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Oxidative Toxicology of Bleomycin: Role of the Extracellular Redox Environment

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Abstract

Bleomycin is a commonly used cancer therapeutic that is associated with oxidative stress leading to pulmonary toxicity. Bleomycin has been used in animal studies to model pulmonary fibrosis, acute respiratory distress syndrome, and pulmonary hypertension secondary to interstitial lung disease. The toxicity with bleomycin is initiated by direct oxidative damage, which then leads to subsequent inflammation and fibrosis mediated by generation of both extracellular ROS and intracellular ROS. While most studies focus on the intracellular ROS implicated in TGF β signaling and fibrosis, the changes in the extracellular redox environment, particularly with the initiation of early inflammation, is also critical to the pathogenesis of bleomycin induced injury and fibrosis. In this review, we focus on the role of extracellular redox environment in bleomycin toxicity, with attention to the generation of extracellular ROS, alterations in the redox state of extracellular thiols, and the central role of the extracellular isoform of superoxide dismutase in the development of bleomycin induced injury and fibrosis.

Keywords

EC-SOD; SOD3; extracellular superoxide dismutase; bleomycin; alveolar inflammation; acute lung injury; pulmonary fibrosis; redox potential

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The authors declare no conflict of interest

Bleomycin is used as a chemotherapeutic agent to treat a variety of cancers such as head and neck squamous cell carcinoma, ovarian cancer, testicular carcinomas, and lymphoma. Bleomycin, a water soluble glycopeptide antibiotic, was initially isolated and purified from *Streptomyces verticillus* [1]. The use of bleomycin in the clinical setting was recognized to cause pulmonary toxicity, predominantly lung fibrosis, attributed to the low levels of the bleomycin-inactivating enzyme, bleomycin hydrolase in the lung [2,3]. Patients who received supplemental oxygen during bleomycin treatment are at a further risk of developing exacerbated bleomycin induced pulmonary toxicity, suggesting an underlying importance of oxygen radicals [4–6]. The incidence of bleomycin induced pulmonary toxicity ranges from 6.8% to 46% of patients and the risk of pulmonary fibrosis limits the utility of bleomycin in cancer treatment [4,7,8]. This review focuses on the oxidative toxicity of bleomycin, with a particular focus on its impact on the extracellular redox environment.

Direct production of ROS by Bleomycin

Administration of bleomycin causes cellular toxicity via production of reactive oxygen species (ROS) and direct oxidative damage to the lung. To generate ROS, bleomycin forms a complex with molecular oxygen and divalent ions including iron. The pyrimidine and imidazole moieties that bind oxygen and iron result in the formation of free radical species such as $O_2^{\cdot-}$, hydroxyl radicals and Fe (III). The bleomycin complex then binds to DNA helix through a bithiazol ring, resulting in DNA strand breaks and lipid peroxidation [9,10]. The result is an increase in cellular injury, which is then followed by an inflammatory response. Iron drives the formation of hydroxyl radical from hydrogen peroxide via the Fenton or Haber-Weiss reactions, therefore several studies have tested the effects of deferoxamine, a free iron chelating agent, on bleomycin lung injury. Reduction in oxidative stress markers as well as partial or complete protection against bleomycin-induced cell or lung injury with deferoxamine supports a key role for iron and iron-dependent ROS production in bleomycin toxicity [11–14].

Use of Bleomycin for Animal Models of Lung Inflammation and Fibrosis

Based on the observation of bleomycin-induced pulmonary toxicity and fibrosis, bleomycin has been utilized extensively in animal models to interrogate mechanisms of pulmonary injury and fibrosis [15,16]. Bleomycin has been administered to animals via systemic and intratracheal routes in single or repeated dosing [17]. Regardless of the route, bleomycin consistently results in pulmonary fibrosis in pre-clinical models. However, the initial site of injury depends upon the mode of exposure, with endothelial injury occurring upon systemic delivery and epithelial injury upon intratracheal delivery. Increasing the dose of bleomycin augments injury, mortality, and to some extent fibrosis [18,19]. There are several discrepancies worth noting. In mice, the development of fibrosis in bleomycin models is variable by strain, with the commonly used C57BL/6 strain susceptible while BALB/c and C3Hf/Kam are resistant to the development of pulmonary fibrosis [20]. It has been suggested that the discrepancy is a result of variable bleomycin hydrolase expression in the lung across strains. With regard to the development of fibrosis, murine bleomycin models also lack key pathological findings of human disease and do not develop fibroblastic foci or parenchymal honeycombing which are the pathologic characteristics of usual interstitial

pneumonia. In addition the disease results in a non-progressive fibrosis that is reversible over time in single-dose models [15].

Intratracheal administration of bleomycin is also used as a model for acute respiratory distress syndrome (ARDS), because the early phase of injury is characterized by pulmonary edema formation, and inflammation [15,17]. Similar to bleomycin, unresolved ARDS is characterized by subsequent fibroproliferative changes [21]. Finally, bleomycin has more recently been adopted as a model of Group III pulmonary hypertension, reflecting late PH developing in the setting of interstitial lung disease. However, one drawback of using bleomycin as a model of ARDS or PH is the lack of direct clinical relevance as bleomycin is not generally a cause of ARDS or PH in patients [16].

Bleomycin Activates Extracellular ROS Production

The first evidence that extracellular ROS play an important role in bleomycin induced pulmonary toxicity and fibrosis comes from a series of early N-acetylcysteine (NAC) studies. NAC is a water soluble, poorly cell-permeable antioxidant that is capable of effectively scavenging $O_2^{\cdot-}$, $\cdot OH$, and $HOCl^-$ [22]. Studies have demonstrated that intraperitoneal injections of NAC attenuated the development of bleomycin induced fibrosis as measured by hydroxyproline and markers of oxidative injury [11,23].

Although highly reactive and unstable, $O_2^{\cdot-}$ has limited direct cellular targets. Thus, while extracellular $O_2^{\cdot-}$ may increase following exposure to bleomycin, its rapid dismutation to H_2O_2 and subsequent production of $\cdot OH$ and other reactive species may be directly responsible for the oxidative injury [24–27]. In addition, increased $O_2^{\cdot-}$ can react with NO to form the toxic nitrating species, $ONOO^-$ as discussed below, which can also mediate injury [24–26].

One major source of extracellular ROS in the setting of bleomycin administration was subsequently shown to be the NADPH Oxidase isoform, Nox2, which generates extracellular $O_2^{\cdot-}$ [28]. Manoury and colleagues demonstrated that absence of Nox2 abrogated PMA stimulated ROS production in BAL cells, reduced hydroxyproline, and increased pro-MMP9/TIMP-1 ratios [28]. Though this study was limited by the use of global rather than cell-specific knockout to address the role of Nox2, the authors reported that the loss of Nox2 was critical for recruitment of cells to the alveolar compartment, where they found an attenuation of macrophage numbers in bronchoalveolar lavage fluid (BAL). These data indicated a critical role for extracellular ROS in recruitment and activation of immune populations [28]. In addition, the study also demonstrated the importance of extracellular $O_2^{\cdot-}$ in activation of pro-fibrotic metalloproteinases central to subsequent fibrosis.

Myeloperoxidase (Mpo) is another enzyme capable of generating extracellular ROS, specifically $HOCl^-$. Similar to Nox2, Mpo is highly expressed in recruited innate immune cells including macrophages and neutrophils [29,30]. The predominant role of Mpo is to generate $HOCl^-$ as part of bactericidal function of these cells. It is well established that upon influx of recruited phagocytes to the alveolar space in response to bleo, their oxidative burst,

releases extracellular $O_2^{\cdot-}$ and $HOCl^{\cdot-}$ augmenting the damage to the cell lining the alveolar spaces and perpetuates further inflammation [31,32].

These limited studies identify distinct sources for extracellular ROS generation following bleomycin, unlike the majority of studies that focused on the intracellular ROS production such as Nox4 or the mitochondria, generated in response to bleomycin. These intracellular sources have been strongly implicated in TGF β signaling [33–35]. While there are links between different intracellular sources of ROS, for example, mitochondrial ROS can activate intracellular NADPH oxidase activity, the link between intracellular and extracellular ROS production has not been carefully studied [36].

Bleomycin and the Extracellular Redox Environment

One largely unexplored mechanism by which extracellular ROS may result in injury is via its effect on the extracellular redox environment. Since most antioxidant defense mechanisms are primarily intracellular, the extracellular redox environment is dictated primarily by the production and consequence of extracellular ROS, as discussed above, and can be assessed by measuring the ratios of key thiol redox couples in the extracellular microenvironment. Redox balance involves a coordinated set of reactions in which electrons are exchanged between reducing and oxidizing agents. The relationship between the reduced and oxidized state of a redox couple is expressed as the Redox potential (E_h), which is calculated using the Nernst equation. In the extracellular compartment, the redox couples include cysteine/cystine, (Cys/CySS), reduced and oxidized glutathione (GSH/GSSG) and reactive protein thiols on the cell membrane. CyS/CySS is the predominant indicator for extracellular redox potential in human plasma. Within the cell, the redox couples include CyS/CySS and GSH/GSSG as well as metabolic redox couples, e.g. NAD(P)H/NA(P)D⁺ or FADH/FAD⁺. An intricate system of enzymes including the thioredoxins (Trx), peroxiredoxins (Prx) glutathione reductase, and glutathione peroxidase regulate the detoxification of hydrogen peroxide and redox state of glutathione and NADH. The regulatory proteins Trx and Prx exist in oxidized and reduced states, which can be assessed by redox western blot [37–40].

In human studies and animal models, an oxidized E_h CySS is associated with an increased risk for cardiovascular disease, aging, and obesity. The extracellular CyS/CySS couples appear to control disease outcomes by modulating pro-inflammatory signaling [39,41,42]. Go and Jones proposed that an oxidized E_h CySS results in subsequent oxidation of thiols on key membrane proteins, which then transduces the signal to the intracellular compartment, activating signaling pathways. This concept is supported by data showing that an oxidized E_h CySS in aortic endothelial cells increases intracellular ROS and this signal is attenuated in cells overexpressing Trx2; implicating ROS derived from mitochondria. In addition, oxidized E_h CySS activates redox-sensitive transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) and nuclear factor erythroid 2-related factor 2 (Nrf2) [42,43].

Only one study has carefully examined how bleomycin impacts the extracellular redox potential in mice. Due to the decreased food intake in bleomycin treated mice, saline treated

mice were pair-fed equal amounts of food to control for the changes in dietary cysteine intake [9]. In this study, GSH/GSSG and CyS/CySS levels were measured in plasma and CyS/CySS in BALF at 1,3, and 7 days post bleomycin associated with the pro-inflammatory phase, and 14 days in the pro-fibrotic phases of lung injury [9]. There was a significant transient decline in plasma GSH levels at day 7, though no change in GSSG, resulting in oxidation of E_h GSSG; E_h GSSG returned to baseline by 14 days [9]. Plasma E_h CySS oxidation was observed at the later fibrotic phase [9]. The authors concluded that the oxidation of plasma E_h GSSG at 7 days reflected impaired GSH homeostasis in the inflammatory phase and an oxidized plasma E_h CySS may contribute to the pathogenesis of bleo-induced fibrosis [9].

In contrast, in the alveolar lining fluid, oxidation of E_h CySS was similar in both bleomycin treated mice and pair-fed controls, thus perturbations in Cys homeostasis in the alveolar compartment was attributed to the decreased food intake. Other studies have also demonstrated GSH depletion during the pro-inflammatory stage but have not investigated CyS/CySS levels or oxidation [44,45]. Given that pulmonary fibrosis is not reversible in human patients as it is in bleo-induced fibrosis in mice, it is plausible that persistent oxidized CyS/CySS and GSH/GSSG states in humans vs. resolution in mice underlie this discordant difference. In addition, pulmonary fibrosis is primarily a disease of “aging”, and oxidized CyS/CySS and GSH/GSSG increase with age, thus these factors collectively can serve as a “second-hit” to promote the persistence of fibrosis [46,47].

Extracellular Superoxide Dismutase – The Sole Extracellular Antioxidant Enzyme

The central role of extracellular ROS in bleomycin lung toxicity is further illustrated by a series of studies interrogating the impact of extracellular superoxide dismutase (EC-SOD) expression on bleomycin induced lung fibrosis. EC-SOD catalyzes the dismutation of $O_2^{\cdot-}$ radicals to H_2O_2 , resulting in detoxification of $O_2^{\cdot-}$ levels and limiting the reaction of $O_2^{\cdot-}$ with nitric oxide to form peroxynitrite (see Figure 1). These reactions serve not only to prevent superoxide and peroxynitrite induced damage, but also to preserve H_2O_2 signaling and nitric oxide bioactivity. Highly abundant in the vasculature and the lung, it is localized to the extracellular spaces via a heparin binding domain (HBD) [48]. A naturally occurring nonsynonymous single nucleotide polymorphism (SNP) within the HBD results in arginine to glycine substitution at position 213 (R213G) [49]. This results in the loss of bound EC-SOD and redistribution of EC-SOD into fluids (e.g. alveolar lining fluid and serum) [49–51]. With an overall low mean allele frequency of 3% in non-Hispanic Europeans and 6% in South Asian populations, carriers of the R213G SNP have decreased risk for inflammatory airway diseases but increased risk for cardiovascular events [49,51].

The level of EC-SOD expression determines the extent of the bleomycin induced injury and fibrosis. Mice lacking EC-SOD exhibit enhanced fibrosis and injury [52]. This was demonstrated by increased levels of hydroxyproline and bronchoalveolar lavage protein respectively [52]. Mechanistically, the loss of EC-SOD in the lung was associated with increased levels of 2-pyrrolidone, a marker of oxidative protein fragmentation [52].

Collectively, the study indicates that in the presence of bleomycin, the loss of bound EC-SOD in the lung further promotes oxidative fragmentation of collagen I, recruitment of activated innate immune cells, and increased fibrosis.

Conversely, mice overexpressing lung EC-SOD are protected against bleomycin induced injury and fibrosis as measured by lung wet/dry ratio and collagen [53,54]. In these mice, EC-SOD overexpression is driven by an SPC promoter to increase EC-SOD in type II alveolar epithelial cell overexpression to increase lung content by 3–4 fold [47,46]. Underlying these findings were reduced levels of redox-sensitive transcription factor, Egr-1, and the pro-fibrotic mediator TGF β [54].

Intriguingly, not only does the expression level of EC-SOD impact the response to bleomycin, but also the distribution of EC-SOD. In the mouse strain harboring the knock-in of R213G EC-SOD, while EC-SOD gene expression and enzyme activity are intact, R213G EC-SOD has a lower matrix binding affinity, resulting in a redistribution of active EC-SOD from the lung and vasculature into the circulation and alveolar lining fluid. In response to bleomycin, these mice demonstrate accelerated resolution of inflammation, decreased fibrosis and protection against subsequent PH [50]. In similar fashion to the studies utilizing overexpression of EC-SOD, R213G mice demonstrated attenuated TGF β mRNA expression, Col1a1 mRNA expression, collagen production, and fibrosis scoring in response to bleomycin[50,54]. With regard to inflammatory cytokines, R213G mice displayed acute increases in Cxcl1 and IL-6 levels, but displayed marked resolution when compared to WT mice [50]. Notably, despite overall low lung levels of EC-SOD, R213G mice exhibited increased ratios of reduced GSH/GSSG in the lung following bleomycin when compared to WT mice [50].

These studies not only demonstrate the importance of EC-SOD in regulating oxidative damage mediated by bleomycin, but also underscores the importance of the local extracellular redox microenvironment. Collectively, increased EC-SOD at the site of bleomycin induced injury, either due to increased production or redistribution of EC-SOD into the alveolar sites of inflammation and oxidative burst, is sufficient to abrogate the oxidative redox potential, accelerate resolution, and attenuate fibrosis.

Conclusions

In conclusion, bleomycin induced toxicity and fibrosis is associated with early direct ROS generation upon exposure. The local injury promotes inflammation, and leads to further extracellular ROS generation via Nox2 and Mpo. Alterations in extracellular ROS alters redox couples such as Cys/CySS and GSH/GSSG ratios, which can perpetuate oxidative signaling. The importance of the extracellular redox environment is highlighted by the protective effects of EC-SOD. EC-SOD is ideally situated in the extracellular spaces and is capable of attenuating bleomycin induced fibrosis through the detoxification of free extracellular O₂^{•-}. Future studies are needed to more fully define the regulation of the extracellular redox environment and determine how the changes in the extracellular environment regulate intracellular ROS signaling in the setting of bleomycin.

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Abbreviations:

ARDS	acute respiratory distress syndrome
Cys	cysteine
CySS	cysteine
EC-SOD	Extracellular Superoxide Dismutase
E_h	redox potential
GSH	glutathione
GSSG	glutathione disulfide
HOCl⁻	hypochlorous acid
H₂O₂	hydrogen peroxide
Nox2	NADPH Oxidase 2
O₂⁻	superoxide
Prx	peroxiredoxin
PH	pulmonary hypertension
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
TGFβ	transforming growth factor beta
Trx	thioredoxin

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Highlights:

- Bleomycin is a toxic chemotherapeutic that is used to model lung injury and fibrosis and is associated with increased production of reactive oxygen species.
- Production of reactive oxygen species occurs due to the direct effect of bleomycin resulting in local injury
- Increased extracellular reactive oxygen species skew the extracellular microenvironment towards an oxidized state, which further augments injury
- Extracellular superoxide dismutase protects against bleomycin induced inflammation and fibrosis

Fig 1.

Proposed summary of how insufficient EC-SOD permits bleomycin induced injury via decreased dismutation of free $O_2^{\cdot-}$ and oxidation of extracellular redox microenvironment.