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- CYTOKINES AND CHEMOKINES IN DIRECT ISCHEMIA REPERFUSION INJURY OF LUNG AND CARDIOTHORACIC TRANSPLANT REJECTION

#### AWARDS

Schilling Lecture, University of Washington, Prize ,2000  
Seattle Surgical Society, Best Presentation, 1999  
Resident Teaching Award 2000

#### FUNDING

Bayer Corporation  
Novartis  
PrimeSource Surgical  
Thoracic Society Directors Association

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 survivals 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. Chemokines of the IL-8 family have been isolated in various models of inflammation and may be involved in mediating reperfusion injury.

The chemokines are a family of chemotactic cytokines with a high degree of specificity for subpopulations of leukocytes. Four groups of chemokines have been characterized based on the structure of the peptides, CC, CXC, CX<sub>3</sub>C, and C. The CC chemokines or the  $\beta$  chemokines have two adjacent cysteine residues, and function primarily as monocyte and lymphocyte chemotactic agents. Members of this family include MCP-1, RANTES and MIP-1 $\alpha$ , MIP-1 $\beta$ , to name just a few. The second group, the CXC chemokines, are also

referred to as the  $\alpha$  chemokines. This group is characterized by the presence of an amino acid between the two cysteine residues, and includes powerful neutrophil chemoattractants, such as IL-8, MIP-2, and CINC. Two recently discovered groups of chemokines include the C and CX<sub>3</sub>C families. These chemokines are chemotactic for T lymphocytes and monocytes and include lymphotactin (C) and fractalkine, also known as neurotactin (CX<sub>3</sub>C).

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. This is strikingly similar to other models of acute lung injury such as immune-complex alveolitis, anti-basement membrane associated injury and secondary lung injury after remote tissue ischemia. A number of cytokines have been identified (i.e. TNF $\alpha$ , IL-1 $\beta$ , PAF) as important mediators in these models and to a lesser degree, in lung reperfusion injury.

Likewise three C-C chemokines, MCP-1, MIP-1 $\alpha$ , and RANTES, have been shown to play roles in the development of several of these models, but only IL-8 has been investigated for any potential role in lung ischemia reperfusion injury. MIP-1 $\alpha$  is upregulated *in vitro* following hypoxic stress and increased MIP1 $\alpha$  messenger RNA is found in liver allografts shortly after reperfusion. Secondary lung injury develops following reperfusion of ischemic limbs, and liver that is at least partially regulated by C-C and potentially C-X-C chemokines. These findings would suggest that chemokines are likely to play some role in regulating direct ischemia reperfusion injury of the lung.

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 mRNA expression for MIP-1 $\alpha$  in reperfusion injury has been suggested by preliminary findings. Unmanipulated control lungs and those from animals undergoing ischemia plus 0.5, 1, 2, 3 and 4 hours of reperfusion were extracted for MIP-1 $\alpha$  mRNA. Message was not detectable in the unmanipulated lung but appeared at 30 minutes of reperfusion and was present throughout the reperfusion period. Using ELISA technology developed in our laboratory, we have also demonstrated increased protein expression MCP-1 (C-C), and CINC (C-X-C) content in BAL fluid from reperfused lungs (data not shown).

Lung injury as assessed by vascular leakage of <sup>125</sup>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 blocking antibody to MIP 1 $\alpha$ , experienced a mean 35% reduction in permeability compared to injured controls (p<.001). The lungs were also analyzed for myeloperoxidase (MPO) content as a measure of tissue neutrophil accumulation.

chemokine blockade at numerous points in the inflammatory cascade.

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 patients 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

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Increased tissue neutrophil content is detectable after two hours of reperfusion, is significant by three hours and is marked by four hours. In contrast, lungs from animals treated with anti-MIP-1 $\alpha$  demonstrated a 42% reduction in MPO content compared to four hours reperfused controls (p=.02). Ongoing studies are also investigating the mechanisms of chemokine regulation of reperfusion injury. 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. We have also developed strategies for blocking multiple chemokine receptors and interfere with common second messenger pathways. These studies should reveal the maximal effectiveness 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 beta chemokines in the development of experimental OB.

In addition to a variety of other mediators, two of the  $\beta$ - 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 PBS or non-

immune rabbit serum; additional animals were treated with polyclonal blocking antibodies against MCP-I 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 analysis were performed to measure upregulation of MCP-I 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-I 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-I and RANTES mRNA were also increased in allograft tracheas but not in isografts. These data suggest that MCP-I 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 IOx operating microscope followed by a hand sewn anastomosis using 8-0 suture. The hearts are explanted at various time points and the laboratory is currently gathering data on the role of chemokine blockade on cytokine expression and abrogation of rejection.

In addition to the *in vivo* work done in the Mulligan lab, there is significant complementary *in vitro* work. All of the chemokines and cytokines discussed previously will be investigated in tissue sample using ELISA and Western Blot for protein analysis and Northern and RPA blots for mRNA analysis. The *in vivo* work is therefore complemented by sophisticated molecular techniques. With this in mind, the lab has embarked on a project to reconstitute reperfusion injury using cell culture. Specifically culture of type II pneumocytes, alveolar macrophages, pulmonary artery endothelial cells and neutrophils will be undertaken separately and in combination to elucidate the specific response of these cells to hypoxia and reoxygenation.

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#### RELATED PUBLICATIONS

1. Mulligan MS, Lentsch AB, Huber-Lang M, Ren-Feng G, Sarma V, Wright CD, Ulich TR, Ward PA. Anti-inflammatory Effects of Mutant Forms of Secretory Leukocyte Protease Inhibitor. *Am J Pathol*, 2000; 156(3):1033-1039.
2. Mulligan MS, Warner RL, Rittershaus C, Thomas LJ, Ryan US, Foreman KE, Crouch LD, Till GO, Ward PA. Endothelial Targeting and Enhanced Anti-inflammatory Effects of Complement Inhibitors Possessing Sialyl Lewis X Moieties. *J Immunol*, 1999; April 15; 162(8):4952-9.
3. Mulligan MS, Lentsch AB, Shanley TP, Miyasaka M, Johnson KJ, Ward PA. Cytokine and Adhesion Molecule Requirements for Lung Injury Induced by Anti-Glomerular Basement Membrane Antibody. *Inflammation*, 1998; 22(4):403-417.
4. Mulligan MS, Warner RL, Lowe LB, Smith PL, Suzuki Y, Miyasaka M, Yamaguchi S, Ohta Y, Tsukada Y, Kiso M, Hasegawa A, Ward PA. In Vitro and in Vivo Selectin-Blocking Activities of Sulfated Lipids and Sulfated Sialyl Compounds. *Intl Immunol* 10, 1998; (5):569-575.
5. Mulligan MS, Miyasaka M, Suzuki Y, Kawashima H, Iizuka M, Suzuki T, Hasegawa A, Kiso M, Warner RL, Ward PA. Anti-inflammatory Effects of Sulfatides in Selectin-Dependent Acute Lung Injury. *Intl Immunol* 10, 1998; 7:10, pp1107-1113.
6. Mulligan MS, Schmid E, Till GO, Friedl HP, Hugli TE, Roth RA, Ward PA. C5a-Dependent Upregulation of P-Selectin in Vivo. *J Immunol*, 1997; Feb. 15; 158(4):1857-61
7. Mulligan MS, Shanley T, Foreback J, Warner R and Ward PA. Protective Effects of IL-4, IL-10, IL-12 and IL-13 in IgG Immune Complex-Induced Lung Injury. *J Immunol*, 1997; 159: 3483-3489.
8. Mulligan MS, Schmid E, Beck-Schimmer B, Till GO, Friedl HP, Rauer RB, Hugli TE, Miyasaka M, Warner RL, Johnson KJ, Ward PA. Requirement for and Role of C5a in Acute Inflammatory Lung Injury in Rats. *J Clin Invest*, 1996; 98:503-12.
9. Mulligan MS, Warner RL, McDuffie JE, Bolling SF, Sarma V, Ward PA. Regulatory Roles of the Th-2 Cytokines IL-4 and IL-10 in Cardiac Allograft Rejection. *J Exp. Mol. Pathol*, 2000.
10. Mulligan MS, McDuffie JE, Shanley TP, Guo RF, Sarma JV, Warner RL, Ward PA. Role of Rantes in Experimental Cardiac Allograft Rejection. *J Experimental Pathology* (in press).

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