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· Cytokines and Chemokines in Direct Ischemia Reperfusion Injury of Lung and Cardiothoracic Transplant Rejection

The UW Distinguished Endowed Professor in Lung Transplant Research

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Lung transplantation, which was introduced into clinical practice nearly twenty years ago, has become an option for selected patients with end stage lung disease. Refinements in patient selection, perioperative care and immunosuppression have resulted in improved three-year survival rates of 70%. Despite these improved outcomes, ischemia-reperfusion, an unavoidable consequence of transplantation, compromises the early and late function of the transplanted lung. Twenty-five percent of transplant recipients experience some degree of reperfusion injury. In addition to acute morbidity, this acute inflammatory injury may compromise the long-term viability of the graft.

Attempts to alleviate immediate reperfusion injury in the grafted lung have focused on improving preservation techniques, minimizing ischemic times and modifying preservation solutions. More recently, a number of studies investigated the role of cytokines and inflammatory peptides in the pathophysiology of reperfusion injury. Roles for several cytokines in reperfusion injury in clinical lung transplantation have been postulated for some time, and animal studies suggest that these mediators may play a critical role. A number of cytokines have been identified (i.e. TNFα, IL-1β) as important mediators in our animal model of lung reperfusion injury. Inhibition of individual cytokines was found to provide only modest protection from injury, however, and has led us to investigate more proximal steps in the proinflammatory signaling cascade initiated by exposure of the lung to oxidative stress.

Reperfusion injury in rat lungs has been shown to be complement-dependent and oxygen radical mediated. It peaks in severity after four hours of reperfusion as assessed by tissue hemorrhage, vascular permeability and accumulation of neutrophils.

A model of hilar isolation for the study of ischemia reperfusion injury of rat lung has been reproducibly established and standardized in our laboratory. A pattern of nuclear factor kappa B (NFkB) and activator protein-1 (AP-1) transactivation has been established and determined to be centrally important to the development of lung injury in our model. We have also found that transcription factor activation is regulated by mitogen-activated protein kinase (MAPK) phosphorylation. MAPK are a group of intracellular signaling proteins activated by multiple stimuli, including inflammatory cytokines (TNFα), lipopolysaccharide, radiation, and ischemic injury. They are highly conserved serine/threonine kinases that require dual phosphorylation to become activated. We have characterized the functional significance of two MAPK in ischemia reperfusion injury: the stress-activated protein kinases (SAPK) p38 and c-Jun N-terminal kinase (JNK).

Lung injury as assessed by vascular leakage of ^{125}I labeled BSA has been determined as a measure of injury severity. The permeability index among negative (unmanipulated) controls is consistently 0.09 ± 0.05. Permeability doubled in animals undergoing only thoracotomy and mechanical ventilation. Ninety minutes of ischemia did not significantly increase mean permeability values; however, four hours of reperfusion resulted in an eight-fold rise in lung permeability to a mean index of 0.75 ± 0.01 ($p < .001$ compared to controls). In contrast, animals treated with a specific p38 inhibitor experienced a mean 50% reduction in permeability compared to injured controls ($p < .001$) while JNK inhibition reduced lung permeability by 35%. The lungs were also analyzed for myeloperoxidase (MPO) content as a measure of tissue neutrophil accumulation. Increased tissue neutrophil content is detectable after two hours of reperfusion, is significant by three hours and is

TLR-4 dependent SAPK activation appears to be the key molecular signaling event leading to the generation of lung ischemia reperfusion injury.

marked by four hours. In contrast, lungs from animals treated with p38 and JNK inhibitor demonstrated a 45% and 20% reduction in MPO content, respectively, compared to four hours in reperfused controls. The alveolar macrophage appears to be the key effector cell early in the reaction, and we are looking at its response to hypoxia and reoxygenation *in vitro* as well.

In addition, we are currently investigating the role of innate immune receptors in the generation of lung ischemia reperfusion injury. Toll-like receptor 4 (TLR-4), well known to initiate inflammatory signaling cascades in response to lipopolysaccharide, has also been suggested to respond to various other stimuli, including oxidative stress and products from injured and necrotic cells. TLR-4 has also recently been implicated in the modulation of reperfusion injury in other vascular beds. These data suggest that TLR-4 is an excellent candidate for initiating signaling in lung reperfusion injury. Utilizing molecular deletion techniques with short interfering RNA (siRNA) in our animal model, we have found that TLR-4 deletion is profoundly protective from reperfusion injury, reducing vascular permeability and MPO content by over 90% compared with positive controls. Western blotting of whole left lung homogenates detected significant reductions in SAPK phosphorylation with TLR-4 molecular deletion, implying that SAPK activation in lung ischemia reperfusion injury occurs via a TLR-4 dependent mechanism.

In addition to the direct lung ischemia reperfusion projects, we have investigated two *in vivo* models of thoracic transplantation. The first of these models investigates the major impediment to long-term survival in lung and heart lung transplantation: chronic rejection, which is histologically defined as obliterative bronchiolitis (OB). OB affects 33–60% of long-term lung and heart lung transplant recipients in recent series and more than 60% of patients in prior reports. Clinically, OB is characterized by progressive dyspnea, non-productive cough, reductions in the FEV-1 and mid-expiratory flow volumes. Treatment typically consists of intensification of immunosuppressive therapy or substitution of medications in a standard post-transplant triple medication regimen. Such therapy is at best capable of slowing the rate of progression, but this disease is characteristically progressive and ultimately fatal.

Recent investigations have attempted to define the mediators involved in the development of OB, but these experiments have been limited by the inability to develop a practical and reproducible model. Whole organ transplants are desirable, but such studies are confounded by technical complications, and the costs can be prohibitive. A technically simple model for airway transplantation with histopathologic features of OB has gained acceptance. This technique, originally described in mice and now adapted to rats, produces an experimental OB that is histologically indistinguishable from human OB. We have used this model to investigate the potential role of β-chemokines in the development of experimental OB.

In addition to a variety of other mediators, two of the β-chemokines, MCP-1 and RANTES, were studied for their potential role in the development of obliterative bronchiolitis. Rat tracheas and main stem bronchi were heterotopically transplanted into the subcutaneous tissue of allogeneically mismatched (BN- LEW) or syngeneically matched (LEW-LEW) recipients. Control animals received daily injections of phosphate-buffered saline (PBS) or non- immune rabbit serum; additional animals were treated with polyclonal blocking antibodies against MCP-1 or RANTES. Tissue was explanted at two weeks and examined histologically to quantify change in airway cross sectional diameter and loss of epithelium. Northern and Western blot analyses were performed to measure upregulation of MCP-1 and RANTES mRNA and protein.

Syngeneic control animals demonstrated mild to moderate peri-tracheal inflammation, but near complete preservation of respiratory epithelium and airway cross sectional area. In contrast, allograft controls demonstrated a dense pan-mural inflammatory response, near complete loss of respiratory epithelium and a 60% reduction in airway cross-sectional area. Animals treated with anti- MCP-1 or anti- RANTES antibodies had more limited histologic

changes, including only a 12% and 26% reduction in cross-sectional area respectively ($p < .001$). Levels of MCP-1 and RANTES mRNA were also increased in allograft tracheas but not in isografts. These data suggest that MCP-1 and RANTES play important regulatory roles in the development of experimental OB.

A heterotopic rat heart transplant model is also being used to determine the role of CC chemokines in heart allograft function and rejection. This model, which is technically challenging, involves a precise dissection of the donor heart using a 10x operating microscope followed by a hand sewn anastomosis using 8-0 suture. The hearts are explanted at various time points. The laboratory is currently gathering data on the role of chemokine blockade on cytokine expression and abrogation of rejection.

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