

# Aprotinin Reduces Interleukin-8 Production and Lung Neutrophil Accumulation After Cardiopulmonary Bypass

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Pulmonary neutrophil entrapment and resultant oxidative injury is thought to be the primary mechanism of cardiopulmonary bypass (CPB) induced lung injury. Interleukin-8 (IL-8), a potent neutrophil chemoattractant induced by cytokines, including tumor necrosis factor- $\alpha$  (TNF), is found in increased concentrations in bronchial alveolar lavage fluid (BALF) in lung inflammation. Since aprotinin reduces TNF release during CPB, the effects of aprotinin on BALF IL-8 concentrations and neutrophil levels were determined after CPB in adult humans. Study patients were equally divided into a control group ( $n = 8$ , Group 1) and an aprotinin-treated group ( $n = 8$ , Group 2). *In vitro* neutrophil chemotaxis was done with volunteer neutrophils using three different chemoattractants: 1) *N*-formyl-1-methionyl-1-leucyl-1-phenylalanine (FMLP); 2) the

supernatant of a human bronchial epithelial cell culture line, A549, after 24 h of TNF stimulation with or without aprotinin or *N*- $\alpha$ -tosyl-L-lysine chloromethyl ketone (TLCK) (a potent protease inhibitor), and 3) BALF. Aprotinin treatment significantly ( $P < 0.05$ ) reduced post-CPB BALF IL-8 concentrations and percentage of neutrophils. *In vitro*, BALF from Group 1 had significantly greater chemotactic ability when compared with Group 2. The TNF stimulated A549 cell culture supernatant had significantly ( $P < 0.05$ ) greater chemotactic ability than control supernatant, while aprotinin and TLCK significantly ( $P < 0.05$ ) reduced this chemotactic ability. These results demonstrate that aprotinin blunts IL-8 production and reduces neutrophil lung accumulation post-CPB.

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**T**he proinflammatory cytokine interleukin-8 (IL-8) is a potent chemoattractant that induces neutrophil chemotaxis (movement toward an inflammatory stimulus) and accumulation in inflamed human airways (1). Lung injury after cardiopulmonary bypass (CPB) is thought to be neutrophil-mediated (2,3); CPB may result in lung injury characterized by neutrophil sequestration, with subsequent impairment of arterial blood oxygenation and an increase in extravascular lung water (3). The human bronchial epithelial cell culture line (A549) (4,5) responds to stimulation by tumor necrosis factor- $\alpha$  (TNF) by producing and releasing IL-8, the only chemoattractant produced by this cell line (4-6). TNF (7) and IL-8 (7,8) are released systemically during human CPB. Aprotinin, a serine protease inhibitor, reduces TNF production and release during and after CPB (9). We investigated the effect of aprotinin on human airway neutrophil accumulation and bronchoalveolar lavage

fluid (BALF) concentrations of IL-8 after CPB, while evaluating *in vitro* the effect of aprotinin on IL-8 production by the human bronchial epithelial cell culture line (A549) after 24 hours of TNF stimulation.

## Methods

After obtaining institutional review board approval and patient informed consent, 16 adult patients scheduled for elective aortocoronary bypass surgery requiring CPB were randomized according to a computer-generated sequence and assigned equally to one of two groups: 1) a control group (Group 1), and 2) aprotinin, 280 mg intravenously (IV) ( $2 \times 10^6$  kallikrein inactivation units [KIU] as a loading dose, 280 mg ( $2 \times 10^6$  KIU) "pump prime," and 70 mg ( $5 \times 10^5$  KIU)/h IV constant infusion until chest closure (Group 2). Patients with a recent history of smoking or a history consistent with bronchial asthma or chronic obstructive lung disease were excluded (1).

On the morning of surgery, each patient was given morphine sulfate (0.1 mg/kg) and scopolamine (0.2-0.4 mg) intramuscularly prior to admission to the operating room. On arrival, a radial

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artery catheter, a right internal jugular vein pulmonary artery catheter, and large-bore IV lines were placed. Standard anesthesia consisting of fentanyl (75–100  $\mu\text{g}/\text{kg}$ ) as a short IV infusion and pancuronium (0.1–0.2 mg/kg) was used. CPB was completed with a centrifugal pump, (Biomedicus, Inc., Eden Prairie, MN), a hollow-fiber membrane oxygenator (Baxter Health Care, Irvine, CA) with arterial line filtration, and mild hypothermia (32°C core temperature). Perfusion flow rate and mean arterial pressure during CPB were maintained between 2.2 and 2.4  $\text{L} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$  and 60–80 mm Hg, respectively. Myocardial preservation was achieved with both antegrade and retrograde administration of cold hyperkalemic blood (8:1 blood to crystalloid mixture) cardioplegia. Anticoagulation was obtained by the administration of bovine lung heparin (300 U/kg), and activated clotting times were maintained at 480 s or greater in both groups by the addition of heparin when necessary. After CPB, protamine was administered in a ratio of 1.3 mg for every 100 U of total heparin administration, and confirmed by the return of the activated clotting times to baseline values.

After CPB termination and protamine administration (usually 20–30 min post-CPB), bronchoalveolar lavage (BAL) was done in two 20-mL saline aliquots through a fiberoptic bronchoscope. The BAL fluid (BALF) was collected as BALF 1 and 2, respectively. The fiberoptic bronchoscope was wedged in the right lower lobe and the BAL technique for cell and BALF handling was done as previously described (10). Each lavage was kept separate and total cell count with the percentage of neutrophils of the total cell count was determined on BALF 1 from each patient. The typical BAL returned 50%–70% of the total volume instilled.

IL-8 levels in BALF 1 and 2 were quantified using a sandwich enzyme-linked immunosorbent assay. Briefly, 96-well flat-bottomed polystyrene microtiter plates (Immulon, Chantilly, VA) were coated with 200  $\mu\text{L}/\text{well}$  of purified goat anti-hIL-8 antibody (R&D, Minneapolis, MN) diluted 1:2000 in Voller's buffer (pH 9.6) for 24 h at 4°Celsius. After washing the plates three times in phosphate-buffered saline (PBS)-Tween, undiluted BALF, and serial dilutions of standard rIL-8 (Sigma, St. Louis, MO) were incubated at room temperature for 90 min. Plates were rinsed three times with PBS-Tween followed by the addition of rabbit anti-hIL-8 antibody (Upstate Biotechnology Incorporated, Lake Placid, NY) diluted 1:4000 in PBS-Tween/BLOTTO (0.2% instant nonfat milk in PBS-T). After a 90-min incubation and three washes, human serum-absorbed peroxidase conjugated goat antirabbit immunoglobulin G (ICN Biomedicals, Costa Mesa, CA) was added at 1:2000 in PBS-T/BLOTTO for a 45-min incubation. The plates were again washed

**Table 1.** Age, Weight, Duration of Cardiopulmonary Bypass (CPB), and Aortic Cross-Clamp Time

Group No.	Age (yr)	Weight (kg)	CPB duration (min)	Aortic cross-clamp time (min)
1	66 $\pm$ 3	79.4 $\pm$ 3	90 $\pm$ 6	44 $\pm$ 5
2	64 $\pm$ 3	76.4 $\pm$ 4	93 $\pm$ 6	42 $\pm$ 4

Values are mean  $\pm$  sd. No significant differences were found between groups in any variable measured.

three times and 200  $\mu\text{L}/\text{well}$  of 10 ng/mL orthophenylenediamine substrate (Sigma) was added. The reaction was terminated with 27.5  $\mu\text{L}/\text{well}$  of 8 M sulfuric acid and plates were read at 492 nm in an automated enzyme-linked immunosorbent assay reader and expressed in picograms per milliliter concentration.

Whole blood was drawn from normal, drug-free volunteers and blood neutrophils were separated by dextran sedimentation followed by Ficoll-Paque centrifugation. The neutrophil suspension was judged to be >95% viable by trypan blue dye exclusion. Chemotaxis determinations were performed as described by others (11) and quantified by the leading front method for chemotaxis determination, and expressed as the mean number of neutrophils per high-powered field.

The chemoattractants used in this study are 1) *N*-formyl-1-methionyl-1-leucyl-1-phenylalanine (FMLP), a known potent chemoattractant peptide, used in this study as a comparison standard; 2) the supernatant of the human bronchial epithelial cell culture line, A549 cells, purchased from American Type Culture Collection (Rockville, MD), after 24-h stimulation by TNF 100 ng/mL, with or without aprotinin 500 U/mL or *N*- $\alpha$ -tosyl-L-lysine chloromethyl ketone (TLCK; Sigma) 100 mM in saline (a known potent serine protease inhibitor); and 3) BALF 1 obtained from patients of either Group 1 or 2.

Data were compared with analysis of variance (effect of treatment) and by Student's *t*-test (comparison of BALF 1 versus BALF 2). Differences were considered significant at  $P \leq 0.05$ .

## Results

No significant differences were found between Groups 1 and 2 in age, weight (kg), duration of CPB (min), or aortic cross-clamp time (min) (Table 1).

The percentage of neutrophils of the total cell count of BALF 1 was significantly reduced ( $P < 0.05$ ) in Group 2 compared with Group 1. The total cell count of BALF 1 was insignificantly reduced in Group 2 versus Group 1 (Fig. 1).

The concentration of IL-8 ( $\text{pg}/\text{mL}^{-1}$ ) (mean  $\pm$  SEM) in BALF 1 was significantly lower in Group 2 ( $P < 0.05$ ) compared with Group 1 (Fig. 2). No significant

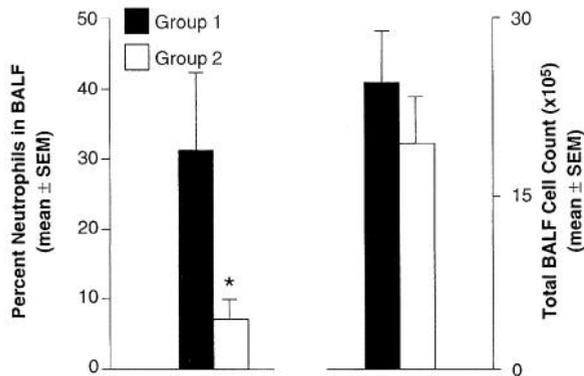


Figure 1. Percentage of neutrophils and total cell counts (mean ± SEM) in bronchoalveolar lavage fluid (BALF) (first lavage) in Groups 1 and 2 after cardiopulmonary bypass. \**P* < 0.05, when compared to Group 1.

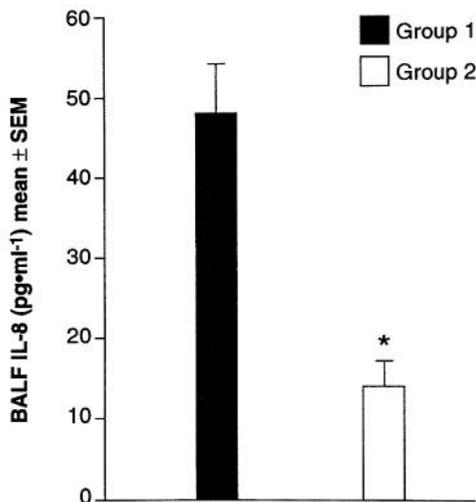


Figure 2. Interleukin-8 concentrations (pg/mL, mean ± SEM) in bronchoalveolar lavage fluid (BALF) (first lavage) in Groups 1 and 2. \**P* < 0.05, when compared to Group 1.

differences were found in BALF 2 between Groups 1 and 2.

### Chemotaxis Data

**FMLP as Attractant.** A significant (*P* < 0.05) increase in neutrophil chemotaxis was noted with FMLP compared with control attractant. Aprotinin as attractant was not significantly different when compared to control. Aprotinin and neutrophils placed on top of the 3-μm chemotaxis filter (separating the neutrophil suspension on top from the chemoattractant on the bottom of the chemotaxis chamber) did not affect chemotaxis with FMLP as the attractant (Table 2).

**The A549 Culture Line Supernatant as Attractant.** After 24-h culture with media alone, the resulting supernatant served as control for this set of experiments. After 24 h of TNF stimulation, the resulting supernatant significantly increased (*P* < 0.05) chemotaxis compared with control. After culturing 24 h with

Table 2. Chemotaxis Data Using *N*-Formyl-1-methionyl-1-leucyl-1-phenylalanine (FMLP), A549 Culture Supernatant, and Bronchoalveolar Lavage Fluid (BALF) (First Lavage) as Chemoattractants

Chemoattractant	Neutrophil count
FMLP	
Control (media only as attractant)	6.1 ± 0.7
FMLP	62.9 ± 2*
Aprotinin as attractant	6.4 ± 1
Aprotinin on top/FMLP on bottom	55.5 ± 2.5*
A549 culture supernatant	
Control	49 ± 4.2
Post-TNF (24 h)	213 ± 12*
Post-TNF plus aprotinin	98 ± 9*†
Post-TNF + TLCK	47.3 ± 2.3‡
BALF	
Group 1 BALF	65.4 ± 10.6
Group 2 BALF	24.9 ± 3.9‡

Values are mean ± SEM.  
 TNF = tumor necrosis factor; TLCK = *N*-α-tosyl-L-lysine-chloromethyl ketone.  
 \**P* < 0.05, when compared to control; †*P* < 0.05, when compared to post-TNF; ‡*P* < 0.05, when compared to Group 1.

TNF plus aprotinin, the resulting supernatant as attractant resulted in chemotaxis significantly greater than control (*P* < 0.05), but significantly less (*P* < 0.05) than TNF alone stimulated A549 cell supernatant. When TNF plus TLCK was cultured 24 h with the A549 cells, the resultant supernatant was not significantly different than control in chemoattractant ability (Table 2).

**BALF 1 as Attractant.** Neutrophil chemotaxis was significantly reduced (*P* < 0.05) when using Group 2 BALF 1 as the chemoattractant compared with Group 1 BALF 1 (Table 2).

### Discussion

IL-8 is a potent proinflammatory chemoattractant cytokine, and can induce blood neutrophils to migrate (chemotax) through vascular endothelium and bronchial epithelium (12). Sputum of patients with inflammatory lung disease, such as cystic fibrosis, bronchiectasis (1), chronic obstructive pulmonary disease, or asthma (13), contain increased concentrations of IL-8 and are characterized by neutrophil accumulation in the airway. Smart and Casak (4,5) demonstrated that TNF stimulation of A549 cells, the same protocol used in this study, results in increased IL-8 supernatant concentrations, and that IL-8, not TNF, is the chemoattractant. Furthermore, Standiford et al. (6) demonstrated that A549 cell culture-generated neutrophil chemotactic bioactivity paralleled IL-8 mRNA levels, and that IL-8 mRNA expression was maximized after 24 h of TNF stimulation. Because these studies (4-6) conclusively demonstrate that the TNF-stimulated

A549 cells produce only IL-8 as a chemoattractant, we did not directly measure IL-8 levels in the A549 cell culture supernatant. Our *in vitro* chemotaxis data demonstrate both aprotinin and TLCK (a known potent serine protease inhibitor) reduce TNF-stimulated A549 cell production of the chemoattractant IL-8. This is further supported by the reduced IL-8 levels in BALF 1 of Group 2 (aprotinin-treated) patients after CPB when compared to the untreated control group.

Endotoxin, found in increased systemic concentrations during CPB (14), stimulates bronchial epithelial cell TNF generation, and TNF in turn induces IL-8 production (1,4-6). TNF also released during CPB (7) is generally not considered a chemoattractant (4,5,12), but acts to induce macrophage, endothelial, or epithelial release of IL-8 (5). Interleukin-1, also released systemically during CPB (7), likewise stimulates IL-8 production and release (15).

Lung injury after CPB is thought to be neutrophil-mediated and is reduced by neutrophil removal using leukofiltration (2,3). Bando et al. (3) demonstrated lung entrapment of neutrophils after CPB, while neutrophil removal by filtration during CPB resulted in reduced lung entrapment and improved pulmonary function post-CPB. Specifically, neutrophil removal has been reported to improve arterial oxygenation, lower pulmonary vascular resistance and extravascular lung water, and on histologic examination, reduce the number of neutrophils, alveolar injuries, and perivascular hemorrhages (3). Neutrophils induce lung injury by release of elastase and oxygen radicals (16). Aprotinin reduces neutrophil elastase release (17). Tumen et al. (18) found that TLCK prevented endotoxin-induced injury in cultured lung endothelium. Other studies (19) demonstrate that inhibition of neutrophil elastase by serine protease inhibitors will reduce neutrophil counts in BALF (also found in this study) as well as subsequent lung edema after endotoxin-induced lung injury in an animal model. These current data lend further support to the concept that serine protease inhibitors, such as aprotinin, may prove to reduce CPB-induced lung injury by reducing lung neutrophil accumulation and lung IL-8 concentrations. Aprotinin is known to inhibit TNF release during CPB (9). TNF is initially synthesized as a membrane-bound precursor, and is cleaved by serine proteases to yield mature TNF (20). Serine protease inhibitors inhibit this cleavage, reducing TNF release by endotoxin-stimulated macrophages (20). Our data from the *in vitro* chemotaxis portion of this study demonstrates that aprotinin has a direct effect on the function of TNF at the cellular level, since incubation of TNF plus aprotinin or TNF plus TLCK inhibits the production of the chemoattractant IL-8, as shown by the resultant change in neutrophil chemotaxis. Hunyadi et al. (21) found direct antagonism by aprotinin on TNF induced cytotoxicity on murine L929 tumor

cells, similar to our findings of a direct antagonism of aprotinin on TNF induced IL-8 release by the A549 cells. Since TNF increases IL-8 gene expression in A549 cells (6), our results suggest that aprotinin may have an inhibitory effect at this level.

Complement activation occurs during CPB (22) and activated complement fragments are known to be potent chemoattractants (23). Human C5a induces IL-8 synthesis in monocytes (24), therefore complement-induced neutrophil chemotaxis may be secondary to IL-8 production (24). We could find no reports of complement products in BALF that could explain our findings of increased neutrophils in BALF after CPB. Similarly, leukotriene B<sub>4</sub> (LB<sub>4</sub>) is a potent chemoattractant and is released during CPB (25), and may explain our *in vivo* findings and the chemoattractant behavior of Group 1 BALF. However, LB<sub>4</sub> also increases chemotaxis secondarily by increased IL-8 induction (26). Also, LB<sub>4</sub> is not found in increased concentration in BALF in other inflammatory lung disease, e.g., asthma (27). The source of IL-8 in BALF is not defined, but thought to be of bronchial epithelial, alveolar macrophage, or of vascular endothelial origin (8). Regardless, the levels of BALF IL-8 found in this study are less than that reported by other investigators. Jorens et al. (8) reported IL-8 BALF levels to be  $386 \pm 73$  pg/mL at four hours post-CPB. Jorens et al. (8) used hypothermic CPB (28°C), a radioimmunoassay IL-8 determination technique, and a longer time interval from CPB termination to BAL, which may explain the higher IL-8 BALF levels than that reported in our study. We have measured IL-8 levels in BALF of several non-CPB, nonsmoking patients undergoing general surgical procedures and found BALF IL-8 levels to be extremely low and usually unmeasurable. The BALF IL-8 concentrations in Group 1 patients apparently contain IL-8 in sufficient amount to be strongly chemoattractant, as demonstrated by the *in vitro* chemotaxis data. This is further supported by the additional finding of the increased neutrophil percentage ( $31\% \pm 10\%$  of total cells present) in the BALF of Group 1. Previous work from this institution (10) demonstrates the percentage of neutrophils from BALF in nonsmoking volunteers is 10% or less. While the increased BALF IL-8 concentration and percentage of neutrophils may suggest a cause and effect relationship, our data do not conclusively prove this relationship.

In summary, aprotinin reduces CPB-induced increased airway IL-8 production and neutrophil accumulation. *In vitro* chemotaxis studies demonstrate aprotinin and TLCK (a potent serine protease inhibitor) both significantly reduce chemoattractant production by a human bronchial epithelial cell line culture after 24 h of TNF stimulation. This study demonstrates that aprotinin

may reduce airway production of the neutrophil chemoattractant IL-8, thereby potentially reducing neutrophil-mediated CPB-induced lung injury.

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