrs. Reporter activity was determined 72 h after transfection.