Depletion of Alveolar Macrophages by Clodronate-liposomes Aggravates Ischemia–Reperfusion Injury of the Lung

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Background: Macrophages play an important role in ischemia–reperfusion injury of various organs. Liposome-encapsulated dichloromethylene diphosphonate (clodronate-liposome) depletes local macrophages in vivo. However, the effect of this approach on alveolar macrophages in pulmonary ischemia–reperfusion injury has not yet been evaluated.

Methods: Clodronate-liposomes in Hanks' balanced salt solution (HBSS) or HBSS alone were given intratracheally to anesthetized male Lewis rats in the clodronate or the control group ($n = 6$/each group). After 3 days, we subjected the lungs to ischemia (37°C, 60 minutes) and reperfusion (60 minutes) in an isolated blood-perfused rat lung model. Analysis during reperfusion included gas exchange, hemodynamics, and airway mechanics. At the end of reperfusion, we determined leukocyte recruitment and macrophage inflammatory protein-2 (MIP-2) in bronchoalveolar lavage fluid.

Results: In the clodronate group, 4 experiments had to be terminated within 10 minutes of reperfusion because of severe lung injury, whereas all lungs of the controls could be studied during the 60-minute reperfusion period ($p < 0.05$). Clodronate significantly decreased dynamic airway compliance ($p < 0.05$) and increased airway resistance. Besides a tendency toward greater pulmonary vascular resistance, this was associated with recruitment of polymorphonuclear neutrophils ($p < 0.05$) and increased MIP-2 concentrations in the bronchoalveolar lavage fluid ($p < 0.05$).

Conclusions: Intratracheal administration of liposome-encapsulated clodronate does not benefit, but aggravates, warm ischemia–reperfusion injury of the lung, increasing MIP-2–associated alveolar neutrophil recruitment and airway mechanical dysfunction. J Heart Lung Transplant 2005;24:38–45. Copyright © 2005 by the International Society for Heart and Lung Transplantation.

Macrophages are one of the main cellular components of the lung and can be divided into several different populations depending on their location.[1,2] The biggest population is that of alveolar macrophages, which play a pivotal role in the primary defense system of the lung,[3,4] such as removal of foreign bodies by phagocytosis.[5] Despite this protective role, pulmonary macrophages also can aggravate tissue injury. Exposure to toxic agents such as ozone or bleomycin results in accumulation of macrophages in the lung, which causes severe injury by hydrogen peroxide, bioactive lipids, and pro-inflammatory cytokines.[6,7]

Recently the role of macrophages in ischemia–reperfusion (I/R) injury of organs has been investigated. In the heart and the liver, local macrophages seem to be actively involved in I/R injury.[8–10] Involvement of macrophages in I/R injury of the lung also has been suggested.[11,12] It is unclear, however, which population of pulmonary macrophages are predominantly active in this process, and the role of alveolar macrophages, the biggest proportion, remains to be investigated.

Dichloromethylene diphosphonate (clodronate) is a bisphosphonate used clinically to treat osteolytic disease.[13] Liposome-encapsulated clodronate (clodronate-liposome) is transported into local macrophages by phagocytosis, and, after digestion, clodronate is released and induces cellular death. This approach has been used to deplete local macrophages in the liver, the spleen, and the testis.[14] In addition, clodronate-liposomes administered intratracheally have been used to deplete alveolar macrophages in the rat[15,16] and in the mouse.[3,17] In these studies, intratracheal clodronate-liposomes had a specific effect on alveolar macrophages

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among the different cell populations in the lung.[3,15–17] The effect of clodronate-liposomes in I/R injury of the lung has not yet been investigated.

Herein, we analyzed the role of alveolar macrophages in lung I/R injury. In an isolated blood-perfused lung I/R model, we studied gas exchange, hemodynamics, airway mechanics, and bronchoalveolar lavage (BAL) fluid after in vivo alveolar macrophage depletion by intratracheal administration of clodronate-liposomes.

METHODS

Experimental Animals and Humane Care

Male Lewis rats (Charles River Laboratories; Sulzfeld, Germany) with a body weight of approximately 300 g were treated with humane care in compliance with the *Principles of Laboratory Animal Care,* formulated by the National Society for Medical Research; the *Guide for the Care and Use of Laboratory Animals,* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985); as well as the German legislation on protection of animals.

Materials

Clodronate (clodronate disodium salt $4H_2O$) was kindly provided by Roche Diagnostics GmbH (Mannheim, Germany). We purchased phosphatidylcholine and cholesterol from Sigma Aldrich (Deisenhofen, Germany) and Reedel-de Haen (Seelze, Germany).

Liposome Preparation

We prepared the liposomes as reported previously[15] with slight modifications. Briefly, phosphatidylcholine (86 mg) and cholesterol (19 mg) were dissolved in chloroform (5.0 ml) in a round-bottom flask. We removed the organic phase using low-vacuum rotary evaporation at 37°C. The lipid film formed inside was dispersed at room temperature for 15 minutes with 10 ml HEPES-buffered saline (HBS; HEPES, 25 mmol/liter; NaCl, 150 mmol/liter; pH 7.4) containing 0.7 mol/liter clodronate. The resulting suspension was incubated for 2 hours at room temperature and then sonicated for 3 minutes in a water bath. After incubation at room temperature for an additional 2 hours, liposomes were diluted in 50 ml HBS and centrifuged twice at 100,000g for 30 minutes at 16°C. The final pellet was washed once with HBS, resuspended in 4 ml HBS, and stored in nitrogen at 4°C.

Liposome Administration and Experimental Groups

After anesthesia with pentobarbital (50 mg/kg, intraperitoneally), the animals were intubated with 14-guage catheters. We randomly allocated the animals to 2 groups ($r = 6$ per group). Calcium- and magnesium-free Hanks' balanced salt solution (HBSS, 1 ml) containing clodronate-liposome suspension (80 μl) was administered intratracheally in the clodronate group, and HBSS (1 ml) alone was administered in the control group, as previously reported.[15] Each administration was followed by mechanical ventilation and auscultation of the chest to ascertain even distribution of the solution. The animals were extubated and allowed free access to food and water after recovery from the anesthesia. Three days later, lungs were harvested from the animals and then subjected to I/R in an isolated rat lung perfusion model.

In additional animals, we analyzed the number of viable alveolar macrophages from BAL fluid 3 days after clodronate ($n = 3$) or HBSS ($n = 3$) application to confirm macrophage depletion. Therefore, bilateral lungs were lavaged 10 times with 5 ml HBSS. All aliquots were centrifuged at 300*g* for 10 minutes at 4°C. The cell pellets from the 10 lavages were resuspended in 50 ml HBSS, centrifuged again, and resuspended in HBSS to a final volume of 0.5 ml. We counted the total number of cells using an automatic cell counter (Act Diff; Coulter Electronic; Krefeld, Germany). Viable alveolar macrophages were counted differentially in May-Grünwald-Giemsa–stained cytocentrifuged preparations, performed using the Hettich-Cyto-System (Hettich Zentrifugen; Tuttlingen, Germany). In each preparation, we counted a total of 500 cells and calculated the numbers of macrophages in alveoli from the total number and the differential counts.

Three days after the administration of clodronate-liposomes and HBSS, anesthetized animals were ventilated mechanically with a positive-pressure ventilation module of the model. The chest was opened with a median sternotomy and heparin (1000 U/kg) was administered intravenously. We cannulated the pulmonary artery and the left atrium and started the isolated perfusion. The heart–lung block was harvested from the animal, installed in the artificial thorax, and negative-pressure ventilation was initiated. In <3 minutes, perfusate flow was increased to the set level (10 ml/min).

Isolated Rat Lung Perfusion

As previously described, an isolated perfused lung using model 829 (Hugo-Sachs Elektronik-Harvard Apparatus; March-Hugstetten, Germany)[18] was modified slightly. The test lung was placed in a glass-made artificial thorax and ventilated with room air at negative pressures at a respiratory rate of 60 cycles/min, peak inspiratory and expiratory chamber pressures of −8 and −4 cm H_2O, and an inspiratory ratio of 50%. The artificial thorax, the circuit of perfusate, and the airway were water-jacketed, and the temperature was maintained at 37°C. We maintained humidity of the air in the airway and in the artificial thorax at 100%.

We used heparinized whole blood diluted with saline and 2% albumin as perfusate. We obtained the blood