Alveolar Macrophage Secretory Products Effect Type 2 Pneumocytes Undergoing Hypoxia-Reoxygenation

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Background. **Activation of the alveolar macrophage is centrally important to the development of lung ischemia reperfusion injury. Alveolar macrophages and type 2 pneumocytes secrete a variety of proinflammatory mediators in response to oxidative stress. The manner in which they interact and how the macrophage may influence pneumocyte responses in lung ischemia reperfusion injury is unknown. Utilizing an in vitro model of hypoxia and reoxygenation, we sought to determine if the proinflammatory response of type 2 pneumocytes to oxidative stress would be amplified by alveolar macrophage secretory products.**

Methods. **Cultured pneumocytes were exposed to control media or media from cultured macrophages exposed to hypoxia and reoxygenation. Pneumocytes were subsequently subjected to hypoxia and reoxygenation and assessed for both nuclear translocation of nuclear factor kappa B and inflammatory cytokine and chemokine secretion. To examine for any reciprocal interactions, we reversed the experiment, exposing macrophages to conditioned pneumocyte media.**

Results. **In the presence of media from stimulated macrophages, production of proinflammatory mediators by type 2 pneumocytes was dramatically enhanced. In contrast, exposure of the macrophage to conditioned pneumocyte media had an inhibitory effect on macrophage responses subsequently exposed to hypoxia and reoxygenation.**

Conclusions. **The alveolar macrophage drives the development of lung reperfusion injury in part through amplification of the inflammatory response of type 2 pneumocytes subjected to hypoxia and reoxygenation.**

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Advances in donor management, organ preservation, surgical technique, postoperative care and immunosuppression have improved overall survival rates after lung transplantation, but the incidence of lung ischemia reperfusion injury (LIRI) remains unchanged at 20% [1–3]. Clinically, LIRI is characterized by pulmonary edema, reduced pulmonary compliance, and poor gas exchange [4]. Treatment options are supportive. Patients surviving the initial insult face an upregulation of MHC class II molecules, which can vary depending on the severity of LIRI, increasing the risk of both acute rejection and obliterative bronchiolitis [5–7]. Characterization of the cellular mechanisms leading to the development of LIRI may lead to therapeutic interventions and improved outcomes.

Studies performed on in vivo rodent models have established the existence of a biphasic response to lung ischemia and reperfusion [8]. The early response occurs after 90 minutes of ischemia and 15 minutes of reperfusion and involves the nuclear translocation of proinflammatory transcription factors with subsequent upregulation of transcription and secretion of inflammatory cytokines and chemokines [9–11]. In the lung there is a transient increase in vascular permeability, together with a brief burst of oxidant release. As this phase has been shown to be neutrophil independent, resident cell types within the lung must be responsible for this early proinflammatory response. This initial release of mediators is critically important to driving the late phase of lung reperfusion injury, characterized by a sustained rise in vascular permeability and accumulation of neutrophils in the lung.

Alveolar macrophages (AM) are a rich source of oxidants, cytokines, chemokines, growth factors, and arachidonic metabolites [12]. Previous studies have implicated the AM as centrally important to the development of LIRI. Within 15 minutes of reperfusion, tumor necrosis factor-alpha (TNF-α) is localized exclusively to the AM but not other lung cell types. The AM depletion with liposomal clodronate or suppression with gadolinium is associated with significant reductions in vascular permeability, nuclear translocation of transcription factors, bronchoalveolar lavage fluid concentrations of TNF-α, macrophage inflammatory protein-2 (MIP-2), cytokine-induced neutrophil chemoattractant (CINC), and macrophage inflammatory protein-1α (MIP-1α), and accumula-

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Abbreviations and Acronyms

AM = Alveolar macrophage
CINC = Cytokine induced neutrophil chemoattractant
ELISA = Enzyme linked immunosorbent assay
EMSA = Electrophoretic mobility shift assay
ERK1/2 = Extracellular signal-regulated kinase
FBS = Fetal bovine Serum
H-R = Hypoxia/Reoxygenation
IL-1β = Interleukin-1 beta
JNK = c-Jun N-terminal kinase
LIRI = Lung ischemia reperfusion injury
MAPK = Mitogen-activated protein kinase
MCP-1 = Monocyte chemotactic protein-1
MHC = Major histocompatibility complex
MIP-1α = Macrophage inflammatory protein-1 alpha
MIP-2 = Macrophage inflammatory protein-2
NFκB = Nuclear factor kappa B
PAEC = Pulmonary artery endothelial cell
PBS = Phosphate buffered saline
PBST = Phosphate buffered saline with tween
RPMI = Roswell Park Memorial Institute Media
T2P = Type 2 pneumocyte
TNF-α = Tumor necrosis factor alpha

tion of tissue neutrophils [13, 14]. These findings indirectly suggest that AM-derived products are important to the development of injury. While the role of the AM in orchestrating inflammatory responses to oxidative stress in the lung is apparent, how they exert that influence has not been fully elucidated.

Another constituent cell population in the lung, type 2 pneumocytes (T2P), help control fluid balance and composition in the alveolar space and can proliferate and differentiate into type 1 pneumocytes to maintain alveolar integrity after lung injury [15]. The T2P have also been shown to upregulate proinflammatory signaling cascades in response to oxidative stress. In vitro studies of T2P established that nuclear translocation of nuclear factor kappa B (NFκB), and secretion of CINC and monocyte chemotactic protein 1 (MCP-1), are all increased in response to hypoxia and reoxygenation (H-R) [16].

The ability of the AM secretory products to augment inflammatory signaling in other lung cell types in response to oxidative stress has recently been described [17]. Chemokine secretion by pulmonary artery endothelial cells (PAEC) when subjected to H-R was shown to be enhanced when cells were exposed to inflammatory mediators secreted by AM. We hypothesized that a similar crosstalk exists between T2P and AM. Utilizing an in vitro cell culture model, we examined if T2P inflammatory signaling in response to oxidative stress was altered when cells were exposed to media collected from AM subjected to H-R. To examine for a reciprocal influence of T2P products on AM, the AM were subjected to the same oxidative stress in the presence of control media or media from T2P previously exposed to H-R.

Material and Methods

Reagents

All reagents were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise specified. Pentobarbital was obtained from the University of Washington Pharmacy.

Alveolar Macrophage Harvest

Pathogen-free, adult male Long-Evans rats (Simonsen Labs, Gilroy, CA) weighing 250 to 300 g were used for all experiments. The University of Washington Animal Care Committee approved all experimental protocols. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and "The Guide for the Care and the Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the Institute of Health.

Animals were euthanized with 120 mg/kg of intraperitoneal pentobarbital. A 14-gauge angiocatheter was inserted into the trachea through a midline neck incision and secured with a 4-0 braided silk suture. A median sternotomy was performed and the heart lung block rapidly excised. Intratracheal lavage of the lungs was performed 15 times with graduated volumes of 3 to 10 mL with cold phosphate-buffered saline (PBS) to minimize damage to lung tissue. This process yielded a 90% lavage recovery. Collected lavage fluid was centrifuged at 1,500*g* for 10 minutes and the cell pellet resuspended in serum free Roswell Park Memorial Institute Media (RPMI: Gibco BRL). Cells counts and viability were assessed by trypan blue exclusion methods, and RPMI added until a density of 500,000 cells per milliliter was reached. One mL of this cellular media was loaded for each well of a 12-well culture plate (Fisher Scientific, Pittsburgh, PA), and AM were incubated at 37°C for 60 minutes to allow adherence. Media was then substituted with fresh RPMI containing 5% heat-inactivated fetal bovine serum (FBS). The concentration of FBS was identical among all experimental and control groups.

Hypoxia and Reoxygenation

We generated three types of AM media for subsequent exposure to T2P. Control media was generated by plating AM and leaving them unstimulated for 6 hours. Early AM media was collected from AM that underwent 2 hours of hypoxia and 15 minutes of reoxygenation, and used to investigate the effects of AM products released during hypoxia or early in reoxygenation. This simulates the "early phase" of LIRI. Late AM media was generated by subjecting AM to 2 hours of hypoxia followed by 4 hours of reoxygenation and used to investigate the influence of AM products generated later in reoxygenation.

Prepared AM were incubated in a humidified hypoxic chamber (Coy Lab Products, Grass Lake, MI) with 0.5% oxygen for 2 hours at 37°C. Reoxygenation was achieved by removing the plate from the hypoxic chamber and placing it into a normoxic humidified incubator for either 15 minutes or 4 hours, which restores normal oxygen tension in the media within 2 minutes. Media was then aspirated and stored at −80°C. Media samples were