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CLUSTERIN PROTECTS THE LUNG FROM LEUKOCYTE-INDUCED INJURY

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ABSTRACT— Clusterin (CLU) is a multifunctional 75- to 80-kDa glycoprotein that is upregulated during cellular stress and might represent a defense mechanism during local cellular damage. Mechanisms discussed are antiapoptotic, antioxidative, and anticomplement properties as well as chaperone-like features protecting stressed proteins. The aim of this study was to investigate potential protective effects of CLU on pulmonary vasculature after *in situ* PMN activation in isolated rabbit lungs. The experiments were performed on 24 isolated and ventilated rabbit lungs that were perfused with 200 mL of Krebs-Henseleit-10% blood buffer with a constant flow of 150 mL/min in a recirculating system. It was tested whether pretreatment with CLU (2.5 µg/mL; n = 8) or catalase (CAT, 5000 U/mL; n = 8) before *N*-formyl-Met-Leu-Phe (fMLP; 10^{-8} M) injection influenced pulmonary artery pressure (PAP) peak airway pressures (PAW) and edema formation as compared with controls (n = 8). Baseline values of PAP were 9–11 mmHg and PAW 11–13 cmH_2O. Application of fMLP resulted in an acute significant ($P < 0.01$) increase of PAP (48 ± 29 mmHg) within 2 min in the control group and PAW increased to 35 ± 7 cmH_2O within 30min. Pretreatment with CLU completely suppressed the PAP and PAW response as a result of the fMLP challenge ($P < 0.001$), whereas a transient PAW increase up to 27 ± 15 mmHg was observed after CAT. Complement factor C3a release was suppressed by CAT, whereas CLU blocked the complement cascade at the level of C5b-9 formation. Moreover, generation of thromboxane A_2 was reduced after CLU and CAT. Lung edema occurred in the fMLP group but was absent ($P < 0.001$) after CLU and CAT treatment. Both CLU and CAT prevented fMLP-induced lung injury. Stabilizing effects of CLU, point towards complement regulating features at the level of the terminal complement sequence. Elevated levels of CLU during inflammation could reflect a compensatory organ protective mechanism. Further studies are required to elucidate the clinical impact of the observed organ-protective properties of CLU.

KEYWORDS—Acute phase response, acute lung injury, chaperone, complement, heat shock proteins, neutrophils, pancreatitis

INTRODUCTION

Ever since first being described in 1983 by Blaschuk (1), clusterin has been thought to play a role in cellular stress reaction. It is a widely expressed multifunctional 75- to 80-kDa glycoprotein that interacts with a number of molecules in biological systems (2). Clusterin upregulation similar to pancreatitis-associated protein upregulation could be a defense mechanism in local cellular damage (3). Custerin RNA overexpression was shown to cause resistance of tumor cells for complement factor mediated cytotoxicity (4, 5). Possible protective mechanisms are considered to be antiapoptotic (6, 7), by blockage of the terminal complement cascade (C5b-9) (2, 8) or by protecting against oxidative stress (9, 10). More recent studies show that clusterin may be a secreted chaperone molecule (11, 12), which works similar to small heat shock proteins (HSP) (13), inhibiting stress-induced precipitation of a very broad range of structurally divergent protein substrates and binding irreversibly via an ATP-independent mechanism to stressed proteins to form solubilized high molecular weight complexes (14). Although lacking detectable ATPase activity, when acting alone, clusterin itself does not refold stressed proteins *in vitro*. Clusterin, however, inhibits stress-induced precipitation of proteins in undiluted human serum and stabilizes stressed proteins in a state in which heat shock protein 70 (HSP70) at physiological levels may refold those stressed proteins (15). In addition, clusterin seems to prevent tumor necrosis factor α-induced cell death either by direct interaction or by the above mentioned pathways, respectively. In contrast, clusterin antisense-transfection makes cells susceptible to tumor necrosis factor α (16) or even induces apoptotic cell death (17, 18).

The aim of the current study was to investigate the potential protective effects of clusterin in leukocyte associated lung failure in the isolated and perfused rabbit lung using published cytoprotective concentrations of clusterin (9). Respiratory burst was evoked by leukocyte stimulation with *N*-formyl-Met-Leu-Phe (fMLP) (19). A quantification of leukocyte induced damage was obtained by measuring pulmonary vascular resistance, edema formation and thromboxane (TX) A_2 by measuring its stable metabolite TXB_2.

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MATERIALS AND METHODS

Isolated rabbit lung

The techniques of preparing and perfusing isolated rabbit lungs have been previously described in detail (20, 21). After approval by the local board for animal protection female chinchilla rabbits (orticolagus caniculus) weighing 2100 ± 196 g (mean ± SD) were anesthetized with ketamine (50 mg kg^{-1}) and xylazine (4 mg kg^{-1}) and anticoagulated with heparin–sodium 1000 U kg^{-1}, injected in the ear vein. After placement of a tracheostomy tube, the rabbits were mechanically ventilated with room air. The thorax was opened via the diaphragm and after a median sternotomy a catheter was inserted into the pulmonary artery. The lung organ-preparation was isolated and suspended from a weight transducer in a temperature-controlled (37°C) and humidified chamber. After the cannulation procedure, the lungs were perfused with 200 mL of Krebs Henseleit hydroxy-ethyl-starch buffer solution (KHB) by a roller pump at a constant volume inflow of 150 mL min^{-1} in a closed recirculatory system with an open reservoir to collect outflow from the left atrium. Pulmonary embolism by circulating particles or air was prevented by a 40-μm filter and a bubbletrap placed right before the pulmonary artery catheter. The lungs were ventilated with 4% CO_2 in air (frequency 25 min^{-1}, tidal volume 25 mL, PEEP 0.5–1.0 cm H_2O). The mean pulmonary arterial (PAP) and airway pressures (PAW) were continuously recorded via Statham strain gauge transducers. Intermittently samples of perfusate were taken from a catheter which collects the effluent from the pulmonary veins, for measurements of pH (blood gas analysis system 288, Ciba Corning, Fernwald, Germany), oncotic pressure (Onkometer BMT 921, Dr. Karl Thomae GmbH, Germany) as well as for determination of TXB_2, C3a, and sC5b-9 formation. Initially the lungs were perfused with KHB solution, using low flow rates in an open circulatory system to remove remaining blood from the vascular bed. The perfusion fluid was then exchanged for fresh buffer via two separate perfusion circuits two minutes after the beginning of the extracorporeal circulation and 15 min later, after the flow was increased to 150 mL/min. After another 30-min steady-state period, these lungs had a constant mean PAP of 8–9 mmHg (zero-referenced at the hilum). The only lungs selected for the study were those that showed a homogenous white appearance with no signs of hemostasis or edema formation and that had no changes in weight during the steady-state period. In previous experiments, the perfusion with KHB has been documented to maintain integrity of the microcirculation for more than five hours in our model, which was assessed by measurements of PAP and weight gain, by biochemical analysis (LDH, AA-metabolites, histamine) as well as by ultrastructural studies (21).

Experimental protocol

Twenty-four lung preparations were randomly assigned to the three different groups. After 10 min of steady state, 10% autologous whole blood was added to all 24 lungs to add serum components like complement and coagulation factors to the system. With respect to previous work of Ermert (22) addition of blood will not significantly elevate the pool of PMN in pulmonary vasculature, since the resident pool of PMN (10^9 PMN) in the lung exceeds the overall number of circulating PMN (10^8 PMN). After 10 more minutes, clusterin (2.5 μg/mL, according to Ref. 9) was added to eight lungs and 5.000 U mL^{-1} catalase was added to eight (according to Ref. 23). The remaining eight lungs were used as controls. PMN respiratory burst was induced after 10 min by an *in situ* concentration of 10^{-8} M of fMLP. PAP, weight gain, airway pressures, as well as formation of TXB_2, C3a, and C5b-9 were monitored. The care and handling of animals were in accordance with the principles expressed in the Helsinki Declaration.

Preparation and purification of clusterin

Recombinant human clusterin was produced as a fusion protein with maltose-binding protein (MBP). Total RNA was prepared from HeLa cells using standard methods (24) and transcribed in cDNA using the Superscript II system (Gibco/Life Technologies). The total cDNA was used as a template for polymerase chain reaction to generate clusterin cDNA under the following conditions: Denaturation at 94°C, 5 min; 30 cycles of 94°C for 10 s, 55°C for 20 s and 72°C for 1 min, and final elongation 72°C for 10 min with the primers 5′Clust-MBP GG_GGA TCC_GAC CAG ACG GTC TCA GAC AAT GAG and 3′Clust-MBP CC_GTC GAC_TCA CTC CTC CCG GTG CTT TTT GCG, which include the restriction sides *Bam*HI and *Sal*I (underlined in the text), respectively. Clusterin cDNA was subcloned into the pMAL-c2 vector (New England Biolabs) using the same restriction sides (*Bam*HI and *Sal*I). The resulting construct was sequenced to ensure in frame reading. JM109 *Escherichia coli* was transformed with the plasmid, grown to an optical density of 0.5 at 660 nm, and the synthesis of the MBP–clusterin fusion protein was induced for 2 h with 1 mM IPTG. Cells were harvested and MBP–clusterin was purified by affinity chromatography on a Maltose-resin column following the recommendations of the manufacturer (New England Biolabs, Frankfurt/Main, Germany). Doses used in the current experiments were shown to have cytoprotective effects (9).

Measurement of TXB_2

Thromboxane B_2 (TXB_2) was assayed from 100 μL of recirculating KHB as stable hydrolysis product of thromboxane A_2 by an enzyme immunoassay (TiterZyme TXB_2 EIA, PerSeptive Diagnostics Inc., Framingham, MA). The immunoassay and the photometric measurement (AR 2001 photometer, Anthos Labtech Instruments, Krefeld, Germany) were performed according to the manufacturer's specifications. The cross-reactivity of TXB_2-antiserum with 2,3-dinor-TXB_2 was 55.8%, 1.5% with 11-dehydro TXB_2, 1.0% with PGB_2 and 0.5% with PGD_2. Other eicosanoids exert cross reactivity less than 0.1%.

Measurement of complement factors

C3a complement factor concentrations were assayed from 100 μL of recirculating KHB measuring C3a-desArg as a stable plasma product by an enzyme immunoassay (QUIDEL C3a EIA, Quidel, Mountain View, CA) utilizing absorbance detection at 450 nm (25).

The terminal complement complex concentrations were analyzed by enzyme immunoassay measurement of SC5b-9 as a soluble, nonlytic form of C5b-9 complement factor (QUIDEL SC5b-9 EIA, Quidel, Mountain View, CA) using absorbance detection at 405 nm (26).

Perfusion buffer

The perfusate consisted of a Krebs–Henseleit 10% blood buffer solution with a colloid oncotic pressure between 23–25 mmHg, yielding final concentrations of: Na^+ 138 mmol/L; K^+ 4.5 mmol/L; Mg^{2+} 1.33 mmol/L; Cl^- 135 mmol/L; Ca^{2+} 2.38 mmol/L; glucose 12 mmol/L; HCO_3^- 12 mmol/L. The osmolality was approximately 330 mosm/kg (Mikro-Osmometer, Roebling Meßtechnik, Berlin). The pH of the buffer solution was adjusted to 7.4 with 1 M $NaHCO_3$. Shortly before lung isolation autologous blood was drawn from the left ventricle.

Catalase

For the recognition of effects derived from oxygen radicals in our model, bovine catalase (Sigma-Aldrich, Deisenhofen, Germany, Lot No 88H72501) was applied. Catalase is a cellular peroxidase, which catalyzes the reaction $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$. (27). At 20°C and pH 7.0 conditions one unit of this enzyme converts 10^{-6} mol of H_2O_2 per minute. The dosage of 5,000 U mL^{-1} was chosen according to previous investigations which showed complete inhibition of free oxygen radical generation (23).

N-formyl-Met-Leu-Phe (fMLP)

In situ activation of adherent neutrophils in pulmonary vasculature was achieved with fMLP in a final concentration of 10^{-8} mol/L (21, 28). On the surface of granulocytes and macrophages fMLP binds to specific receptors, activating phospholipase C and downstream intracellular pro-inflammatory signal cascades (20, 21). FMLP was purchased from Sigma-Aldrich (Deisenhofen, Germany, Lot H5915), dissolved in DMSO and stored deep frozen until use.

Statistical analysis and data presentation

Data are presented as means ± standard deviations (SD). Because the lungs in the current study were perfused in a closed circuit, the stable metabolites C3a-desArg and sC5b-9 accumulate over the observation period in the perfusate. Thus, release rates were calculated per minute as the difference between the complement factor concentration and the respective previous value divided by the time in minutes.

Differences between groups were tested by one-way analysis of variance (ANOVA) followed by a Bonferroni correction for multiple comparisons. Observations between groups (between-subject-factor) over time (within-subject-factor) were tested with the general linear model for repeated measurements (GLM), according to a two way ANOVA. Endpoints for organ survival comparison with the Kaplan–Meier–test was lung weight gain of >20 g or occurrence of edema fluid in the endotracheal tube, which are indicators for severe and irreversible lung injury in our isolated lung model, lacking chest wall compliance and respective counterpressure. For the determination of bivariate correlation between measured parameters Pearson's correlation coefficient was calculated. Statistical significance was accepted for all procedures at $P < 0.05$. Statistical analysis was performed with SPSS software for MS Windows (Release 10.0.7, SPSS Inc., Chicago, IL.)

RESULTS

Before induction of respiratory burst all lungs showed mean pulmonary arterial pressures (PAP: 9-11 mmHg) and ventilation pressures (peak PAW: 10.5–12.5 mbar) similar to prior studies (20, 21). Within 2 min after granulocyte *in situ* stimulation an acute rise in PAP to 48 ± 29 mmHg (Fig. 1A; $P < 0.01$) was observed. Although clusterin application nearly completely suppressed PAP reaction, transient pressure changes up to 27 ± 15 mmHg were noted in the catalase group. These changes normalized to baseline measurements after 20

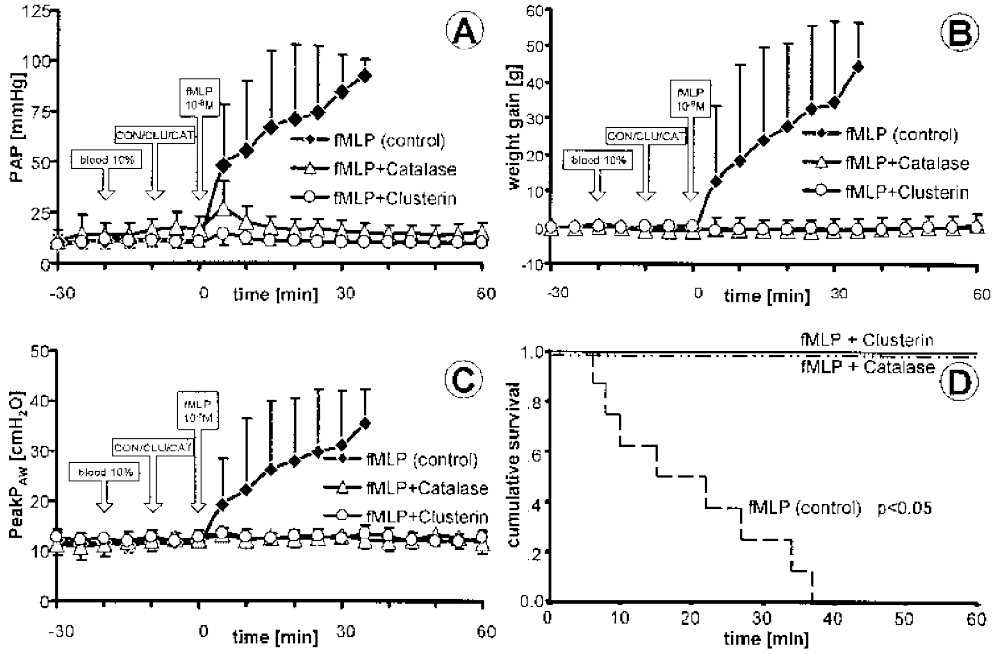

FIG. 1. **(A) PAP (mmHg); (B) lung weight gain (g); and (C) peak pulmonary airway pressure (P_{AW}, cmH_2O) means ± SD of lungs after application of 10^{-8} M fMLP in the three groups: controls (n = 8), addition of 2.5 μg mL^{-1} clusterin (n = 8) and 5000 U mL^{-1} catalase (n = 8).** $P < 0.001$ vs. fMLP (GLM). In six of the eight controls, observation (and therefore graphical representation) ceased after 30 min because of massive edema. D, Kaplan–Meier analysis of organ survival. Endpoints for organ survival were lung weight gain of >20 g or occurrence of edema fluid in the endotracheal tube. *$P < 0.05$ fMLP vs. clusterin and catalase.

min. The clusterin and catalase groups showed a significant suppression of PAP compared with controls ($P < 0.001$ GLM). In controls PMN stimulation with fMLP induced edema (measured in pulmonary weight gain, Fig. 1B) of 44 ± 12 g over the duration of the experiment. The clusterin and catalase groups both showed a complete suppression of pulmonary edema ($P < 0.001$ GLM).

Baseline peak airway pressure of 9–12 mbar (Fig. 1C) remained stable during the steady-state phase of observation under tidal volumes of 22–25 mL. In controls fMLP activation led to a rise in peak PAW to 35 ± 7 mbar. The clusterin and catalase groups suppressed any change in airway pressure ($P < 0.001$ GLM). A Kaplan-Meier-Analysis (Fig. 1D) showed a significant difference ($P < 0.05$) in organ survival in the clusterin and catalase groups as compared with controls.

TXB_2 baseline concentrations at the end of the steady-state phase did not differ significantly between the groups (Fig. 2A). After application of 10^{-8}M fMLP a significantly increase of TXB_2 concentrations was observed in controls ($P < 0.05$, Fig. 2A) within 5 min in comparison to the clusterin and catalase groups. TXB_2 release rates and PAP correlated significantly ($r^2 = 0.33$; $P < 0.01$).

Baseline complement factor C3a concentrations were 14.8 ± 6.7 ng mL^{-1} and 39.2 ± 4.7 ng mL^{-1} for C5b-9. Inflammatory activation induced a rise in complement factor generation as shown in Figure 2B and C. Catalase application significantly reduced anaphylatoxin C3a release versus controls ($P = 0.014$ GLM). In the clusterin group slightly reduced C3a liberation remained below the control concentrations. This difference showed no statistical significance ($P = 0.054$ GLM). On the other hand clusterin application showed a significant reduction in C5b-9 release rates 5 min after fMLP stimulation. During the remaining observation no group differences could be noted.

The appearance of downstream products (e.g., C5b-9) of a cascade system should closely correlate with respective indicators of their activation (e.g., C3a), in the case that the cascade runs entirely and is not inhibited. Lack of correlation might vice-versa indicate blockage of the cascade. Due to the initial differences in C3a concentrations between the groups a linear regression analysis was performed to differentiate statistical dependencies between C3a and C5b-9 for each of the three groups. Positive correlations between C3a and C5b-9 in both, the fMLP ($r^2 = 0.44$; $P = 0.005$) and catalase groups ($r^2 = 0.72$; $P < 0.0005$) were found. No significant correlation, however, was detected in the clusterin group ($r^2 = 0.08$; $P = 0.13$).

DISCUSSION

To verify the effect of clusterin on inflammatory pulmonary vascular response we chose a clusterin concentration of 2.5 μg mL^{-1} described as being protective in oxygen radical (ROS)-induced cellular damage (9). This concentration lies one to two decades below the serum concentration as found in meningococcal sepsis (10–130 μg mL^{-1}) or in septic patients (60–290 μg mL^{-1}) (29). Clusterin-induced blockage of complement activation was a subject of discussion in recent years (2, 8). To be able to evaluate the significance of the latter effect, 10% autologous whole blood was added to *ex vivo* isolated perfused and ventilated rabbit lungs. Whole blood was added to the perfusate mainly for the reason to include serum components. Because the resident pool of sequestrated neutrophils in the lung is much higher than the complete pool of circulating

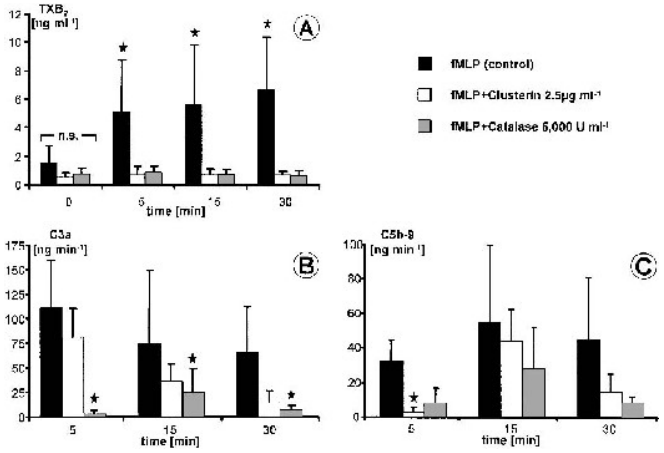

FIG. 2. **(A) TXB_2 generation (ng ml^{-1}); (B) complement factor C3a; and (C) terminal complement complex (C5b-9) generation (mean ± SD) after application of 10^{-8} M fMLP in the three groups: controls (n = 8), addition of 2.5 µg mL^{-1} clusterin (n = 8) and 5000 U mL^{-1} catalase (n = 8).** *$P <$ 0.05 vs. fMLP, GLM. ±SD.

neutrophils (22), addition of 10% blood has no major impact on the number of neutrophils in the pulmonary vasculature. Inclusion of one group of 8 animals receiving catalase as an ROS scavenger enabled the distinction between sole antioxidant and possible additional effects of clusterin. Next to a biochemical analysis of TXB_2 (the stable metabolite of TXA_2) complement factors C3a and sC5b-9 were regularly quantitatively measured. Pulmonary arterial pressure reactions in response to fMLP (Fig. 1a) correlated with those observed by other groups, which noted a maximal vasoconstriction 5 min (30). From previous findings, it can be supposed that pulmonary hypertension, edema, and bronchoconstriction were caused by the release of vaso- and bronchoconstrictive eicosanoids (20, 21, 31), complement activation products (32, 33), ROS (23, 34, 35), proteases (36) and cytokines (37). Later stages of pulmonary edema with fluid displacement and consequent loss of lung volume may also secondarily reduce airway compliance (21, 36).

Our data show a significant reduction in pulmonary hypertension and edema due to a protective effect of clusterin in granulocyte induced pulmonary injury. The influences can be explained by reduced TXA_2 concentrations in animals under treatment with clusterin and catalase (Fig. 2a). To distinguish relevant anti-complement effects (2, 4) in our model we evaluated complement cascade activation by measuring C3a release (Fig. 2b). In addition, C5b-9 generation was measured to evaluate complement induced cytolysis (Fig. 2c) and possible inhibitory effects of clusterin on the terminal complement sequence.

Interestingly enough, catalase application after inflammatory activation resulted in a reduced complement activation in comparison to controls (Fig. 2b), whereas clusterin did not significantly modify complement activation. Reasons for this finding must be assumed in the key role of ROS in complement activation after fMLP-induced granulocyte burst, which were scavenged by catalase. In our setting the antioxidative capacity of clusterin in a concentration of 2.5 μg/mL (9) might play a minor role.

Even though an initial complement activation in clusterin application could be observed, a significantly reduced C5b-9 release was measured against controls (Fig. 2c). To statistically verify this observation a group regression analysis was performed showing a significant dependence of complement activation and membrane complex only in controls and catalase application. Clusterin showed no correlation between C3a and C5b-9. Our explanation for these results is that clusterin inhibits terminal complement complex formation regardless of the degree of complement cascade activation (2, 4).

The initial C5b-9 formation corresponds to TXA_2 generation. Seeger and Suttorp showed that C5b-9 induces TXA_2 and other prostanoid production in lung tissue, (38, 39) this then being the main factor in complement induced lung damage (32). The observed significant correlation between TXA_2 release and pulmonary arterial pressure supports those findings (32, 39). Besides this, Hammerschmidt has demonstrated that myeloperoxidase activity is closely related ($r = 0.97$; $P < 0.001$) to pulmonary artery pressure in fMLP induced injury in the present model (34). This observation points towards a crucial pathogenetic role of ROS in the current setting.

As the measured concentrations of soluble C5b-9 had no impact on lung function and TXA_2 concentrations during the remaining course of the experiments (15–30 min), early C5b-9 formation after fMLP stimulation seems to be mainly responsible for the extent of lung damage. This finding may be explained by membrane associated rather than circulating C5b-9 being responsible for our observations (32). In the course of the initial inflammatory activation the amount of circulating C5b-9 reflects the cell membrane situation. Later, however, this is no longer the case, due to cell disruption and the resulting release of formerly membrane bound C5b-9.

The protective effects of clusterin on the pulmonary vasculature therefore seem to be based on C5b-9 regulation. Compared with the catalase-suppressed C3a activation clusterin's antioxidative properties (9) seem to play a minor role in granulocyte-induced pulmonary damage.

Concerning the experimental model, effects resulting from endotoxin contamination of the perfusate were excluded in previous cell-free experiments (40). In the present setting, however, inclusion 10% blood in the perfusion buffer theoretically bears the risk of endotoxin contamination and subsequent priming of resident neutrophils in the pulmonary vasculature, thus, enhancing reagibiliy to fMLP. Because blood was drawn under aseptical conditions right before extracorporeal circulation, contamination can practically be excluded. Moreover, all experimental groups received blood, hence, observed between group differences may not be attributed to this fact.

Conclusions on the exact molecular mechanisms of the observed effects, such as antiapoptotic properties (6, 7) or modifications of heat shock reactions, cannot be drawn from our data. A more obvious explanation for the observed quick effect would be an interaction via a modification of the extracellular complement cascade rather than via intracellular mechanisms. Nevertheless, long-term tissue protection can be explained by an HSP-mediated accelerated neutrophil apoptosis (41, 42). Assumptions that clusterin as a soluble-HSP

modulates the structure of stressed proteins in the present lung model remain, however, speculative.

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