ORIGINAL ARTICLES

Interleukin-8 Expression in Lung Tissue During Ischemia–Reperfusion

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OBJECTIVE: Local cytokine production is a pathogenic factor in ischemia–reperfusion injury in early graft dysfunction. This study analyzed interleukin 8 (IL-8) messenger RNA (mRNA) expression in lung tissue and the association between IL-8 mRNA levels and interstitial lung changes in an experimental model of warm lung ischemia–reperfusion.

MATERIAL AND METHODS: We studied 16 New Zealand rabbits divided into 3 groups: control, ischemia (tissue taken from right lower lobe after 1, 2, or 3 hours of ischemia), and reperfusion (tissue taken from right upper and middle lobes after 1 hour of ischemia and 1, 2, or 3 hours of reperfusion). Expression of IL-8 mRNA was determined by reverse transcription and polymerase chain reaction. Interstitial infiltration by polymorphonuclear neutrophils was determined. The Mann–Whitney U-test was used for statistical comparisons, with *P*<.05 considered to indicate a significant result.

RESULTS: During ischemia, IL-8 mRNA levels were elevated at the end of hour 1 (*P*=.009) with respect to the control group, but not thereafter. Interstitial changes were minimal. IL-8 mRNA levels during reperfusion were similar to those observed during ischemia, with a slight increase at the end of hour 2. There were no significant differences between hours 1, 2, and 3. Polymorphonuclear neutrophil recruitment occurred at the beginning of reperfusion (*P*=.014), but no significant differences were observed at hours 2 or 3. Progressive thickening of alveolar septa and edema was documented.

CONCLUSIONS: Changes in IL-8 mRNA expression during ischemia precede interstitial infiltration by polymorphonuclear neutrophils during reperfusion, suggesting that the 2 processes are related. Quantification of IL-8 mRNA expression could facilitate early diagnosis of graft dysfunction.

Key words: *Ischemia–reperfusion. Interleukin 8. Polymorphonuclear leukocytes. Lung transplantation.*

Estudio de la expresión de interleucina-8 en el tejido pulmonar durante la isquemia-reperfusión

OBJETIVO: La generación local de citocinas es un factor patogénico en el daño por isquemia-reperfusión en la disfunción precoz del injerto. Este estudio analiza la expresión en tejido pulmonar de ARN mensajero de interleucina-8 (ARNm de IL-8) y su relación con los cambios intersticiales pulmonares en un modelo experimental de isquemia-reperfusión pulmonar normotérmica.

MATERIAL Y MÉTODOS: Se estudiaron 16 conejos de la raza Nueva Zelanda en 3 grupos de estudio: a) basal; b) isquemia (lóbulo inferior derecho tras isquemia de 1, 2 o 3 h), y c) reperfusión (lóbulos superior y medio derechos tras 1 h de isquemia y 1, 2 o 3 h de reperfusión). Se determinó la expresión del ARNm de IL-8 mediante transcripción inversa y reacción en cadena de la polimerasa y estudió la infiltración intersticial por polimorfonucleares (PMN). Para el análisis estadístico se empleó el test de la U de Mann-Whitney aceptando como significativo un valor de p < 0,05.

RESULTADOS: Durante el período de isquemia se observó respecto al basal elevación del ARNm de IL-8 al final de la primera hora (p = 0,009), pero no durante el resto del período isquémico. Los cambios intersticiales fueron mínimos. Durante la reperfusión los valores de ARNm de IL-8 fueron semejantes a los observados durante la isquemia, con una ligera elevación al final de segunda hora; no hubo diferencias significativas entre la primera, segunda y tercera horas. Hubo reclutamiento de PMN al inicio de la reperfusión (p = 0,014), sin observarse diferencias significativas en la segunda y tercera horas. Se objetivó un engrosamiento progresivo de los tabiques interalveolares y edema.

CONCLUSIONES: Los cambios en la expresión del ARNm de IL-8 durante la isquemia preceden a la infiltración intersticial de PMN durante la reperfusión, lo que señala una relación entre ambos procesos. La cuantificación del ARNm de IL-8 podría ser un procedimiento para el seguimiento diagnóstico de la disfunción precoz del injerto.

Palabras clave: *Isquemia-reperfusión. Interleucina-8. Leucocitos polimorfonucleares. Trasplante pulmonar.*

This study was realized with the scientific help and the financiation of the Sociedad Española de Neumología y Cirugía Torácica. (SEPAR).

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Manuscript received August 16, 2004. Accepted for publication October 24, 2006.

Introduction

On reestablishing blood flow during ischemia–reperfusion, the damage initiated during the ischemic period is accentuated and some of the characteristics of acute inflammatory tissue reaction, such as infiltration by polymorphonuclear neutrophils (PMNs), are mimicked.[1] Thus, a key factor in the pathogenesis of ischemia–reperfusion syndrome and in early nonspecific graft dysfunction may be the appearance of leukocyte chemoattractants, and chemokines in particular, in tissues. Interleukin-8 (IL-8) is the main member of an extensive family of chemotactic cytokines (chemokines). This chemokine initiates highly selective recruitment of PMNs into tissue from peripheral blood.

Given the structural characteristics of the lung interstitium, this is the site where the essential processes in the inflammatory response take place and where PMNs accumulate and are activated, as indicated by the findings of different animal models of acute inflammatory injury.[2-4] However, alveolar macrophages are a key cell element for triggering the response. It is possible to envisage a pathogenic mechanism in which macrophages generate chemotactic factors that recruit PMNs after adherence and migration through the endothelial cells. The PMNs are thus concentrated in the lesion site, where they release inflammatory mediators responsible for tissue damage. Such a mechanism can account for tissue damage due to ischemia–reperfusion in at least 25% of the recipients of transplanted lungs.[4,5]

IL-8, initially identified as a chemoattractant of PMNs, triggers 3 basic responses in these cells, namely, changes in morphology and transendothelial migration, in degranulation, and in oxidative activation.[6] Furthermore, exposure of cells to oxidative stress such as ischemia–reperfusion induces the local production of IL-8.[7] Clarification of the sequence of production and action of IL-8 during lung ischemia–reperfusion could be of interest in understanding the mechanism by which tissue damage occurs and help in the development of possible treatments.

With this in mind, the aim of this study was to determine the pattern of IL-8 production and how this pattern was related to the presence of PMNs during lung ischemia–reperfusion in a warm-lung animal model of ischemia–reperfusion.

Material and Methods

The rabbit was chosen as the experimental animal in view of its anatomic and physiologic characteristics, availability, cost and ready access to appropriate facilities, and our previous experience in experimental models.[8-10] Sixteen white New Zealand rabbits (weight range, 2.5-4 kg) underwent the operation in accordance with an established protocol. The animals were killed at the end of the experiment by barbiturate overdose in accordance with current legislation (European Union Directive 86/608/EC and Royal Decree 223/1988).

Experimental Design

Intramuscular administration of ketamine hydrochloride (30 mg/kg) assured sedation and hypnosis of the animals. The animals also received an additional subcutaneous injection of atropine (0.12 mg/kg). General anesthesia was achieved by intravenous administration of sodium thiopental (10 mg/kg) and pancuronium bromide (0.5 mg/kg). With the animal in dorsal decubitus, a median cervicotomy and tracheotomy were undertaken. During the procedure, animals received respiratory support in the form of mechanical ventilation (Engstrom Respiratory System 300; type ER 311, Engstrom, Bromma, Sweden), with a fraction of inspired oxygen of 0.21 (35 mL/ kg/min) at a frequency of 30 breaths/min. The internal jugular vein was cannulated for administration of the medication and measurement of central venous pressure. The contralateral common carotid artery was cannulated for continuous arterial blood pressure monitoring and for arterial blood gas analyses at different times during the experiment.

A median sternotomy was performed, with removal and separation of the thymus. Both pleura were opened to allow both pulmonary hila to be isolated with vascular and bronchial exclusion of the additional central lobe. A vessel loop was placed around the right pulmonary hilum and also around the corresponding inferior lobe to facilitate control and subsequent exclusion. Full anticoagulation of the animal was achieved through administration of sodium heparin (1 mg/kg).

Lung Ischemia–Reperfusion

Once the hemodynamic and respiratory stability of the animals had been confirmed, right lung ischemia was induced by total bronchial blockade (main bronchus) and interruption of pulmonary blood flow (pulmonary and bronchial artery and veins). At the same time, circulation through the hilum of the right lower lobe was interrupted. With this approach, it was possible to simultaneously measure IL-8 messenger RNA (mRNA) levels and determine histologic variables in the same animal after different ischemic periods of the right lower lobe (1, 2, and 3 hours) and after ischemia (1 hour) followed by different periods of reperfusion (1, 2, and 3 hours) of the middle and upper lobes.

Study Groups

In accordance with the aims of the experiment, 3 study groups were established:

– Control: lung tissue removal without inducing ischemia–reperfusion (4 animals)
– Ischemia group: lung tissue taken from the right lower lobe after ischemia lasting 1, 2, or 3 hours (4 animals for each ischemic period)
– Reperfusion group: lung tissue removed from the right upper and middle lobes after 1 hour of ischemia and 1, 2, or 3 hours of reperfusion (4 animals for each reperfusion period—the same animals as the ischemia group)

Assessment Procedures

Lung tissue samples were taken to measure the expression of IL-8 mRNA and to determine the histologic changes in the animals of the different groups. For the histologic study, a fragment was separated from each sample and preserved in formaldehyde; the lung fragments for measuring IL-8 mRNA levels were immediately immersed in liquid nitrogen and stored at a constant temperature of –80ºC until use.

Measurement of IL-8 mRNA levels. For extraction of cellular RNA, the lung tissue was disrupted mechanically with a mechanical Polytron tissue homogenizer (Kinematica AG, Littan-Lucerne, Switzerland) for 30 seconds at 4ºC. Total RNA was extracted using guanidine thiocyanate.

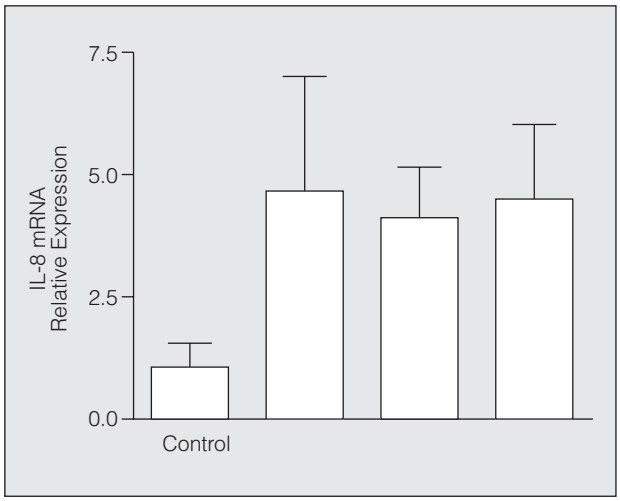

Figure 1. Changes in expression of interleukin-8 (IL-8) messenger RNA (mRNA) during ischemia. RNA samples were extracted from rabbit lungs at different times after the onset of ischemia and analyzed using reverse-transcription polymerase chain reaction with primers designed for rabbit IL-8 and glyceraldehyde–phosphate–dehydrogenase. Changes at different times are expressed relative to control, which is assigned the value of 1. Significant differences can be seen between the first hour of ischemia and control (*P*=.09) but not between the different ischemic periods (*P*>.05).

The first strand of complementary DNA was synthesized from total RNA by reverse transcription. The reaction mixture contained 0.2 μg/μL of total RNA, 2.5 μL of diethyl-polycarbonate–treated water, 20 U of RNasin ribonuclease inhibitor, 4 μL of buffer 5×, 2 μL of dichloro-diphenyl-trichloroethane 0.1 M, 4 μL of deoxyribonucleotide triphosphate (dNTP) 2.5 mM, 1 μL of a mixture of hexanucleotides 0.1 mM, and 200 U of reverse transcriptase of the Moloney simian leukemia virus. The reaction mixture was incubated for 1 hour at 37ºC in a volume of 20 μL. The complementary DNA was amplified by polymerase chain reaction (PCR) in a reaction mixture containing 5 μL of reverse transcriptase, 14 μL of water, 2.5 μL of buffer 10×, 0.75 μL of magnesium chloride 50 mM, 1.0 μL of dNTP 2.5 mM, 1.25 μL of each primer, and 0.25 μL of Taq-DNA polymerase 5 U/μL. This solution (25 μL) was placed in a thermocycler. The amplification program included an initial denaturing cycle at 94ºC for 5 minutes and 30 amplification cycles as follows: denaturing at 94ºC for 30 seconds, annealing at 59ºC for 30 seconds, elongation at 62ºC for 30 seconds, and final extension at 72ºC for 7 minutes. The PCR products were separated by agarose gel electrophoresis and quantified by densitometry. Expression of glyceraldehyde-phosphate-dehydrogenase was used to confirm that the loads of the different lanes were homogeneous and for normalization of the results.

Histologic assessments. Presence of alveolar neutrophil and macrophage infiltrate, fibrinous exudate, septal infiltration, intravascular neutrophil aggregation, edema, and destruction of lung architecture were investigated in all samples. Samples were taken from different areas, and those with findings indicative of artefacts from surgical manipulation of the parenchyma were discarded. Cells were counted in a visual field 600 μm in diameter under a Zeiss microscope using a ×40 lens. For regions considered as valid, the definitive result was taken to be the average percentage in the case of each of the variables studied.

Statistical Analysis

The data were entered into a database (SPSS version 11.5) for statistical analysis. In view of the small sample size, group comparisons of IL-8 mRNA levels and neutrophil counts were performed using the Mann-Whitney nonparametric U test.

Results

Measurement of Interleukin-8 Messenger RNA

A significant elevation in IL-8 mRNA levels (*P*=.0090) was found after the first hour of ischemia (mean [SD], 4.75 [2.31]) compared to control values (1 [0.57]). This significant difference was maintained throughout the 3-hour ischemic period. There were no statistically significant differences between IL-8 mRNA values observed at the different ischemic times (2nd hour, 4.12 [1.02]; 3rd hour, 4.53 [1.56]; *P*=.5637 and *P*=.3865, respectively) (Figure 1).

At 1 hour after the onset of reperfusion, IL-8 mRNA levels (4.42 [1.43]) were very similar to those observed at the end of the period of ischemia (*P*>.05). A second increase in IL-8 mRNA levels was found at the end of the second hour of reperfusion (4.97 [0.54]), followed by a slight decrease in the third hour (3.63 [1.18]). However, there were no statistically significant differences between IL-8 mRNA levels found during ischemia and those found at the end of the first, second, and third hours of reperfusion (*P*>.05) (Figure 2).

Histologic Assessments

In the control group, histologic analysis of the lung samples revealed a structured lung parenchyma, with thin alveolar septa. Edema and interstitial cells were not observed. The mean PMN and alveolar macrophage counts per field were 3.8 (0.84) (range, 3-5) (Figure 3) and 1.2 (0.84) (range, 0-2), respectively.

During the ischemic period of the lungs, microvascular changes and a minimal PMN accumulation in the interstitial space could be discerned. There were no significant differences in the PMN count between the control period and the ischemic period, or between values observed at the end of the first, second, and third hours of ischemia (3.75 [1.5] cells per field [cpf], 3.33 [0.58] cpf, and 4 [1.41] cpf, respectively) (*P*>.05) (Figure 3). Likewise, for macrophage counts, no significant differences were found between the control group and the end of the first, second, and third hours of ischemia (3.75 [1.5], 1 [0.81], 2.25 [2.63], respectively) (*P*>.05).

Rapid recruitment of PMNs to the interstitial space occurred at the start of reperfusion. During the first hour of reperfusion, the PMN count increased to a mean value of 20.75 (10.87) PMNs per field (range, 9-30) with significant differences with respect to the mean control value (*P*=.014) and the one observed at the end of the first hour of ischemia (*P*=.018). In the second hour of reperfusion, a slight decrease in the PMN count (13 [2.65]) was recorded, but this value was still significantly different from control values (*P*=.013) and that recorded at the end of the first hour of ischemia (*P*=.017). In the third hour of

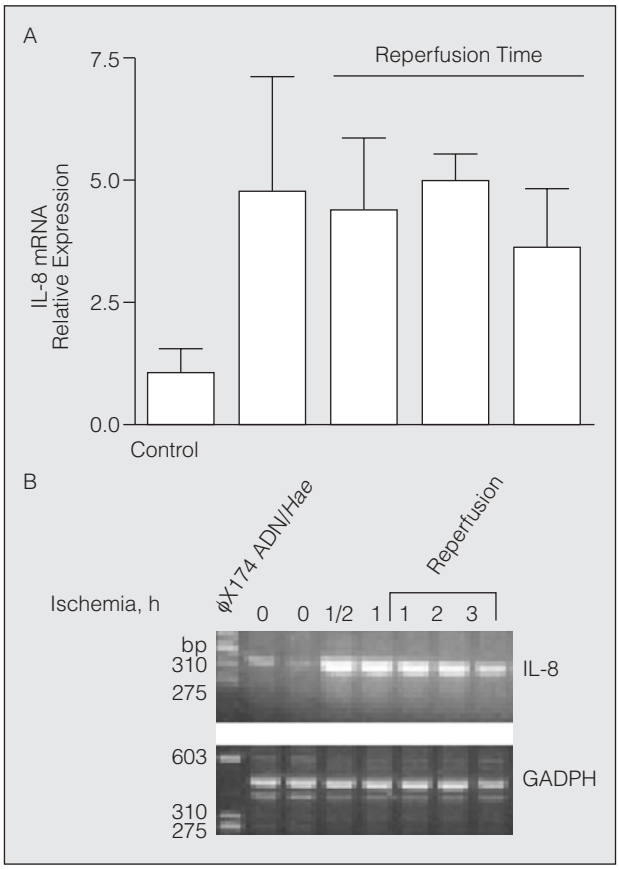

Figure 2. Expression of interleukin-8 (IL-8) messenger RNA (mRNA) during ischemia–reperfusion. The lungs of the animals were maintained in ischemic conditions for 1 hour and then reperfused for the indicated periods. At the end of these periods, lung tissue was obtained for RNA extraction and analyzed by reverse-transcription polymerase chain reaction (RT-PCR). No significant differences were observed between the values at the end of ischemia and those found during the additional reperfusion (*P*>.05). Panel B shows the result of analysis of the products by RT-PCR in a representative experiment. The left lane shows the position of the markers of different numbers of base pairs (bp). GADPH indicates glyceraldehyde-phosphate-dehydrogenase.

reperfusion, leukocytes once again accumulated in the interstitial space (mean count, 26.75 [15.54] cpf); nevertheless, there were no significant differences between mean neutrophil counts at the end of the first hour of reperfusion and counts at the end of the second and third hours (*P*=.2454 and *P*=.6631, respectively) (Figure 4). Macrophages did not show the same behavior: their levels during reperfusion were very similar to those observed for control animals and during ischemia (first hour, 3.25 [1.89] cpf; second hour, 1.25 [1.26] cpf; third hour, 3 [2.94] cpf).

During the reperfusion period, progressive thickening of the alveolar septa, vascular congestion, and edema were reported at the same time as elevated PMN counts in the interstitial space. The formation of hyaline membranes was only observed in the latest stages of reperfusion (Figure 5).

Discussion

In acute inflammatory processes, PMN infiltration of tissues occurs when PMNs adhere to endothelial cells following the signal of a chemotactic factor concentration gradient. Given that many characteristics of acute inflammatory reaction are also found in ischemia–reperfusion tissue, the action of different chemotactic factors implicated in this process has been extensively investigated. The most widely studied of these factors are the chemoattractant cytokines, such as tumor necrosis factor α (TNF-α), IL-1, and IL-8, anti-inflammatory cytokines such as IL-10 and IL-12, and other inflammatory mediators such as interferon-γ (IFN-γ) and platelet activating factor. IL-8, identified initially as a PMN chemoattractant, also causes changes in PMN morphology, transendothelial migration, degranulation, and oxidative activation.[11] Levels of the protein itself as well as its mRNA have been studied in various processes, such as abdominal

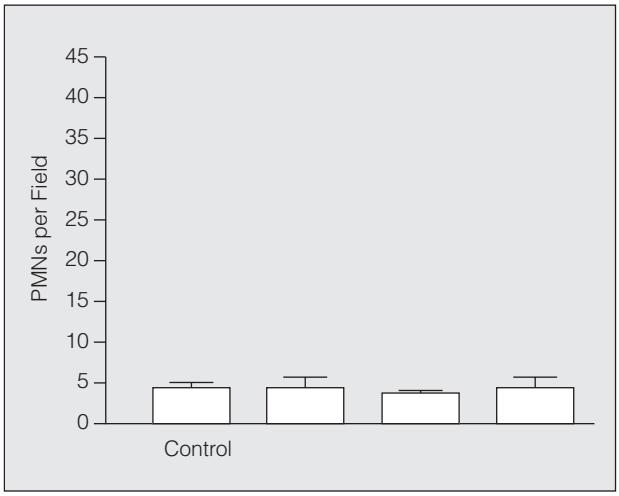

Figure 3. Polymorphonuclear (PMN) cell count in lung tissue during ischemia. There were no significant differences compared to control.

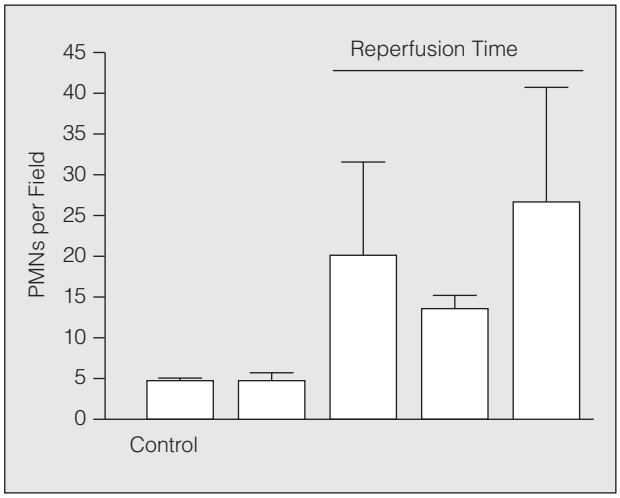

Figure 4. Polymorphonuclear (PMN) cell count in lung tissue during ischemia–reperfusion. Significant differences between the first hour of ischemia and the first hour of reperfusion were observed (*P*=.018) but not during the remaining reperfusion period.

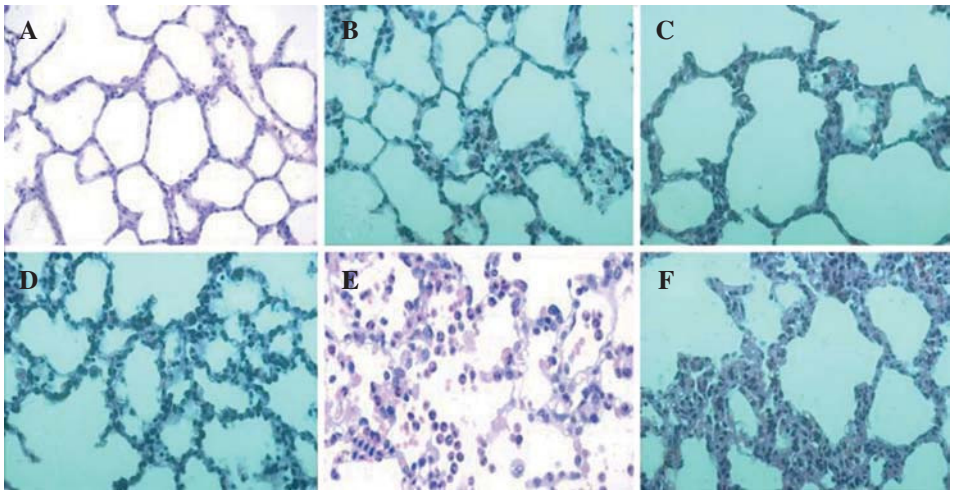

Figure 5. Histologic changes in the lung interstitium: A, control; B, after 1 hour of ischemia; C, after 3 hours of ischemia; D, after 1 hour of reperfusion; E, after 2 hours of reperfusion; F, after 3 hours of reperfusion. Significant samples were chosen in which progressive appearance of interstitial edema and infiltration by polymorphonuclear leukocytes can be seen.

aortic surgery[12] and liver transplantation,[13] in which the consequences of tissue ischemia–reperfusion are of utmost importance.

The lung interstitium, in view of its histologic and structural characteristics, is the site of inflammatory reaction and its response to different chemotactic factors has been studied in different models of acute inflammatory damage.[2,3] Clarification of the production sequence is essential to extend our knowledge of the mechanism of lung ischemia–reperfusion and tissue damage.

It is generally accepted that pathological and functional evidence of tissue damage due to lung ischemia–reperfusion first appears after reperfusion. This observation suggests that the pathological mechanism is the result of the kinetics of chemotactic factors as the process is triggered by their production. The concentrations of IL-8 during ischemia reported in the literature are inconsistent. In the animal models studied by Sekido et al[14] and Sakuma et al,[15] IL-8 concentrations increased after 2 hours of lung ischemia but never significantly, even after extending the ischemic period to 5 hours.[14,15] In contrast, De Perrot et al,[16] in a study of bronchoalveolar lavage (BAL) samples from lung transplant donors, reported significant increases in IL-8 concentrations and also in the concentrations of other factors such as TNF-α, IFN-γ, IL-10, IL-12, and IL-18. When mRNA was measured as an indicator of IL-8 levels, the results seemed to confirm its production during the ischemic period. In the clinical setting, Fisher et al[17] reported significantly higher concentrations of IL-8 mRNA in donor lungs compared to lungs from healthy control subjects. Metinko et al[7] found significantly elevated IL-8 levels in BAL samples from donor lungs after tissue ischemia of 3, 6, 9, and 12 hours. This elevation correlated with a parallel increase of IL-8 mRNA levels in lung tissue at 30 and 90 minutes of ischemia.

In our study, ischemic damage was associated with significantly elevated IL-8 mRNA levels during the first hour with respect to the control group. These values remained high from the first through third hour after ischemia, with no significant variations during the 3-hour ischemic period.

The effect on tissue of elevated levels of IL-8 or its corresponding mRNA during ischemia has also been studied. Recent studies with animal models show that increases in IL-8 levels in the interstitium are only accompanied by minimal changes in lung tissue after 2 to 5 hours of lung ischemia.[14,18-20] In our experiment, histologic analysis of controls revealed a structured lung parenchyma, with thin alveolar septa. No edema was observed but a modest number of interstitial PMNs were present. These findings are not suggestive of significant changes at any point during the ischemic period.

Most experimental studies show a continuous increase in IL-8 synthesis during reperfusion, both in homogenized lung tissue and BAL samples.[14] Clinical studies such as those by the Toronto Lung Transplant Program[21] have concluded that IL-8 concentrations tend to increase gradually from the beginning of ischemia and then during reperfusion whereas concentrations of TNF-α, IFN-γ, IL-10, IL-12, and IL-18 also increased in parallel during the ischemic period but decreased after reperfusion. In addition, a correlation between mortality due to early graft failure and increased expression was only established for elevated IL-8 levels during ischemia with further increases during reperfusion; no correlation was observed for the other factors.[16,17]

In our study, during reperfusion, IL-8 mRNA levels remained similar to those found at the end of the ischemic period. Given that IL-8 mRNA is a precursor for IL-8 synthesis, we observed a slight decrease during the first hour of reperfusion but then a slight increase at the end of the second hour. In our opinion, this observation is evidence of a negative feedback mechanism in the synthesis of IL-8 after IL-8 elevation or, alternatively, of the disappearance of the trigger.

With regard to the histologic changes described for reperfusion, most authors agree that rapid recruitment of PMNs to the lung interstitium occurs.[14,16,17,19] In our study, at 2 hours after the onset of reperfusion, we observed moderate neutrophil and macrophage infiltration of the

interstitium and alveoli. In studies that included analysis of BAL samples, the findings were also in agreement with the histologic study.[14]

In our experiment, a substantial accumulation of PMNs in tissue occurred during the first hour of reperfusion. During the second hour, a slight decrease in the PMN count was observed, a decrease followed by the production of an intense infiltration of the alveolar septa and intravascular PMN aggregates at the end of the third hour. No significant increase in the number of macrophages was observed with respect to those accumulated during the ischemic period. The accumulation of PMNs was accompanied by changes in the lung interstitium, such as edema and thickening of the alveolar septa, congestion of the pulmonary vessels, and presence of hyaline membranes in the latest phases of reperfusion. These findings are in agreement with those of other studies[14,22] (including those in which the PMN count was determined by other methods such as measurement of myeloperoxidase levels[22]) and support the idea that PMNs are the main type of cells recruited to the interstitial space in lung damage due to ischemia–reperfusion. The results of tissue ischemia–reperfusion experiments done with leukocyte-depleted blood showed that the role of PMN interstitial infiltration was the main determinant of the extent of tissue damage.[15,18,20]

These findings reflect the importance of IL-8 —a cytokine that acts as a chemoattractant of PMN—in the development of interstitial damage due to ischemia–reperfusion. Concentration changes during the process point to production during the ischemic period and progression during reperfusion. In our experiment, measurement of IL-8 mRNA levels was found to be a reliable marker of the production of IL-8 itself and to correlate with pathological findings.

Even in the absence of significant histologic changes, elevated IL-8 and/or IL-8 mRNA in donor lungs at the end of ischemia has been shown to be associated with poor initial graft function and greater early mortality of the recipient.[17] During ischemia, the possibility of determining a prognostic marker which predicts subsequent tissue damage could be very useful for avoiding the onset of such damage. IL-8 mRNA could be a candidate for such a marker given that it is an indicator of a potent mediator.

In summary, in an animal experiment, it has been possible to show that administration of IL-8-specific antibodies at the beginning of lung reperfusion markedly reduces tissue damage.[14] Lines of research focused on the donor and recipient, quantifying levels of IL-8 mRNA as a precursor of IL-8 production, and investigating factors that inhibit its action could contribute to improved short-term function and prognosis of transplanted lungs.

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