HHS Public Access

Author manuscript

Free Radic Biol Med. Author manuscript; available in PMC 2016 November 01.

Published in final edited form as:

Free Radic Biol Med. 2015 November ; 88(0 0): 268–274. doi:10.1016/j.freeradbiomed.2015.04.035.

NRF2 promotes survival following exposure to ionizing radiation

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Abstract

NRF2 is a transcription factor that promotes antioxidant and drug-metabolizing gene expression. It also regulates the transcription of genes involved in carbohydrate and lipid metabolism, NADPH regeneration, heme and iron metabolism, as well as proteasome metabolism. Emerging research has identified NRF2 as a critical factor for promoting survival of mammalian cells subjected to ionizing radiation. At a mechanistic level, NRF2 promotes the repair of DNA damage and drives detoxification of superoxide that is generated hours to days after irradiation. This review summarizes research in these areas and discusses targeting of NRF2 in radiation resistant cancer and NRF2's role in mitigating Acute Radiation Syndrome.

Keywords

Nrf2; ionizing radiation; reactive oxygen species; DNA repair; cell and tissue damage

Introduction

Electrophiles and reactive oxygen intermediates, as well as reactive nitrogen species, can contribute significantly to the etiology of many chronic human diseases. This knowledge has driven a major research effort that focused on providing mechanistic insight and guidance for the development of redox-based therapeutic strategies. The effort, however, was hampered by the structural diversity of electrophilic and oxidative compounds. It took the pioneering work of Talalay and colleagues [1-3] and Pickett and associates [4, 5], as well as research from Violet Daniel's laboratory [6, 7], to provide a fundamental molecular framework that ultimately was used to explain how a thiol-based protein sensor distinguished between different types of chemistries and translated the information into physiological responses. The sensor is kelch-like ECH-associated protein 1 (Keap1), originally characterized by Itoh et al. [8]. The murine protein contains 25 and the human contains 27 cysteine residues that function as redox sensors capable of integrating diverse chemistries [9], including radiolytically-generated hydroxyl radical (•OH) and hydrogen

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peroxide [10], into a common signal: activation of nuclear factor erythroid 2-like 2, or NRF2 (reviewed in [11].

NRF2 (HGNC:7782), encoded by *NFE2L2*, is a member of the cap 'n' collar (CnC) family of basic leucine zipper transcription factors [12] that are conserved in mammals [13], birds [8], fish [14], insects [15], and worms [16], but not expressed in plants and fungi [13]. The family is composed of the transcription factors SKN-1, NRF1, NRF2, NFE2, NRF3, CncC, BACH1 and BACH2 [13]. These proteins are characterized by a leucine zipper protein-protein dimerization domain as well as CnC and abasic domains that confer DNA binding activity [17]. An NMR solution structure of the DNA binding domain may be found at http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=105542.

Nrf2 was originally identified as a key regulator of canonical antioxidant and drug-metabolizing gene expression [18, 19]. Nrf2 heterodimerization with MAF-G [20] or the JUN CnC-bZIP factor [21] licenses binding to *cis* antioxidant response elements (AREs) located in proximal promoters of Nrf2 target genes that now number over 500 [20], including those involved in carbohydrate and lipid metabolism, NADPH regeneration, heme and iron metabolism, as well as proteasome metabolism [19, 22]. The functional ARE has recently been defined as TMANNRTGACTCAGCRWWWW, where M = A or C, R= A or G, and W = A or T [20]. Emerging research now shows that Nrf2-mediated transcription can protect cells and tissues from the pathogenic consequences of hydroxyl radicals that are directly generated by ionizing radiation as well as the hydrogen peroxide and superoxide that are generated as a secondary consequence of irradiation.

NRF2 promotes survival of irradiated cells

Preclinical cell culture models have been used to address the question of whether Nrf2 impacts survival of irradiated cells. Keap1$^{-/-}$ mouse embryo fibroblasts (MEFs) constitutively overexpress Nrf2 and Nrf2 target genes and are characterized by low levels of intracellular reactive oxygen species (ROS) and a radiation-resistant phenotype compared to wild-type MEFs [23]. The generalized term ROS is used in this review when the initial oxidizing species were not identified in the cited papers [24] and has been defined by C Winterbourn as "those initial species generated by oxygen reduction (eg, superoxide) as well as all secondary reactive products. The definition includes overlapping reactive nitrogen species" [25]. Relative to wild type MEFs, Nrf2$^{-/-}$ MEFs express high levels of intracellular ROS and are intrinsically radiosensitive [23, 26]. Activation of Nrf2/NRF2 signaling due to electrophilic adduction of Keap1 or a deficiency in the expression/function of Keap1 has been shown to lower intracellular ROS and confer radioresistance in fibroblasts [27], bronchial and breast epithelial cells [28], DU145 prostate cells [29], squamous cell lung cancer [30], and glioblastoma cells [31]. RNA interference (RNAi) or pharmacological targeting of NRF2 in DU145 prostate cancer cells [29, 32], non-small cell lung cancer A549, H460, or H1299 cells [23, 33], or gliobastoma cells [31] elevates ROS and produces a corresponding radiosensitive phenotype. Taken all together, these investigations support a hypothesis that NRF2 promotes a pro-survival response in irradiated cells.

Molecular effects of ionizing radiation

Initial events

The term ionizing radiation describes a photon or particle with sufficient energy to displace orbital electrons from atoms, thereby yielding ions and ionized electrons [34]. Coulomb interactions occur between ionized charged particles (e.g., an electron) moving through a medium such as a cell and the orbital electrons of the constituent atoms. These interactions result in a transfer of kinetic energy from the ionized charged particles to the electrons in the medium [35] and are quantified as absorbed dose (D), which is defined as the absorption of energy in a medium of known mass by ionizing particles. [35]The units of D are Gy (the SI unit; 1 Gy = 1J/kg) or rad, which is equal to 0.01 Gy. In the case of X- or γ-irradiation, 70% of photons traversing a cell interact with water molecules that ultimately decompose into hydroxyl radicals (•OH), hydrogen radicals (•H), hydrogen peroxide, superoxide, and solvated electrons (e_{aq}^-) [36]. The hydroxyl radical can react at diffusion controlled rates with all four purine and pyrimidine bases, as well as 2-deoxyribose. However, neither superoxide nor hydrogen peroxide reacts significantly with DNA bases or 2-deoxyribose [37] and as discussed below, radiation-induced damage to DNA is a critical event. Thus, the initial reactions relevant to this review can be described as follows [38-40].

$$H_2O \rightarrow H_2O^{+\bullet} + e_{aq}^- \quad (1)$$

$$H_2O \rightarrow H_2O^* \quad (2)$$

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

$$H_2O^* \rightarrow \bullet OH + \bullet H \quad (4)$$

The radical cation in eq 1 ($H_2O^{+\bullet}$) can donate a proton to a nearby water molecule within 10^{-14} seconds to yield H_3O and the hydroxyl radical (•OH), eq 3 while H_2O^* (eq 2) can decompose into •OH + •H (eq 4). These reactions are complete on a time scale of milliseconds.

G-value is a term used to quantify the chemical effects of ionizing radiation. The term was originally defined as the number of molecules transformed, produced, destroyed, or changed per 100 eV of energy absorbed. In SI units the G-value is assigned a value of mol/J. G-values are energy-dependent. For example, Cobalt 60 emits two monoenergetic photons of 1.17 and 1.34 MeV. The G-value for the hydroxyl radical was calculated to be 2.74 molecules formed per 100 eV absorbed [38-40]. The $LD_{50/60}$ is a term to describe the mean lethal dose that will produce 50% mortality in a population over 60 days. For humans the $LD_{50/60}$ is approximately 4 Gy [34]. The G-values discussed above allow one to calculate the concentration of hydroxyl radicals generated by a given dose of radiation. For example, 4 Gy will generate approximately 20.4 μmol of hydroxyl radicals in a person who weighs 190 pounds.

Radiation-induced damage to DNA

While all cellular macromolecules are susceptible to attack by •OH, damage to DNA represents a critical injury with pluripotent consequences: cell death, tissue injury, and disease. Hydroxyl radicals can abstract a hydrogen atom from the methyl group of thymine and from each carbon atom of the 2-deoxyribose moiety [41]. [41][42]Hydroxyl radicals are able to add to the double bonds in DNA bases to generate hydroxyl DNA base radicals [41]. These and other reactions can result in apurinic/apyrimidinic (AP) sites, single strand breaks [42], double strand breaks (DSBs), and protein DNA crosslinks, as well as other types of DNA damage. It is estimated that 4 Gy will damage more than 2000 base pairs and generate 2000 single strand breaks per cell nucleus, as well as approximately 80 DNA DSBs per nucleus. In addition to hydroxyl-mediated attack on chromatin, 30% of DNA damage is a consequence of direct interaction with ionizing particles.

NRF2 promotes repair of damaged DNA

Base excision repair

Damaged bases, abasic sites, and single strand breaks are repaired by the base excision repair pathway. This pathway can be briefly summarized as follows: a) base recognition and excision by lesion-specific DNA glycosylases, b) incision by endonucleases [43] is followed by formation of an abasic site, c) replacement of the excised base, and d) appropriate end-terminal processing and ligation [44]. These steps are performed with the aid of enzymes such as DNA Polymerase β, poly(ADP-ribose) polymerase 1 (PARP1), and XRCC1/LIG3 [42].

8—oxo-7,8-dihydroguanine (8-oxoGua) is a highly mutagenic DNA lesion that is detected in x-irradiated cells [45{Chen, 2001 #108][45, 46] . It can cause GC to TA transversion mutations [47]. 8-oxoGua lesions are repaired by the enzyme 8-oxoguanine DNA glycosylase (OGG1). Human OGG1 is located on chromosome 3p25 [48]. Alternative splicing of OGG1 results in expression of mitochondrial and nuclear proteins [48]. Dhenaut et al. [49] have shown that the human OGG1 promoter harbors an ARE 29 base pairs from the transcriptional start site. Analysis of 2 kb of the OGG1 promoter region using a luciferase fusion reporter provided evidence of a functional ARE [49]. Chromatin immunoprecipitation (ChIP) assays confirmed that Nrf2 binds to the OGG1 promoter[50] and RNAi experiments demonstrated that NRF2 deficiencies suppressed OGG1 expression [50]. The research of Hyun et al. [51] has shown that OGG1 deficiencies increase the radiation sensitivity of human cells, thus supporting a hypothesis that posits a mechanistic link between Nrf2, repair of DNA base damage, and radiation sensitivity.

Repair of DNA double strand breaks

Radiation-induced lethal damage is a consequence, in large part, to a DNA DSBs. The majority of DNA DSBs are generated by 2 independent hydroxyl radicals that interact in close proximity [34]. DSBs are repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR). In brief, canonical NHEJ, which operates in all phases of the somatic mammalian cell cycle but represents the major pathway in G_0/G_1 cells [52-54], is activated by DNA DSBs which the Ku70/Ku80 complex binds to and stabilizes [55].

DNA-dependent protein kinase is then recruited to the breaks, becomes activated, and phosphorylates target proteins, including the endonuclease Artemis that processes DNA ends with overhangs. Finally, the XRCC4-DNA ligase 4 (LIG4) complex is recruited and ligates the DNA strand with the help of the XRCC4-like factor (XLF). In addition to XRCC4-LIG4–dependent C-NHEJ, at least one alternative end-joining (A-EJ) pathway exists. This pathway involves microhomology and is mainly used in cells with defects in C-NHEJ [52-54].

HR, which is restricted to late S and G2 cells, begins with resection of the 5'-strands within the DNA DSB, yielding 3'-single stranded DNA overhands that are coated with replication protein A (RPA) [55, 56]. RAD51 then displaces RPA, forming a nucleoprotein filament (one RAD51 monomer for every 3 nucleotides). The filament aligns with homologous DNA sequences on a sister chromatid, forming a structure termed a D-loop. Strand invasion, followed by the capture of a second 3'-single strand DNA overhang that was created by the resection process, forms a Holliday junction. Finally, the junction is dissolved (the major pathway) or cleaved to generate double strand DNA. Cleavage can produce non-crossover and crossover products. Experimental evidence acquired to date indicates that non-crossover products predominate. In addition, there is an alternative HR pathway termed synthesis-dependent strand annealing that is independent of Holliday junction formation.

The choice of repair pathway is impacted by whether or not the broken DNA ends undergo extensive 5' to 3' nucleolytic resection that generates 3' DNA overhangs [57]. Resection promotes initiation of the HR pathway. The question of whether resection will be regulated by the interactions of 53BP1 and breast cancer type 1 susceptibility protein (BRCA1) was reviewed in [58]. A model has been proposed in which 53BP1 is phosphorylated in G1 cells by ataxia telangiectasia mutated (ATM), localizes to sites of DNA breaks, and attenuates HR. In S and G_2 cells the protein CtIP is phosphorylated by CDK, inducing the formation of a complex with BRCA1 and Mre11/Rad50/Nbs1. This complex displaces 53BP1 and initiates resection [58].

Emerging research has shown that transcriptional regulation of 53BP1 is regulated in part by Nrf2. Kim et al. [59] examined 53BP1's proximal promoter region for *cis* regulatory elements and found 3 putative AREs in normal human colonic epithelial cells. ChIP assays confirmed Nrf2 binding to all 3 *cis* acting AREs. RNAi-mediated suppression of Nrf2 prevented the electrophilic triterpenoid bardoxolone methyl from protecting human colonic epithelial cells from radiation-induced cytotoxicity [59]. These results are consistent with the work of others who have shown that 53BP1 deficiencies increase radiation sensitivity [60-62].

BRCA1 has several significant roles in the DNA DSB repair process, including regulation of CtIP-mediated DNA end resection [55]. BRCA1 deficiencies result in elevation of ROS and a corresponding radiation hypersensitivity phenotype [63]. Thus, BRCA1 promotes high fidelity DNA repair while suppressing genotoxic ROS. The mechanism involves BRCA1's ability to bind to Nrf2's proximal promoter and positively regulate Nrf2 mRNA expression [64, 65]. In addition, BRCA1 physically interacts with and stabilizes Nrf2, thereby

promoting Nrf2-mediated promoter transactivation [65]. These concepts are summarized in Figure 1.

Loss of BRCA1 increases the risk of breast, ovary, and fallopian tube cancers (see reference [66] for review). Loss of BRCA1 (or BRCA2) in cancer can be exploited by targeting PARP1, a facilitator of DNA repair [67]. Recently, Wu et al. [68] found that PARP1 interacts directly with small Maf proteins, NRF2's heterodimeric partners, and that this complex augments Nrf2's ARE-specific DNA binding, thereby enhancing Nrf2-dependent gene transcription. Thus, one may hypothesize that the therapeutic efficacy obtained by molecular targeting of PARP1 in cancer could be due in part to suppression of Nrf2-mediated DNA repair.

At a biochemical level, acquisition of DNA damage in mitotically active mammalian cells activates the Mre11/Rad50/NBS1 sensor complex whose binding and processing of DNA damage initiates ATM and/or ATR activation, which are followed by transduction of signals to downstream effector pathways [69]. One of these effector proteins, TP53 (p53), is an important regulator of radiation-induced cell cycle checkpoint signaling and apoptosis [70]. ATM-mediated activation of p53 has been shown to suppress Nrf2-mediated target gene expression and contribute to an enhanced apoptotic response [71].

Nrf2 and induction of apoptosis following X-irradiation

Although it is well established that hydroxyl radical-induced nuclear DNA damage is responsible for a significant proportion of cell death following exposure to ionizing radiation, it appears that superoxide generated hours to days after irradiation can also impact radiation sensitivity. Gao et al [72]found that radiation resistance was increased in human glioma cells over expressing SOD1. Similarly, Petkau [73] found that SOD1 activity increased the recovery of hematopoietic myeloid progenitor cell recovery in X-irradiated mice. The mechanisms appears to be a consequence of suppression of superoxide-mediated induction of apoptosis [72]. Although radiation-induced apoptosis is not considered to play a large role in cell death sub-routine execution, computerized video analysis of cell death by Dewey and colleagues demonstrated that apoptosis during interphase was the primary mode of cell death initiated by mitotic catastrophe [74, 75]. SOD1 is a Nrf2 target gene [76]. Thus, one may hypothesize that elevated levels of Nrf2 would lead to increased SOD1 expression and radioprotection.

In total, these studies show that NRF2 contributes to a pro-survival response due to an enhanced detoxification of pathogenic superoxide and promotion of •OH-mediated DNA damage repair. As many human cancers are characterized by elevated NRF2 and NRF2-mediated gene expression [23, 77, 78], the question of targeting NRF2 for treatment of radioresistant disease gains importance. However, this question needs to be addressed in the context of the radiation response of normal tissue.

The radiation response of normal tissue is NRF2-dependent

Stem and progenitor cells are defined by their self-renewal capacity and ability to differentiate. The intrinsic radiosensitivity of mammalian hematopoietic stem and progenitor

cells (defined as LIN^{-} $SCA1^{+}$ $c\text{-}KIT^{+}$) dictates the LD_{50} dose. Irradiated stem cells can undergo apoptosis, cell cycle arrest, or senescence, all initiated by DNA damage [79, 80], and these responses can negatively impact cell survival and homeostasis, which are critical for prevention/mitigation of the life-threatening Acute Radiation Syndrome (ARS). Recently it has been shown that the $Nrf2^{-/-}$ mouse exhibits a greater radiation sensitivity compared to the wild-type mouse and that the severity of ARS can be partially mitigated by pharmacological activation of NRF2 [59, 79].

Life-threatening radiation-induced pulmonary fibrosis, characterized by the loss of parenchyma, the progressive accumulation of myofibroblast progenitors, the development of fibrosis, and the subsequent remodeling of lung interstitium, presents 6 to 24 months after absorption of ionizing energy ([81] and references therein, Figure 2). Transforming growth factor (TGF)-β and ROS contribute significantly to the pathogenesis of radiation-induced pulmonary fibrosis [82, 83]. Travis et al. [81] found that Nrf2 binds to CAGA elements in the proximal promoter of the TGF-β1 target gene plasminogen activator inhibitor (PAI)-1 and suppresses its expression. Others have found that transduction of TGF-β1/Smad2/3 target genes collagen 1A1, fibronectin-1, tissue inhibitor of matrix metalloproteinase 1, and PAI-1 were suppressed by elevated levels of Nrf2, a consequence of genetic targeting of Keap1 or electrophilic isothiocyanate inactivation of Keap1 [84, 85]. Consistent with these molecular studies, Travis et al. [81] found that PAI-1 expression in lung was elevated in thoracic irradiated $Nrf2^{-/-}$ C57BL mice compared to irradiated wild-type mice. Irradiated $Nrf2^{-/-}$ mice had fewer alveoli compared to their wild-type counterparts and these were more distended. Most importantly, the life span of $Nrf2^{-/-}$ mice was shortened by thoracic irradiation [81].

Rana et al. [86] found that Nrf2 deficiency was associated with a drastic overall decrease in bone volume after irradiation, as quantified by microCT analysis. Loss of bone volume in $Nrf2^{-/-}$ mice was associated with a decrease in osteoblast mineralization and an increase in osteoclasts. RT-PCR analysis of calvarial osteoblasts revealed that in the absence of Nrf2, expression of RANKL was increased after irradiation but could be suppressed by treatment with N-acetyl cysteine, implicating a role for ROS in radiation-induced bone injury.

Inflammatory cytokines are critical to the radiation response of normal tissue (reviewed in [87]) and Nrf2's role in regulating cytokine expression in inflammatory disease is well characterized ([88] and references therein). Although not well studied, one may hypothesize that part of Nrf2's role in promoting cell survival following irradiation is linked to modulation of the cytokine response.

Summary

Nrf2 promotes cell survival in irradiated cells and tissues through ROS detoxification, supporting DNA repair and potentially modulating cytokine responses. Although overexpression of NRF2 in human cancer cells may produce a radioresistance phenotype, there is a strong possibility that targeting NRF2 will result in significant normal tissue toxicity. However, administration of NRF2 activators has the potential for ARS mitigation following deployment of a radiological dispersal device.

Acknowledgments

This work was supported in part by NIH/NHLBI grant RO1HL112286. We wish to thank Melissa Stauffer, PhD for her careful reading of the manuscript.

Abbreviations

ARE	antioxidant response element
ARS	Acute radiation syndrome
ATM	Ataxia telangiectasia mutated
BRCA1	Breast cancer type 1 susceptibility protein
ChIP	Chromatin immunoprecipitation
CnC	Cap ‘n’ collar family of basic leucine zipper transcription factors
DSB	Double strand break
HR	Homologous recombination
Keap1	Kelch-like ECH-associated protein 1
LD_{50}	Lethal dose for 50% of a population
MEF	Mouse embryo fibroblast
NADPH	Nicotinamide adenine dinucleotide phosphate
NHEJ	Non-homologous end joining
NRF2	Human nuclear factor (erythroid-derived 2)-like 2 protein
Nrf2	Mouse nuclear factor (erythroid-derived 2)-like 2 protein
OGG1	8-Oxoguanine DNA glycosylase
•OH	Hydroxyl radical
PARP1	Poly(ADP-ribose) polymerase 1
RNAi	RNA interference
ROS	Reactive oxygen species
RPA	Replication protein A

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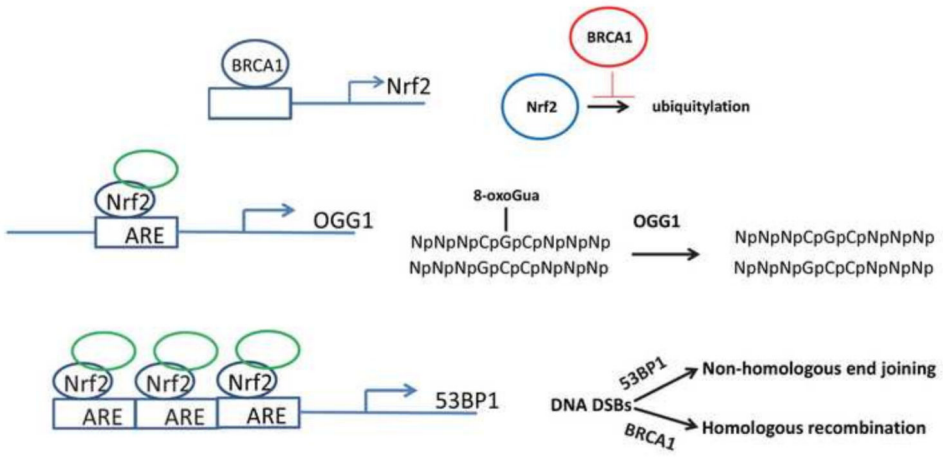

Figure 1.

Role of Nrf2 in repair of DNA damage. BRCA1 can promote Nrf2 expression by both increasing transcription and inhibiting Nrf2 degradation. Nrf2 has been shown to bind to AREs located in the proximal promoters of the OGG1 gene and 53PB1, thereby increasing expression of these DNA repair proteins.

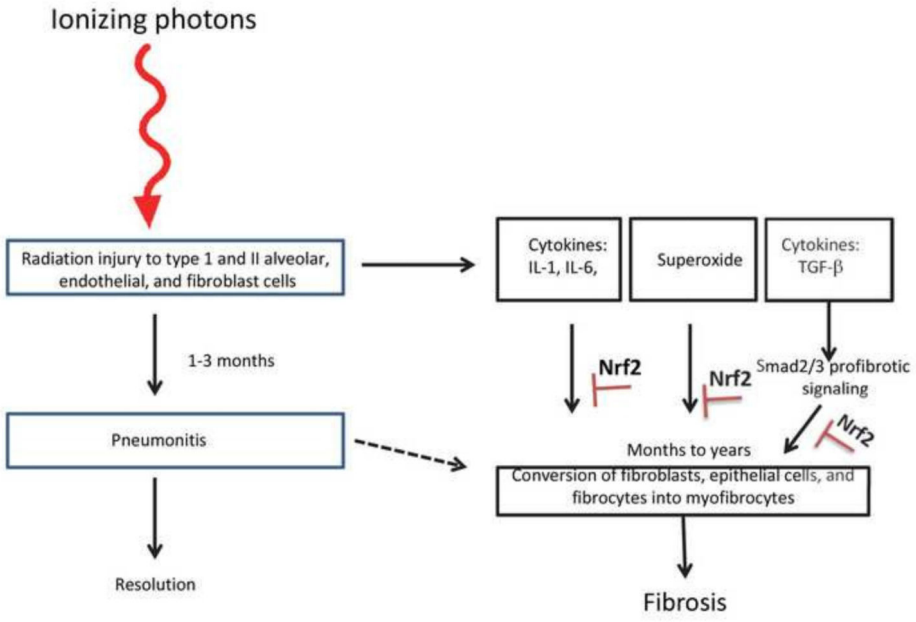

Figure 2.

Brief overview of the development of radiation-induced pulmonary fibrosis, summarized from refs [89, 90]. Low linear energy transfer ionizing radiation injures epithelial and stroma cells. One to 3 months later pneumonitis can develop, which can either resolve, convert to chronic inflammation or convert to fibrosis. The relationship, if any, between pneumonitis and fibrosis is not well characterized. Irradiation results in cytokine production, exemplified by IL-1, IL-6 and TGF-β. Additionally, chromic superoxide generation is observed. Cytokines and superoxide contribute to myofibroblast generation, which promotes collagen deposition and the development of fibrosis. Nrf2 can inhibit fibrosis due to its anti-inflammatory activity, it ability to induce antioxidant enzymes and its suppression of R-Smad-dependent expression of pro-fibrotic gene expression.