Published in final edited form as:

Free Radic Biol Med. 2012 July 15; 53(2): 337–346. doi:10.1016/j.freeradbiomed.2012.04.014.

Temporal expression of hypoxia-regulated genes is associated with early changes in redox status in irradiated lung

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Abstract

The development of normal lung tissue toxicity after radiation exposure results from multiple changes in cell signaling and communication initiated at the time of the ionizing event. The onset of gross pulmonary injury is preceded by tissue hypoxia and chronic oxidative stress. We have previously shown development of debilitating lung injury can be mitigated or prevented by administration of AEOL10150, a potent catalytic antioxidant, 24 hours after radiation. This suggests that hypoxia-mediated signaling pathways may play a role in late radiation injury, but the exact mechanism remains unclear. The purpose of this study was to evaluate changes in the temporal expression of hypoxia-associated genes in irradiated mouse lung and determine whether AEOL10150 alters expression of these genes. A focused oligo array was used to establish a hypoxia-associated gene expression signature for lung tissue from sham-irradiated or irradiated mice treated with or without AEOL10150. Results were further verified by RT-PCR. 44 genes associated with metabolism, cell growth, apoptosis, inflammation, oxidative stress and extracellular matrix synthesis were upregulated after radiation. Elevated expression of 31 of these genes was attenuated in animals treated with AEOL10150, suggesting that expression of a number of hypoxia-associated genes are regulated by early development of oxidative stress after radiation. Genes identified herein could provide insight into the role of hypoxic signaling in radiation lung injury, suggesting novel therapeutic targets, as well as clues to the mechanism by which AEOL10150 confers pulmonary radioprotection.

Keywords

radiation-induced lung injury; oxidative stress; hypoxia-inducible factor; inflammation

INTRODUCTION

The risk of radiation-induced normal tissue toxicity limits the therapeutic dose of radiation that can be used in treating human cancers. This limitation is a significant barrier to

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors.

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CONFLICT OF INTEREST

Isabel L. Jackson is a consultant for AEOLUS Pharmaceuticals, Inc. All other authors declare no conflict of interest.

achieving successful tumor control in all tumor types, but it is particularly confounding in treating tumors of the thoracic region because of the extreme radiosensitivity of the lungs. Although the mechanisms underlying radiation-induced normal tissue injury have been partially elucidated, the way in which radiation affects specific inter- and intracellular signaling pathways and inflammatory mediators is not completely understood.

It is well known that the initial ionizing event not only directly damages DNA causing apoptosis or mitotic catastrophe, but also affects cellular macromolecules, and lipid membranes. The initial cellular damage begins a series of reactions that ultimately result in chronic oxidative stress, tissue hypoxia, inflammation, and fibroproliferation. If left unresolved, these conditions may result in tissue damage, diminished quality of life, and complete organ failure.

The development of chronic oxidative stress within irradiated tissue begins during the initial exposure and is perpetuated by molecules activated within minutes or hours post irradiation (1). Transforming growth factor- β (TGF- β 1), a pro-inflammatory and pro-fibrogenic cytokine, is a prominent effector in the response of tissue to radiation. Within seconds of radiation exposure, TGF- β 1 is activated by cleavage of the active protein from its latency-associated complex by hydroxyl radical (2). Once activated, TGF- β 1 can induce synthesis of NADPH oxidase, an oxidant-generating enzyme (3, 4). The increase in active NADPH oxidase causes chronic ROS/RNS overproduction, creating an imbalance in free radical production and the antioxidant capabilities of the cell. This self-perpetuating increase in production of radical species in the cell causes continuous activation of pro-apoptotic, hypoxia-mediated, pro-inflammatory, and pro-fibrogenic pathways long after the initial radiation exposure.

Continuous activity of these pathways contributes significantly to vascular dysfunction and tissue hypoxia (1, 5). As areas of hypoxia develop, inflammatory cells, most notably macrophages, are recruited to the site of injury where they undergo the respiratory burst further increasing tissue levels of ROS/RNS(6). These high levels of ROS/RNS are responsible for activation of molecules involved in radiation-induced normal tissue injury, including TGF- β 1, NF- κ B, and HIF-1 α (7, 8). The cyclic nature of these events creates a self-perpetuating pathologic environment characterized by chronic oxidative stress, tissue hypoxia, and continual activation of redox-regulated signaling molecules and pathways involved in inflammation and fibroproliferation.

In our previous studies, we have shown the potent catalytic antioxidant, AEOL10150 (9) can mitigate or prevent radiation-induced lung injury in a rodent model when given prior to or following radiation exposure(10, 11). In these studies, treatment with AEOL10150 significantly reduced oxidative damage to DNA (8-OHdG), TGF- β mediated fibrosis, and expression of hypoxia-inducible factor-1 α (HIF-1 α) and its downstream products CAIX and VEGF (11).

In this study we hypothesized the development of radiation-induced lung injury is facilitated by genes upregulated by hypoxia and that expression of these genes is affected by redox-mediated signaling. To investigate this hypothesis, we used a hypoxia signaling pathway specific Oligo DNA microarray to examine hypoxia-associated gene expression in C57BL/6J mouse lungs 1 day, 3 days, 1 week, 3 weeks, 6 weeks, and 6 months after a single dose of 15 Gy to the whole thorax. Next, we evaluated whether the potent catalytic antioxidant AEOL10150 could influence expression of select hypoxia-associated genes when administered for four weeks following radiation exposure.

The transcriptome profile for hypoxia-associated genes identified in this study provides valuable insight into the sequence of events associated with development of normal tissue

injury following radiation exposure. We hypothesize that the protective effect of AEOL10150, shown in our previous studies (10, 12), is due to its effects on redox-regulated cell signaling, which may include, but is not limited to, hypoxia induced gene expression. Additional evaluation of hypoxia-associated genes not affected by AEOL10150 may lead to a better understanding of the pathological mechanisms underlying the development of injury and may identify new targets for therapeutic intervention.

MATERIALS AND METHODS

Animal Irradiation

Female C57BL/6J mice (8–10 weeks) received a single 15 Gy dose of x-ray irradiation (Therapax 320, Pantak Inc., East Haven, CT) at a dose rate of 67cGy/min using a beam energy of 320 kV (75 SSD, HVL $\approx$ 2.00 mm Al). Radiation was delivered to the whole thorax through adjustable apertures (1.25–1.5 cm) with 8 mm lead shielding of the rest of the body. Sham-irradiated animals were subjected to the same scenario, but the radiation source was not turned on. All mice were anesthetized before irradiation with an intraperitoneal (i.p.) injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture. Radiation dosimetry was performed using a calibrated ionization chamber and single-use MOSFET radiation detectors placed in tissue equivalent material or within the chest cavity of non-study related C57BL/6J mice (13, 14). The variation in dose rate across the field profile was less than 6%. Animals were euthanized and tissue harvested at pre-determined time points of 1 day, 3 days, 1 week, 3 weeks, 6 weeks, and 6 months. At the time of euthanasia (>250mg/kg sodium pentobarbital, i.p.), lungs were snap frozen in liquid nitrogen and stored at -80°C (n = 7/group). All studies were performed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center.

AEOL10150 regimen

To determine whether AEOL10150 affects the transcriptome profile of hypoxia-associated genes, a separate cohort of animals was randomized into the following groups: a) sham-irradiation, b) AEOL10150 alone, c) 15 Gy whole thorax irradiation (WTI), and d) 15 Gy WTI + AEOL10150. Each group contained 6 animals. AEOL10150 was supplied by Aeolus Pharmaceuticals (San Diego, CA). Animals receiving AEOL10150 were given a loading dose of 40 mg/kg/day by subcutaneous injection 2 hrs after radiation. For the remainder of the study animals were treated with a maintenance dose of 20 mg/kg every other day for 4 weeks.

Histopathology and Immunohistochemistry

To visualize histopathologic damage in sham-irradiated and irradiated tissue at each of the predetermined time points, hematoxylin and eosin (H&E) staining was carried out as previously described (15). Immunostaining for carbonic anhydrase was performed as previously described by Gauter-Fleckenstein et al (16). Briefly, 5 micron thick paraffin embedded tissue sections were deparaffinized and rehydrated using xylene and graded alcohol (100–80%) concentrations. Hydrogen peroxide (10%) was used to block endogenous peroxidase activity and antigen retrieval performed with citrate buffer (Biogenex, San Ramon, CA). Slides were incubated at 4°C overnight with a rabbit polyclonal antibody to carbonic anhydrase IX (1:200; Abcam, Cambridge, MA) followed by washing in phosphate buffered saline and incubation for one hour at room temperature with a donkey anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA). Slides were counterstained with Harris hematoxylin and visualized under light microscopy using a 40x objective.

Pimonidazole

Pimonidazole hydrochloride (70 mg/kg i.p, Chemicon International Inc, Temecular, CA) was injected three hours prior to euthanasia and immunostaining performed as previously described(17).

RNA isolation

Snap frozen whole lung tissue was homogenized in 5 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with the PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). The total RNA was isolated using TRIzol reagent following the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The RNA was repeatedly purified until the A260:A280 was greater than 2.0 and A260:A230 was greater than 1.7.

Probe preparation

cDNA synthesis, cRNA synthesis, and cRNA labeling were performed using the TrueLabeling-AMP 2.0 kit (SA Bioscience, Frederick, MD, USA) according to the manufacturer's manual. Briefly, 10 µg of pooled total RNA was used for the cDNA synthesis. The RNA pool was produced by mixing equal amount of total RNA from each sample in the same group. The cDNA was then subjected to cRNA synthesis and labeling with Biotinylated-UTP overnight at 37°C. After purification with SA Biosciences ArrayGrade cRNA cleanup kit (SA Bioscience, Frederick, MD, USA), 15 µg of biotin-labeled cRNA was used for hybridization.

Hybridization

A mouse hypoxia signaling pathway specific Oligo GE Array DNA microarray (SA Bioscience, Frederick, MD, USA, Cat#: OMM-032) was used to determine temporal changes in the expression of 113 genes following thoracic irradiation. Prior to hybridization, array membranes were pre-wetted with 5 ml of deionized water for 5 minutes and then pre-hybridized with 2 ml pre-warmed GEAhyb hybridization solution (SA Bioscience, Frederick, MD, USA, Cat#: H-01) for 4 hrs at 60°C. The array membrane was hybridized with 15 µg of purified biotin-labeled cRNA probes overnight at 60°C with continuous agitation. The membranes were then washed with Wash Solution 1 (2x SSC, 1% SDS) for 20 min at 60°C and followed by Wash Solution 2 (0.1x SSC, 0.5% SDS) for 15 min at 60°C with continuous agitation.

Chemiluminescent detection and data analysis

The signal was visualized using the Chemiluminescent Detection kit (SA Bioscience, Frederick, MD, USA, Cat#: D-01). Briefly, after blocking, the membranes were bound with alkaline phosphatase-conjugated streptavidin. After washing, the membranes were incubated with Chemiluminescent substrate for 5 min and exposed to x-ray film. Gene spots with signal intensity higher than the blank on the same membrane were considered detectable. The densitometry of GAPDH in the radiation group was normalized with that in control group. Densitometry for each gene spot was then normalized with the adjusted GAPDH. For the time point experiment, the average increased densitometry from two repeated experiments was graded in 5 expression degrees. One represents a weak increase and five represents the highest increase. If the gene spot was undetectable in both the control and irradiated mice in same time point, it was designated as 0. For the AEOL10150 regimen, the densitometry of each gene in 4 groups was directly compared. The percentage of reversal by AEOL10150 was calculated.

RT-PCR

Total RNA isolation was performed as described above. Reverse transcription was performed by incubating 3 µg of pooled total RNA, 2.5 µM of random primer, and 200 units of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), for a total reaction volume of 20 µL, for 10 min at 25°C followed by 60 min at 50°C. PCR amplification was carried out using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and two pairs of primers in one reaction, one amplifying the target gene fragment and another amplifying a 1 kb GAPDH fragment to serve as an internal control. PCR products were visualized on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. The ratio of PCR product of target gene to GAPDH better reflect the RNA level in the samples. Primer sequences and the size of PCR products are listed in Table 1.

Western blotting

Snap frozen left lungs were prepared for western blot analysis by immersing the lung in homogenization buffer, containing protease and phosphatase inhibitors (Roche Applied Sciences, Indianapolis, IN), and sonicating samples to disrupt cell membranes. Samples were centrifuged to remove undigested tissue. The supernatants were diluted 1:1 in Laemmli Sample Buffer (95% LSB, 5% β-Mercaptoethanol) (BioRad, Berkeley, CA). The prepared samples were then boiled and centrifuged prior to loading (10 µL per well) onto 10% SDS-PAGE gels. Following electrophoresis, protein was transferred onto polyvinylidene fluoride (PVDF) membranes and probed with a specific antibody to HIF-1α (Novus Biologicals, Littleton, CO), HIF-2α (Abcam, Cambridge, MA), or HIF-3α (Novus Biologicals, Littleton, CO) diluted in 5% non-fat milk in TBS+0.1% Tween20. Following secondary antibody incubation (Cell Signaling, Beverly, MA), signals were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Films were scanned using an HP ScanJet 3110. Band intensity was quantified using ImageJ Software (NIH, Bethesda, MD). Measured intensities were normalized using α-tubulin expression and then averaged within groups. Comparisons between irradiated and non-irradiated controls at each time point were made using a student's t-test.

RESULTS

Temporal changes in tissue oxygenation

Positive staining for carbonic-anhydrase IX, an endogenous marker of hypoxia, and the exogenous hypoxia marker, pimonidazole, was evident three days after radiation and was strongly increased at one week (Figure 1). The development of tissue hypoxia occurred prior to the overt onset of histopathologic damage (H&E stain, Figure 1). By six months, architectural distortion and tissue fibrosis can be seen.

Changes in HIF-1α, 2α, 2β, 3α mRNA expression after radiation

Differences in expression of HIF-1α, 2α, 2β, and 3α were examined by comparing RT-PCR of pooled total RNA from the lungs of the control and irradiated mice sacrificed at each time point (n= 7). HIF-1α mRNA expression was constant across all of the time points examined, whereas expression of HIF-2α was upregulated in irradiated mice at multiple time points. HIF-2β expression was upregulated in irradiated animals beginning 1 week after radiation whereas expression of HIF-3α was not upregulated until 6 months post-radiation (Figure 2).

HIF-1α, 2α, 2β, 3α protein expression

Western blot analysis suggests a dynamic role for HIF-1α, -2α, and -3α. Assessment of protein expression showed that HIF-1α protein expression is elevated in irradiated animals

24 hours after radiation, but does not differ significantly at any other time point until 6 months post-irradiation, when HIF-1 α expression is reduced relative to untreated controls (Figure 3A). HIF-2 α expression was elevated 24 hours post-irradiation and again 6 months following radiation. Differential expression was also observed at the one-week time point, but was not different between irradiated and control animals at any intervening time points (Figure 3B). No differential expression of HIF-3 α protein was observed at any point following radiation (Figure 3C).

Genes with elevated post-radiation expression at all time points

Oligo array assay revealed that beginning one day after radiation, messenger RNA expression of *Adm*, *Agpat2*, *Ard1*, *CTGF*, *Eno1*, HIF-2 α , *Gna11*, *Prkaa1*, *Sdh1*, and *Tuba3* was elevated in irradiated animals relative to control. This increase continued throughout the six-month follow-up period (Table 2). One week after radiation increased expression of *Agtppb1*, *Angptl4*, HIF-2 β , *Nmyc1*, *Ppar- α* , and *Th* was observed. Expression remained elevated in all later time points (Figure 4).

Genes exhibiting a bi-phasic increase after radiation

As shown in Table 2, mRNA expression of *Cygb* and *Dapk3* is briefly increased at two time points, the first beginning one day after radiation and lasting no more than a few days, and the second beginning 6 weeks after radiation and continuing to the 6 month time point. *Dctn2*, *Gap43*, *Man2b1*, *Rora*, and *Rps7* mRNA expression was elevated for periods of differing duration 3 days after radiation and again 6 weeks post-radiation.

Genes expressed during the development of symptomatic injury

Six weeks after radiation, an increase in mRNA expression of *Car12*, *Cdc42*, *Eef1a1*, *Htatip*, IL-1 β , *Lep*, *Lipe*, *Mmp14*, *Plod3*, and TGF- β 1 was observed in all irradiated animals. Expression of *Dr1*, *Fabp4*, *Fnbp3* and *Slc2a1* was elevated only at 6 months after post-radiation (Table 2).

AEOL10150 normalizes hypoxia-associated gene expression 6 weeks after radiation

To further investigate changes in the hypoxia-associated transcriptome, we analyzed expression of the same 113 genes, 6 weeks after radiation and treatment with AEOL10150, using a hypoxia signaling pathway specific Oligo DNA microarray. Thirty of the 31 genes observed to be upregulated in mice following radiation alone showed no detectable increase in expression following treatment with AEOL10150 (Table 3). The only hypoxia gene that remained upregulated even when AEOL10150 was administered following radiation was *Add1*, the gene encoding adducin- α (Table 3). Messenger RNA expression was further confirmed by RT-PCR for 11 genes (Figure 5). Both RT-PCR and microarray showed no changes in *HIF-1 α* mRNA.

DISCUSSION

The development of tissue hypoxia is a key step in the progression of radiation-induced lung injury (1, 17, 18). In the current study, we aimed to identify genes induced by tissue hypoxia between the time of irradiation and development of lung injury. We furthermore sought to determine whether the introduction of a potent antioxidant, AEOL10150, during the first four weeks post-irradiation could mitigate expression of those genes six weeks into the course of disease development. This study found dynamic changes in hypoxia-inducible gene expression in lung tissue during the six months between the time of thoracic irradiation and development of pulmonary injury. Furthermore, we found hypoxia-inducible gene expression could be modulated by changes in the cellular redox environment resulting in

normalization of 30 of 31 genes upregulated after radiation. These findings are consistent with previous studies by other groups, who have suggested oxidative stress is a potent inducer of hypoxia-associated genes(19).

It is believed that the first signs of tissue hypoxia can occur within days following thoracic irradiation due to endothelial cell swelling leading to capillary occlusion (20). One of the most prominent gene families regulated by hypoxia are the hypoxia-inducible factors (HIF). HIF proteins are transcriptional complexes composed of alpha and beta subunits that can activate a wide number of downstream genes involved in cellular response to stress. The alpha subunit of the HIF complex is the main hypoxia sensor. Under normoxic conditions, the alpha subunit is rapidly degraded by the proteasome(21). However, under hypoxic conditions or oxidative/nitroxidative stress, the alpha subunit is stabilized and associates with its binding partner, HIF-1 β to form an activated transcriptional complex (21, 22). Three alpha subunit isoforms have recently been identified in mammalian tissues: HIF-1 α , HIF-2 α and HIF-3 α , all of which were evaluated in this study.

HIF-1 α and HIF-2 α are closely homologous and recognize hypoxia-responsive elements (HREs) in the promoter regions of a vast array of genes, including many of those found to be upregulated in this study (23–26). One of the most interesting findings in the current study was the contrast in mRNA expression between HIF-1 α and HIF-2 α following radiation. Although no increase in HIF-1 α mRNA was observed in irradiated lung tissue at any of the time points evaluated, HIF-2 α mRNA was strongly increased as early as 24 hours post-radiation. However, an increase in protein stabilization of both HIF-1 α and HIF-2 α subunits was observed. The stabilization of the alpha subunits of both HIF proteins was coupled with a mild increase in tissue hypoxia measured by CA-IX and pimonidazole staining. This data provides evidence that hypoxia develops in irradiated mouse lung within the first few days after exposure.

Recent studies using HIF-1 α or HIF-2 α knockout mice revealed different characteristics and functions of the two isoforms(27, 28). Unlike HIF-2 α -knockout mice, which develop multiple-organ deficiency and biochemical abnormalities (29), HIF-1 α -knockout mice mainly show abnormal vascular development and embryonic lethality(30). Furthermore, distinct transcriptional targets for HIF-1 α and HIF-2 α have also been proposed(31). Thus, the regulation of HIF exhibits a complex pattern in irradiated lung. Further clarification of the mechanisms and consequences of HIF activation in lung post-radiation is warranted.

One of the primary mechanisms by which the alpha subunit of HIF is stabilized under both normoxic and hypoxic conditions is through s-nitrosylation of a cysteine residue (Cys533) in the oxygen dependent domain(32). It is thought that oxidative stress is a more potent stabilizer of HIF than the prolyl hydroxylase pathway(33). Chronic oxidative stress is an important feature of radiation-induced lung injury and can be exacerbated by tissue hypoxia(1). Thus, it is not surprising that many of the genes induced after radiation in this study were those coding for oxidoreductases such as cytoglobin (Cygb), sorbitol dehydrogenase (Sdh1), procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 (Plod3), and tyrosine hydrolase (Th). Treatment with AEOL10150 resulted in reduced expression of both Sdh1 and Th (Table 3). Increased expression of oxidoreductases is consistent with the known increase in oxidative/nitroxidative stress in the lung following radiation exposure and attenuation of this increase in oxidative stress with antioxidant therapy has been shown to mitigate lung injury(1, 11, 16, 34, 35).

The induction of the gene encoding for cytoglobin at one to three days and again at six weeks to six months was particularly interesting. Cytoglobin is an oxygen-binding globin predominantly expressed in activated fibroblasts under hypoxic conditions (36). The primary

role of cytoglobin is to maintain the redox status of the cell during periods of oxidative stress by scavenging hydrogen peroxide, nitric oxide, and peroxynitrite (37–39). Several recent studies have evaluated cytoglobin as a therapeutic target in fibrotic disease (38–41). Xu et al. (39) found viral-mediated overexpression of cytoglobin in primary hepatic stellate cells prevented H₂O₂ mediated fibroblast differentiation, TGF- β 1 expression, and collagen synthesis. Thus, cytoglobin may prove to be a potential therapeutic target in the lung and warrants further exploration.

Our prior studies have shown a continuous and persistent increase in cell death between twenty four hours and six months post-radiation (42, 43). In this study, increased expression of the gene encoding for death-associated protein kinase (Dapk), a protein involved in programmed cell death, autophagy, and inflammation (44), was observed one and three days post-radiation and again at six weeks and six months. Death-inducing signals, such as TGF- β , IFN- γ , and Myc, as well as DNA damaging agents, can influence Dapk gene expression and increase Dapk-mediated apoptosis and autophagy of nutrient-starved cells (44). Dapk expression was reduced by more than 99% in animals treated with AEOL10150 after radiation. This observation is consistent with our previous studies, which demonstrated a significant decrease in cell death six weeks post-radiation in animals treated with AEOL10150 (42, 43).

Other interesting genes upregulated after radiation were those encoding dynactin subunit 2 (Dctn2) and growth associated protein-43 (Gap43), both of which are involved in tissue regeneration and cell proliferation/differentiation and genes involved in cell cycle progression (Cdc42), and DNA damage repair (Dr1). AEOL10150 mitigated expression of both Dctn2 and Cdc42, genes primarily involved in cytoskeletal reorganization and biogenesis, by more than 99%.

It is well known that hypoxia can stimulate both resident lung cells and inflammatory cells, such as macrophages, to secrete pro-inflammatory cytokines, chemokines, and growth factors (6, 45, 46). Interleukin-1 β (IL-1 β), a cytokine produced by activated macrophages, is an important mediator of cell proliferation, differentiation, and apoptosis as part of the inflammatory response (47–49). The gene encoding IL-1 β , as well as those encoding fatty acid binding protein 4 (Fabp4) and Leptin, both of which are involved in the inflammatory response, were all upregulated at six weeks after radiation. TGF- β 1 and matrix metalloproteinase-14 (MMP-14), which are involved in collagen deposition and fibrogenesis were also upregulated in this time frame. These later changes are indicative of initiation of late injury disease processes. Expression of peroxisome proliferator activated receptor- α (Ppara), which plays a role in the anti-inflammatory response by negatively regulating macrophage foamy cell differentiation (50), is upregulated at all time points after radiation. Increased Ppara expression is likely a compensatory response to the increase in foamy macrophages observed in C57BL/6J mice following radiation (51). Expression of IL-1 β , TGF- β 1, and Ppara was reduced in irradiated lungs of mice treated with AEOL10150 suggesting a reduction in overall pulmonary inflammation, which is consistent with the reduced lung weights and decreased macrophage infiltrate seen in rodents treated with AEOL10150 (11).

In conclusion, upregulation of hypoxia-associated genes begins following radiation and continues at least throughout the first six-month post-exposure. These genes participate in a variety of physiological and pathological functions, including hypoxia response, inflammation, oxidative stress, transcriptional regulation, signal transduction, cell metabolism, proliferation and differentiation, and apoptosis. Radiation-induced overexpression of 31 hypoxia related genes was suppressed six weeks after radiation following four weeks of post-radiation administration of AEOL10150, a potent catalytic

antioxidant. It has been previously demonstrated that AEOL10150 can attenuate development of late radiation-induced pulmonary injury in rats following single dose(10) or fractionated radiation(12). A relationship between early signaling changes and late injury would support the initiation of therapeutic interventions in the first few weeks post-exposure in order to protect the lungs from developing acute and chronic lung damage. This may not be the only therapeutic option, however, as the biphasic wave of gene expression observed in this study and in previous studies (52) suggests that there may be a larger window of opportunity for intervening in the progression of disease processes. More importantly to this study, however, was that the observed reversal of radiation-induced gene expression following treatment with AEOL10150 suggests that these genes may be involved in the cellular response to oxidative stress or the generation of ROS/RNS. Identifying the roles of these genes in pulmonary radiation injury could provide insight into the role of hypoxia-associated genes in radiation lung injury, widen the temporal window for therapeutic interventions, and introduce novel therapeutic targets.

Acknowledgments

We would like to thank Ross McGurk and Greta Toncheva for performing radiation dosimetry, validation, and quality assurance. This study was supported by the National Institutes of Health Grant U19-AI067798-06 and by AEOLUS Pharmaceuticals.

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Highlights

- Oxidative stress and tissue hypoxia underlie the pathogenesis of radiation injury in lung tissue
- Hypoxia-associated gene expression is dynamically altered in lung tissue after radiation
- mRNA expression and protein stabilization of HIF-1 alpha and HIF-2 alpha differ with respect to time
- AEOL10150 reduces expression of 31 of 32 hypoxia-associated genes elevated in response to radiation

Bars represent 100 μ m

Figure 1. The temporal progression of tissue hypoxia in lung after radiation

Tissue hypoxia is first noticeable around three days (CAIX, Pimonidazole) post-radiation and progressively increases throughout the follow-up period (6 months). At six weeks, the first histopathologic lesions are seen (H&E). These are generally focal in nature and are characterized by thickening of the alveoli wall and increased inflammatory cell infiltrate. By six months, tissue damage had considerably worsened and a greater number of focal lesions were observed. Error bars represent 100 μ m.

Figure 2. Temporal expression of HIF mRNA

The amplified PCR fragments were visualized on 1.5% agarose gel containing 0.5 $\mu\text{g/ml}$ ethidium bromide. The GAPDH and HIF genes were amplified in the same reaction. The top band shows a 1 kb GAPDH fragment and the bottom band shows the HIF gene fragment.

A

B

C

Figure 3. Western blot analysis illustrates the dynamic changes in stabilization of HIF-1 α (A) and HIF-2 α (B) proteins changes over time following irradiation, while HIF-3 α (C) remains constant

Western blot bands were visualized and normalized based on α -tubulin expression. The relative intensities were measured and averaged within groups and irradiated animals were compared to time matched controls. 1d: 1 day, 3d: 3 days, 1wk: 1 week, 3wk: 3 weeks; 6wk: 6 weeks, 6m: 6 months, C: control, R: radiation.

Figure 4. Representative Oligo GEArray hybridization images

The control for all of these time points shows a similar pattern. Arrows indicate gene spots with significant increases.

Figure 5. Expression of genes after AEOL10150 treatment

The amplified PCR fragments were visualized on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. The GAPDH and target gene were amplified in the same reaction. The top band shows a 1 kb GAPDH fragment and the bottom band shows the targeted gene fragment.

Table 1

RT-PCR primers and product size

Gene Symbol	Primer sequence	Fragment size (bp)
Adm-F	5'-AGCTGGTTTCCATCACCTG-3'	502
Adm-R	5'-CCTGGGAGGACTCCACAGTC-3'	
Angpt14-F	5'-GTGAGGACACAGCCTACAGC-3'	333
Angpt14-R	5'-AGGCTGCTGTAGCCTCCATG-3'	
CTGF-F	5'-CTGCCTACCGACTGGAAGAC-3'	495
CTGF-R	5'-ACATCTTCCTGTAGTACAGG-3'	
Eno-1 α -F	5'-GCCAGAGAGATCTTTGACTC-3'	443
Eno-1 α -R	5'-CCGTTGATCACATTGAAAGC-3'	
Gna11-F	5'-CAGAGCGCAGGAAGTGATC-3'	427
Gna11-R	5'-ACTCCTTCAGGTTTCAGCTGC-3'	
HIF-1 α -F	5'-CTCAGTTTGAACAACTGGAC-3'	680
HIF-1 α -R	5'-GTCGTGCTGAATAATACCAC-3'	
HIF-1 α -F	5'-CGTGGTGACCCAAGACGGTGA-3'	630
HIF-2 α -R	5'-AGCATCCGGTACTGGCCAGA-3'	
HIF-2 β -F	5'-CAGAACATATCCCAGATCTC-3'	439
HIF-2 β -R	5'-GGTCAGCAAAGTCCTCGATG-3'	
Ppara-F	5'-TGAAGCCATCTTCACGATGC-3'	446
Ppara-R	5'-ATCTCTTGCAACAGTGGGTG-3'	
Rora-F	5'-TCATTACGTGTGAAGGCTGC-3'	495
Rora-R	5'-CCAGATGCTGGTGTGTAGTC-3'	
Sord-F	5'-GAACAAGGTCCTTGTGTGTG-3'	427
Sord-R	5'-GTCCTCGATGCAAGCATGGA-3'	
GAPDH-F	5'-GGTGAAGGTCGGTGTGAACG-3'	990
GAPDH-R	5'-TGGAGGCCATGTAGGCCATG-3'	

Table 2

Increased ratio of gene expression

Gene Symbol	Description	Increased fold					
		1d	3d	1w	3w	6w	6m
Adm	Adrenomedullin	1	3	4	1	5	5
Apat2	1-acylglycerol-3-phosphate O-acyltransferase 2	2	3	4	1	5	5
Agtpbp1	RIKEN cDNA A230056J06 gene	0	0	1	2	5	2
Angptl4	Angiotensin-like 4	0	0	1	1	4	2
Ard1	N-acetyltransferase ARD1 homolog	1	3	5	1	3	2
HIF-2 β	Aryl hydrocarbon receptor nuclear translocator 2	0	0	2	1	2	3
Car12	Carbonic anhydrase 12	0	0	0	0	1	2
Cdc42	Cell division cycle 42 homolog	0	0	0	0	1	2
CTGF	Connective tissue growth factor	4	3	5	2	2	4
Cygb	Cytoglobin	1	1	0	0	2	4
Dapk3	Death-associated kinase 3	1	1	0	0	2	3
Dem2	Dynactin 2	0	1	0	0	2	3
Dr1	Down-regulator of transcription 1	0	0	0	0	0	3
Eef1a1	Eukaryotic translation elongation factor 1 alpha 1	0	0	0	0	1	3
EGFR	Epidermal growth factor receptor	0	0	1	1	0	0
Eno1	Enolase 1, alpha non-neuron	4	5	5	2	5	5
HIF-2 α	Endothelial PAS domain protein 1	5	5	5	3	5	5
Fabp4	Fatty acid binding protein 4, adipocyte	0	0	0	0	0	1
Fbnp3	Formin binding protein 3	0	0	0	0	0	1
Gap43	Growth associated protein 43	0	1	0	0	1	3
Gna11	Guanine nucleotide binding protein, alpha 11	2	3	4	2	5	3
Htatip	HIV-1 tat interactive protein, homolog	0	0	0	0	1	3
IL1p	Interleukin 1 beta	0	0	0	0	1	1
HIF-3 α	Hypoxia inducible factor 3, alpha subunit	0	0	0	0	0	1
Lep	Leptin	0	0	0	0	1	2
Lipe	Lipase, hormone sensitive	0	0	0	0	1	1
Man2b1	Mannosidase 2, alpha B1	0	1	1	0	3	4
Mmp14	Matrix metalloproteinase 14	0	0	0	0	1	3

Gene Symbol	Description	Increased fold					
		1d	3d	1w	3w	6w	6m
Nmyc1	Neuroblastoma myc-related oncogene 1	0	0	1	1	1	1
Plod3	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	0	0	0	0	1	2
Ppara	Peroxisome proliferator activated receptor alpha	0	0	2	1	5	3
Ppp2cb	Protein phosphatase 2a, catalytic subunit, beta isoform	0	0	2	0	1	2
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	1	1	4	1	5	4
Rora	RAR-related orphan receptor alpha	0	1	3	0	5	4
Rps7	Ribosomal protein S7	0	2	3	0	3	4
Sdh1	Sorbitol dehydrogenase 1	3	4	4	2	5	5
Slc2a1	Solute carrier family 2, member 1	0	0	0	0	0	1
Slc2a8	Solute carrier family 2, member 8	0	0	2	0	1	1
Snrp70	U1 small nuclear ribonucleoprotein polypeptide A	0	0	3	0	2	4
Sod2	Superoxide dismutase 2	0	0	1	0	0	0
TGF-β1	Transforming growth factor, beta 1	0	0	0	0	1	1
Th	Tyrosine hydroxylase	0	0	1	2	1	2
Tuba1	Tubulin, alpha 1	0	0	2	1	0	1
Tuba3	Tubulin, alpha 3	3	4	5	2	4	5

* d: day, w: week, m: Month. 0 = undetectable, 1 = weak increase, 5 = highest increase.

Table 3

The increased ratios of gene expression and reversal by AEOL10150

Gene Symbol	Description	Increased folds with radiation	% reversal with AEOL
Add1 α	Adducin 1 (alpha)	10.1	0
Adm	Adrenomedulin	11.6	84.1
Agpat2	1-acylglycerol-3-phosphate O-acyltransferase 2	10.8	85.2
Agtpbp1	RIKEN cDNA A230056J06 gene	8.6	94.2
Angptl4	Angiopietin-like 4	8.8	>99
Ard1	N-acetyltransferase ARD1 homolog	9	91
HIF-2 β	Aryl hydrocarbon receptor nuclear translocator 2	5.7	>99
Car12	Carbonic anyhydrase 12	1.3	>99
Cdc42	Cell division cycle 42 homolog	1.6	>99
CTGF	Connective tissue growth factor	1.7	>99
Dapk3	Death-associated kinase 3	1.6	>99
Dctn2	Dynactin 2	2.9	>99
Eef1 α 1	Eukaryotic translation elongation factor 1 alpha 1	1.2	>99
Eno1	Enolase 1, alpha non-neuron	11.2	76.3
Hif-2 α	Endothelial PAS domain protein 1	11.4	80.8
Gna11	Guanine nucleotide binding protein, alpha 11	8.1	95.2
IL1b	Interleukin 1 beta	1.7	>99
Man2 α b1	Mannosidase 2, alpha B1	2.8	>99
Mmp14	Matrix metalloproteinase 14	1.5	>99
Nmyc1	Neuroblastoma myc-related oncogene 1	1.3	>99
Ppara α	Peroxisome proliferator activated receptor alpha	7.1	>99
Ppp2c β	Protein phosphatase 2a, catalytic subunit, beta isoform	1.7	>99
Prka1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	3.7	>99
Rora α	RAR-related orphan receptor alpha	5.1	96.3
Rps7	Ribosomal protein S7	1.9	>99
Sdh1	Sorbitol dehydrogenase 1	5.9	95.7
Snrp70	U1 small nuclear ribonucleoprotein polypeptide A	1.5	>99
Sumo2	SMT3 suppressor of mif two 3 homolog 2	1.7	>99
TGF- β 1	Transforming growth factor, beta 1	1.4	>99
Th	Tyrosine hydroxylase	1.6	>99
Tuba3	Tubulin, alpha 3	5.5	89.4