Original Paper

Journal of Biomedical Science

J Biomed Sci 2003;10:746–756
DOI: 10.1159/000073962

Received: February 13, 2003
Accepted: June 1, 2003

Apoptotic Neutrophils Undergoing Secondary Necrosis Induce Human Lung Epithelial Cell Detachment

Chien-Ying Liu[a] Yun-Hen Liu[b] Shu-Min Lin[a] Chih-Ten Yu[a]
Chun-Hua Wang[a] Horng-Chyuan Lin[a] Chien-Huang Lin[c] Han-Pin Kuo[a]

Departments of [a]Thoracic Medicine II and [b]Thoracic Surgery, Chang-Gung Memorial Hospital,
[c]Department of Medical Technology, Taipei Medical University, Taipei, Taiwan, ROC

Key Words
Apoptosis · Neutrophil · Protease · Phagocytosis · Lung injury

Abstract
Clearance of apoptotic neutrophils by alveolar macrophages plays an important role in the resolution phase of lung inflammation. If not cleared, apoptotic neutrophils are postulated to release histotoxic granular contents. Since numerous cellular proteins are degraded during apoptosis, we sought to determine whether functional serine proteinases are indeed released by apoptosing neutrophils in vitro. In a coculture system, cytokine-activated neutrophils induced detachment in the human epithelial cell line, A549. This process was CD18- and serine proteinase-dependent. Early apoptotic neutrophils induced significant detachment, but live, senescent, resting neutrophils and terminal, secondary necrotic neutrophils had a different effect. This detachment process was CD18-independent but serine proteinase-dependent. Similarly, detachment occurred with primary human small airway epithelial cells. Notably, epithelial cell detachment correlated with the transition of early apoptotic neutrophils to secondary necrosis and with the accumulation of elastase in the supernatant. The membrane integrity of lung epithelial cells was damaged in advance of significant cell detachment. These observations suggest that not only live activated neutrophils but also apoptosing neutrophils can reveal functional elastase activities. Furthermore, the rapidity of the transition emphasizes the importance of the prompt clearance of apoptotic neutrophils before they progress to secondary necrosis at the site of lung inflammation.

Copyright © 2003 National Science Council, ROC and S. Karger AG, Basel

Introduction

The utilization of neutrophilic proteinase is an important host defense mechanism against pathogenic microorganisms [7, 41, 53]. However, abundant literature documents the potential hazards of these proteolytic enzymes

C.Y.L. and Y.H.L contributed equally to the work on this project as first authors.

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2003 National Science Council, ROC
S. Karger AG, Basel
1021–7770/03/0106–0746$19.50/0
Accessible online at:
www.karger.com/jbs

Dr. Han-Pin Kuo
Professor and Head, Department of Thoracic Medicine II
Chang-Gung Memorial Hospital and Chang-Gung University
199 Tunhwa N. Road, Taipei 105, Taiwan (ROC)
Tel. +886 3 3281200 (ext. 8467), Fax +886 3 3272474, E-Mail q8828@ms11.hinet.net

in the pathogenesis of inflammatory organ injury. Circulating mature human neutrophils are committed to die, even when their life spans are prolonged by various endogenous and exogenous factors. Apoptotic neutrophils are recognized by professional phagocytes and semiprofessional phagocytic parenchymal cells. Although definitive in vivo studies are lacking, apoptosing neutrophils are postulated to be removed without further augmentation of the existing inflammation [49, 50]. This process is believed to be rapid, efficient and important during the resolution phase of inflammation [10, 19]. Coincident with the highly controlled process of neutrophil apoptosis is the shutdown of the degranulation response [24]. If not phagocytosed, apoptotic neutrophils will undergo secondary necrosis, an end-stage cellular disintegration characterized by breached membrane integrity. Consequently, proteinases residing in apoptotic neutrophils may be passively released and, in turn, contribute to tissue injury.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α, both common at inflammation sites, accelerate macrophage clearance of neutrophils undergoing apoptosis in vitro [6, 18, 46, 51]. However, several factors associated with chronic inflammatory diseases can markedly inhibit the ability of macrophages to recognize and engulf apoptotic neutrophils. For example, a number of granulocyte enzymes with an extracellular presence in inflammatory diseases are extremely cationic [41]. These cationic molecules, along with a low pH, are common features of chronic inflammation and have been demonstrated to significantly inhibit recognition of apoptotic neutrophils by macrophages [50]. In patients with cystic fibrosis, *Pseudomonas aeruginosa* products have been reported to inhibit macrophage phagocytosis [36, 37]. Moreover, the ability of alveolar macrophages to phagocytose apoptotic neutrophils was found to be significantly impaired in smoke inhalation injury, a condition associated with a high incidence of both bacterial pneumonia and adult respiratory distress syndrome (ARDS) [26], and in children suffering recurrent respiratory tract infections [43]. Other investigations have suggested that macrophages which had previously ingested apoptotic cells subsequently have impaired ingestion capacity [18]. In this situation, apoptotic neutrophils can undergo secondary necrosis, presumably resulting in the release of histotoxic granular contents [52]. If the proteinases released remain functional, they can then escalate the inflammatory response and exacerbate lung injury. Surprisingly, few studies have examined the release of functional proteinases from apoptotic neutrophils.

Neutrophils express several serine proteinases, including neutrophil elastase, cathepsin G and proteinase 3. Among these, elastase is the major proteinase, and has been demonstrated to be critical in neutrophil-mediated tissue injury and extracellular proteolysis [1, 8, 34, 40]. Previous studies showed that serine proteinase inhibitors blocked A549 cell detachment induced by activated neutrophils, and that exogenous elastase restored this detachment [3]. Because the disruption and desquamation of airway and lung epithelial cells have been widely observed in a variety of immunological, infectious and inflammatory pulmonary diseases [2, 8, 10, 23], we used lung epithelial cell detachment as a bioassay in this study [9] to investigate the effect of early apoptotic neutrophils on airway epithelial cells and the role of elastase activity in causing the effect. Because CD18 is a β2 integrin subunit critical to the firm adhesion and the subsequent degranulation response of activated neutrophils [5, 57], and because apoptotic neutrophils have been demonstrated to accumulate CD18 on the cell surface [16], we also investigated the role of the integrin CD18 in the cytotoxic effect of apoptosing neutrophils.

Methods

Reagents

Secretory leukocyte proteinase inhibitor (SLPI), TNF-α and GM-CSF were purchased from R&D Systems (Minneapolis, Minn., USA). The caspase inhibitor zVAD-fmk and the calpain inhibitors zLLY-fmk and PF150606 were purchased from Calbiochem (San Diego, Calif., USA). Human CD18 blocking monoclonal antibody (mAb) 60.3 is a murine IgG2a which was obtained from Serotec (Raleigh, N.C., USA) [44]. All culture medium and additives were purchased from BioWhittaker (Walkersville, Md., USA) unless otherwise specified.

Neutrophil Isolation

Human neutrophils were isolated from freshly drawn heparinized blood by dextran sedimentation and centrifugation on a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden), as previously described [17]. Additionally, contaminating erythrocytes were lysed using 0.9% buffered ammonium chloride. Purified neutrophils of varying concentrations were then suspended in RPMI with or without 10% heat-inactivated fetal bovine serum (FBS; HyClone Sterile, Logan, Utah, USA), depending on assay conditions. Isolated neutrophils were consistently >98% pure according to Lukostat staining (Fisher Scientific, Pittsburgh, Pa., USA), and >99% viable according to the trypan blue exclusion test.

Cell Culture

A549, a human bronchoalveolar carcinoma cell line, was purchased from the American Type Culture Collection (Rockville, Md., USA). Cells were then maintained in minimum essential Eagle medium supplemented with 10% FBS, 5 m*M* glutamine (Life Tech-

nologies, Carlsbad, Calif., USA), 1.9 m*M* sodium pyruvate and 0.1 m*M* nonessential amino acids at 37°C in a 5% CO_2 incubator. The cells were passaged at 80–100% confluence using 0.25% trypsin. Human primary small airway epithelial cells (SAECs) were purchased from Clonetics (Walkersville, Md., USA). The SAECs were expanded and maintained in SAEC basal medium supplemented with bovine pituitary extract, hydrocortisone, human recombinant epidermal growth factor, epinephrine, insulin, retinoic acid, triiodothyronine, gentamicin, amphotericin B and bovine serum albumin (termed SAEC growth medium) at 37°C in a 5% CO_2 incubator. To minimize the phenotypic changes in epithelial cells, SAECs were used in a maximum of 3 passages. Screening tests for *Mycoplasma* contamination at the beginning and at the end of this study showed no positive reaction. All cells used in this study grew well for the determined times.

Detachment Assay

A549 cells were plated in 96-well plates at 4×10^4 cells/well in 100 µl of minimum essential Eagle medium supplemented with 10% FBS. Following overnight culturing, confluent cells were labeled with 20 µ*M* CellTracker Green CMFDA (Molecular Probes, Eugene, Oreg., USA) for 30 min at 37°C and in darkness. Cells were washed twice and incubated in fresh medium for another 30 min at 37°C. At this point, the culture medium was replaced with 200 µl of supernatant from neutrophils cultured in phenol red-free, serum-free RPMI-1640 medium. Alternatively, fresh or cultured neutrophils resuspended at varying concentrations in fresh medium were added to the A549 cell monolayer. Following 6 h of coincubation, the fluorescence intensity (FI) in the wells was measured using CytoFluor (PerSeptive Biosystems, Framingham, Mass., USA) (with excitation at 485 nm and emission at 530 nm). Detached and loosely adherent cells were then removed by gently washing the plate 3 times with phenol red-free medium. Next, the remaining fluorescence in the wells was determined. After subtracting background fluorescence (phenol red-free medium alone without cells), detachment was computed using the following formula:

$$\left[1 - \frac{\text{FI in test well after washing/FI in test well before washing}}{\text{FI in control well after washing/FI in control well before washing}}\right] \times 100\%,$$

where the control well contained the A549 monolayer with RPMI-1640 supplemented with 10% FBS for A549 cells and SAEC growth medium for SAECs. Detachment of the SAEC monolayer was determined similarly, except that SAEC growth medium was used.

Annexin V-Based Fractionation of Cultured Neutrophils

For spontaneous apoptosis, freshly isolated neutrophils were suspended in RPMI-1640 supplemented with 10% FBS and incubated at a concentration of 5×10^6 cells/ml in 6-well tissue culture plates for 16 h. Apoptotic neutrophils were isolated using a MACS apoptotic cell isolation kit (Miltenyi Biotech, Auburn, Calif., USA) according to the instructions of the manufacturer. Briefly, neutrophils in culture were resuspended in 80 µl of binding buffer and 20 µl of annexin V-conjugated paramagnetic microbeads/10^7 cells. Cells were then incubated for 15 min at 6°C. After a single washing, cells resuspended in 1 ml of binding buffer were loaded onto a Midi-MACS column, and the column was then irrigated with binding buffer. Both the annexin V-selected (with a magnet) and flow-through fractions were collected.

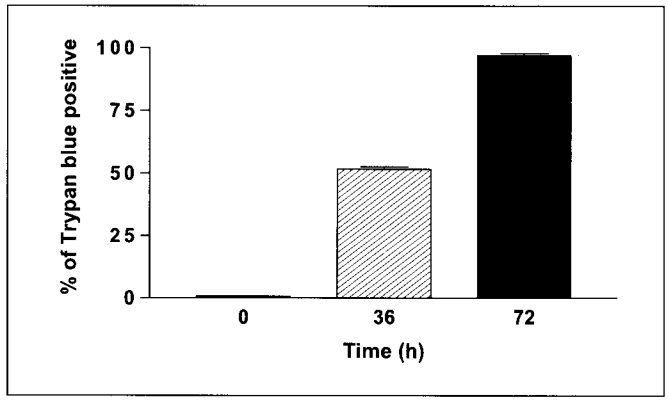


Fig. 1. Fresh neutrophils in serum-free media. When fresh neutrophils at a concentration of 2×10^6/ml were cultured in serum-free media, approximately 50% of cells lost their membrane integrity within 36 h, based on positive trypan blue staining, and 95% of cells did within 72 h.

To ensure quality control, the purity of each fraction was determined in parallel with experiments by double labeling with annexin V-FITC (PharMingen, San Diego, Calif., USA) and propidium iodide (Molecular Probes). Briefly, neutrophils in each fraction were washed once with phosphate-buffered saline (PBS). Cells were then incubated with annexin V-FITC and propidium iodide in binding buffer containing 10 m*M* HEPES, 1.5 m*M* $MgCl_2$ and 2.5 m*M* $CaCl_2$ in normal saline for 15 min at room temperature, and analyzed by flow cytometry (Epics XL, Coulter, Hialeah, Fla., USA).

Neutrophil Elastase Activity Assay

Elastase activity in cell-free supernatant was assessed by a modified method described previously [13] using methoxysuccinyl-Ala-Ala-Val-7-amid-4-methyl-coumarin (Calbiochem). Sample activity was then compared with a standard curve determined using known concentrations of purified human neutrophil elastase (Calbiochem).

Propidium Iodide Staining and Flow Cytometric Analysis

To determine the cell membrane integrity of the adherent lung epithelial cells, propidium iodide staining was used [9]. At each evaluation point, floating cells were removed. The remaining adherent cells were washed twice with PBS and stained with propidium iodide (5 µg/ml in RPMI-1640) for 5 min at 37°C. After 2 gentle washes, cells were examined with fluorescence microscopy using a 590-nm filter and then trypsinized for flow cytometric analysis (with excitation at 488 nm and emission at 585 nm). The percentage of propidium iodide-stained cells in the gated lung epithelial cell region on flow cytometric plots was determined in order to quantify the damage to cell membrane integrity.

Statistical Analysis

Data are expressed as the mean ± SEM. For normally distributed data, a t test was used to evaluate differences between sets. For non-normally distributed data, the Mann-Whitney U test was used. GraphPad Prism (version 2.01, GraphPad Software, San Diego, Calif., USA) was used for all statistical analyses. Statistical significance was defined as $p < 0.05$.

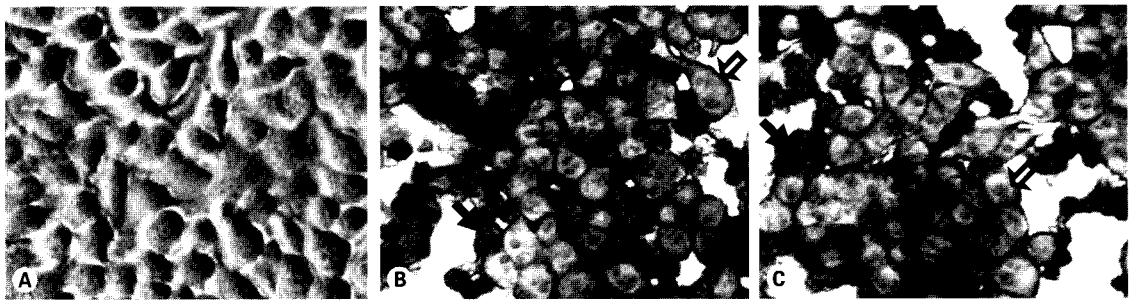


Fig. 2. Fresh neutrophils cultured with a human lung epithelial cell line. **A**, **B** When cocultured on a monolayer of a human lung epithelial cell line (A549, in 96-well plates), fresh neutrophils (1×10^6) were found to induce epithelial detachment within 24 h. **C** Moreover, the time required for comparable detachment to occur was significantly reduced to 6 h if the neutrophils had previously been cultured for 36 h. Solid arrows indicate neutrophils; open arrows indicate A549 cells. Original magnification ×400.

Results

Resting Cultured Neutrophils Induce A549 Lung Epithelial Cell Detachment

When cultured in serum-free media, approximately 50% of the fresh neutrophils lost their membrane integrity with positive trypan blue staining within 36 h, and 95% of cells within 72 h (fig. 1). Fresh neutrophils, when cocultured on a monolayer of the human lung epithelial cell line A549, were found to induce epithelial detachment within 24 h (fig. 2A, B). Moreover, the time required for comparable detachment to occur was significantly reduced to 6 h if the neutrophils had previously been cultured for 36 h (fig. 2C), suggesting a process related to neutrophil senescence and possibly cell death. Next, the underlying mechanism was examined to determine whether it was soluble factor-dependent. As shown in figure 3A, supernatant from neutrophils cultured for 36 h induced A549 cell detachment after 6 h of coculturing. Supernatant from neutrophils cultured for 72 h also caused significant detachment. In parallel experiments, freshly isolated neutrophils or neutrophils cultured for 72 h and resuspended in fresh serum-free medium induced minimal detachment. In contrast, neutrophils cultured for 36 h induced significant A549 cell detachment in a cell number-dependent manner without exogenous neutrophil-activating stimuli (fig. 3B).

There was prominent A549 cell detachment within 6 h when freshly isolated neutrophils were activated with TNF-α (fig. 4). In this coculture system, detachment was noted to be almost completely abrogated by mAb 60.3, a CD18-blocking antibody. To determine whether detachment induced by resting cultured neutrophils requires CD18 engagement, the effect of mAb 60.3 on A549 cell

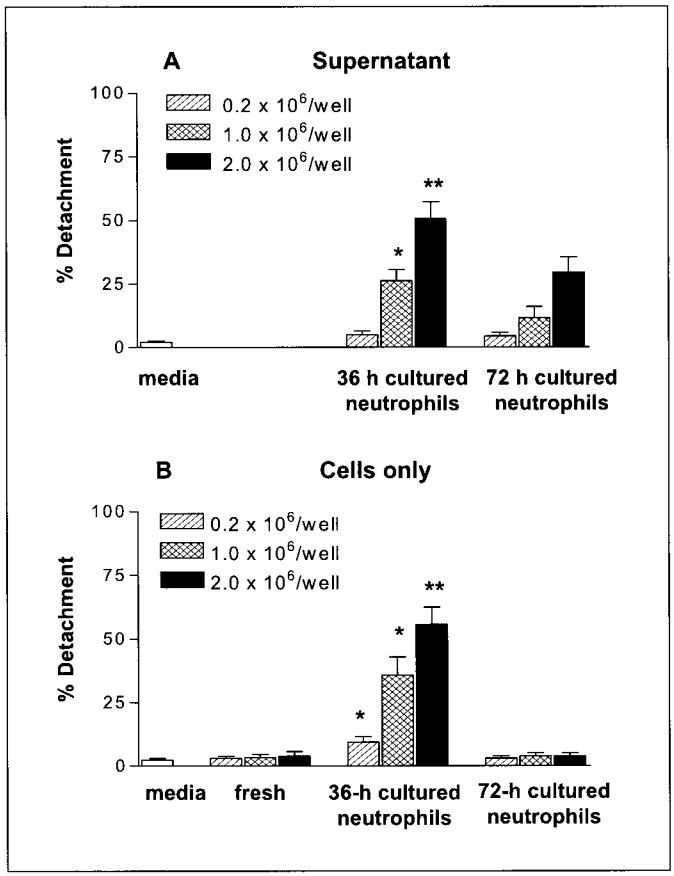


Fig. 3. Cultured resting neutrophils induce A549 cell detachment. Neutrophils were cultured in serum-free medium at the indicated cell densities in order to induce spontaneous apoptosis. After centrifugation, the supernatant (**A**) or cultured neutrophils resuspended in the same volume of serum-free fresh medium (**B**) were added onto a fluorescence-labeled A549 cell monolayer. Detachment of A549 cells was measured after 6 h of coincubation. Results are expressed as the mean ± SEM of 4 experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ compared with medium alone.

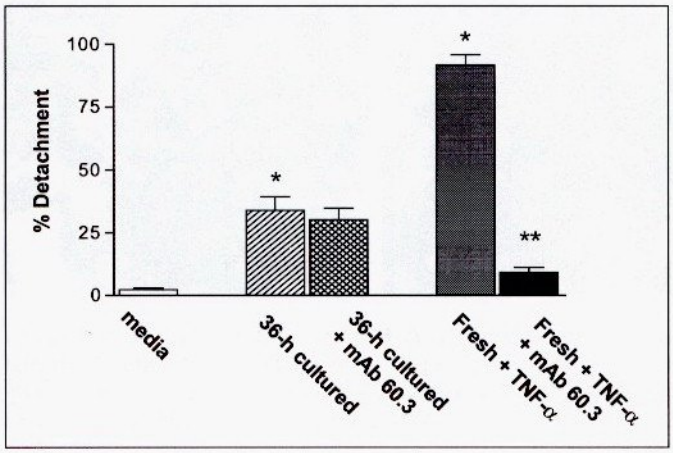


Fig. 4. Detachment of A549 cells by cultured neutrophils is CD18-independent. Neutrophils (5×10^6/ml) were cultured in serum-free medium for 36 h and then collected and centrifuged. Cells were resuspended in the same volume of serum-free fresh medium. Two hundred microliters of cell suspension was added to each well (1×10^6 cell equivalents/well) and cocultured with A549 cells in the presence or absence of mAb 60.3 (10 μg/ml). Fresh neutrophils (1×10^6/well) stimulated with 10 ng/ml TNF-α were also cocultured with A549 cells in both the presence and absence of mAb 60.3 (10 μg/ml). Detachment was measured after 6 h of incubation. Results are expressed as the mean ± SEM of 4 experiments performed in triplicate. *$p < 0.01$ compared with medium alone. **$p < 0.01$ compared with neutrophils treated with TNF-α alone.

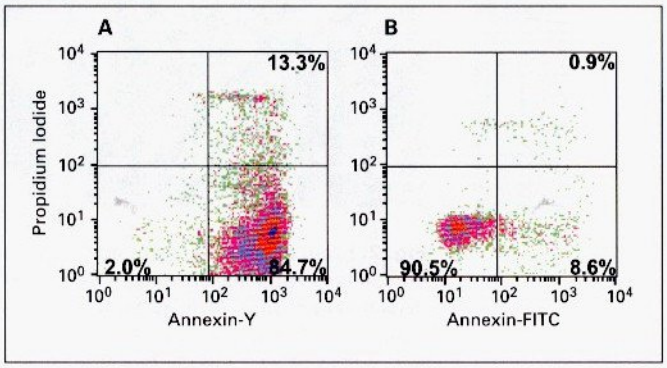


Fig. 5. Annexin V-conjugated magnetic beads separate cultured neutrophils into fractions enriched with nonapoptotic and early apoptotic neutrophils. Freshly isolated neutrophils were cultured for 16 h in order to induce spontaneous apoptosis. Neutrophils were then separated into fractions enriched with either early apoptotic neutrophils (**A**) or nonapoptotic ones (**B**) using annexin V-conjugated paramagnetic beads. Following double staining with annexin V-FITC and propidium iodide, cells were analyzed by flow cytometry. A representative flow cytometric analysis is displayed. The lower left quadrant represents live, nonapoptotic cells, while the lower right quadrant represents early apoptotic cells, and the upper right quadrant represents secondary necrotic cells.

detachment was tested. Figure 4 confirms the suspicion that A549 cell detachment induced by resting neutrophils cultured for 36 h was not affected by mAb 60.3.

Annexin V-Enriched Apoptotic Neutrophils Induce Detachment of Human Lung Epithelial Cells

Neutrophils incubated in vitro undergo apoptosis constitutively. Flow cytometric analysis of the approximately 50% trypan blue-positive neutrophils cultured for 36 h revealed that over 90% of cells were annexin V positive. Thus, we hypothesized that apoptotic neutrophils, if allowed to undergo secondary necrosis, would contribute to A549 cell detachment. To test this hypothesis, neutrophils cultured for 16 h were separated into an annexin V-selected fraction and a flow-through fraction, each of which was then cocultured with A549 cells for 6 h. Neutrophils in the annexin V-selected fraction mainly occurred early in apoptosis (>80% annexin V positive, propidium iodide negative, fig. 5A) and induced significant detachment within 6 h of coculturing in a cell number-dependent manner. Meanwhile, >85% of neutrophils in the flow-through fraction were alive (annexin V negative, propidium iodide negative, fig. 5B) and had a minimal effect at best (fig. 6A). Utilizing human primary lung epithelial cells, SAECs, also revealed significant detachment (fig. 6B). This detachment was not affected by mAb 60.3 (fig. 7). However, the apoptosing neutrophil-induced detachment of airway epithelial cells was completely abrogated by exogenous SLPI, a specific inhibitor of serine proteinases (fig. 7A). Additionally, the supernatant from the apoptosing neutrophils was able to induce A549 cell detachment, which could be attenuated by SLPI (fig. 7B).

Apoptosing Neutrophils Release Functional Elastase Activity

To determine whether early apoptotic neutrophils release functional elastase, we examined elastase activity in supernatant from the annexin V-selected fraction cultured in vitro, and the data revealed that elastase activity continually increased with time (fig. 8A). Parallel to the determination of elastase activity, the membrane integrity of cells was evaluated using trypan blue. The release of functional elastase activity from apoptosing neutrophils was temporally correlated with the progression to second-

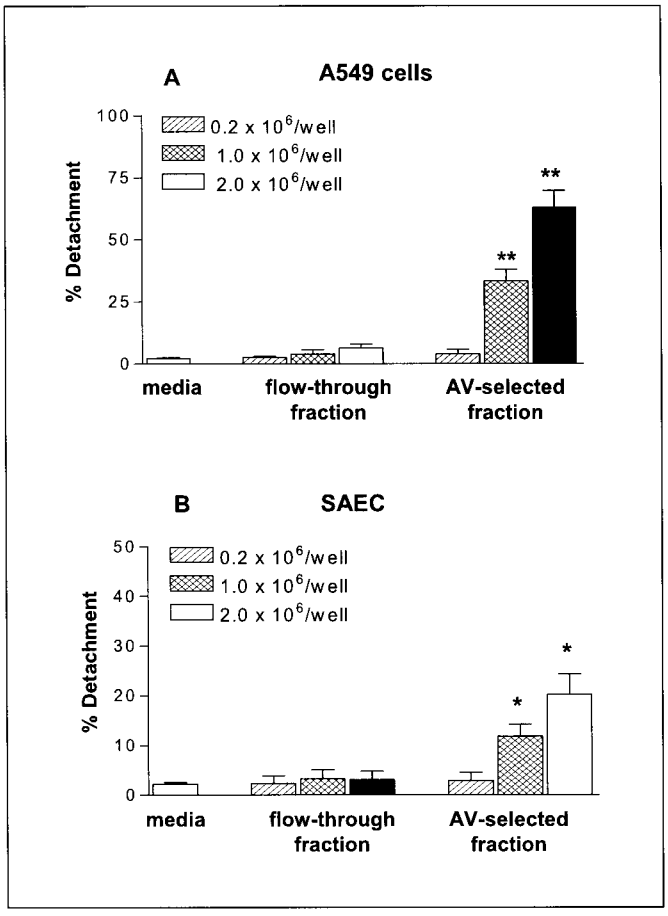


Fig. 6. The fraction enriched with early apoptotic neutrophils induces A549 cell and SAEC detachment. Following annexin V (AV)-conjugated paramagnetic bead-mediated separation, neutrophils cultured for 16 h in each fraction were resuspended in serum-free RPMI-1640 or SAEC growth medium, and added to an A549 cell (**A**) or an SAEC (**B**) monolayer, respectively. Detachment was measured after 6 h of incubation. Results are expressed as the mean ± SEM of 4 experiments performed in triplicate. * $p < 0.05$ compared with neutrophils at the same cell density in the flow-through fraction. ** $p < 0.01$ compared with medium alone.

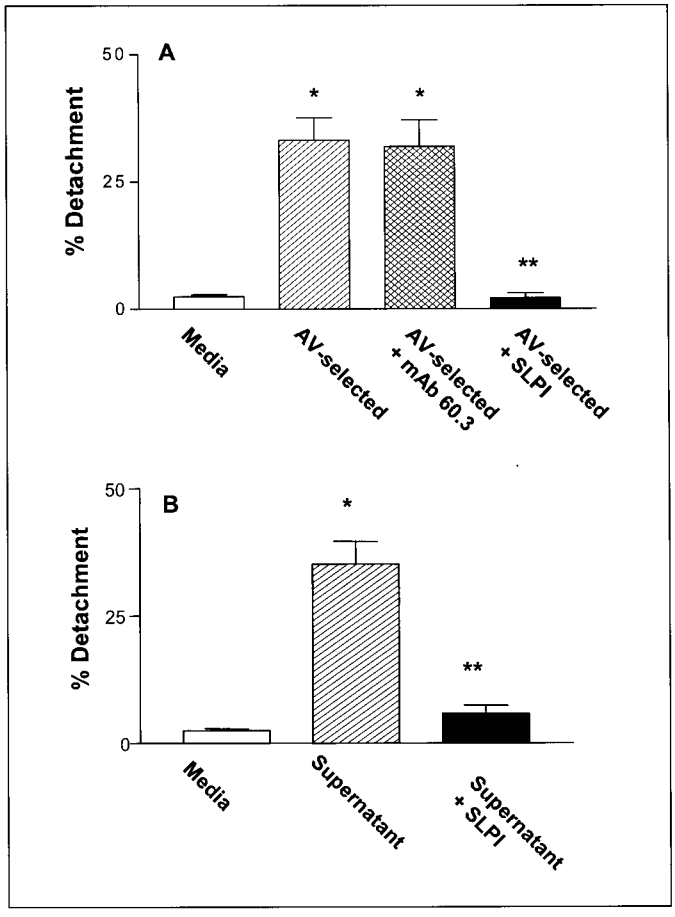


Fig. 7. Early apoptotic neutrophil-induced A549 cell detachment is CD18-independent and serine proteinase-dependent. Neutrophils cultured for 16 h in the annexin V (AV)-selected fraction were resuspended in serum-free medium at a concentration of 5×10^6/ml. Two hundred microliters of cell suspension (1×10^6/well) (**A**) or supernatant from the annexin V-selected fraction cultured for a further 6 h (**B**) was added to each well and cocultured with A549 cells in both the presence and absence of mAb 60.3 (10 μg/ml) or SLPI (10 μg/ml). Detachment was measured after 6 h of incubation. Results are expressed as the mean ± SEM of 4 experiments performed in triplicate. * $p < 0.01$ compared with medium alone. ** $p < 0.01$ compared with the annexin V-selected fraction.

ary necrosis (fig. 8B). In separate dose-response experiments using purified human neutrophil elastase, it was observed that a threshold concentration of 1.25 μg/ml was required to induce significant A549 cell detachment within 6 h. In our experiments, elastase activity in the supernatant of the annexin V-selected fraction reached this level after only 4 h. Although the annexin V-negatively selected flow-through fraction also released elastase, the level of its activity was below the threshold concentration (fig. 8A, B).

The Cell Membrane Integrity of Lung Epithelial Cells Was Damaged by Apoptotic Neutrophils in Advance of Cell Detachment

To further explore the effect of early apoptotic neutrophils on adherent lung epithelial cells, the annexin V-selected fraction of neutrophils cultured for 16 h was coincubated with A549 cells for 6 h. As shown in figure 9, the percentage of propidium iodide-stained adherent A549 cells increased with time, in advance of significant cell detachment, suggesting that early apoptotic neutrophils

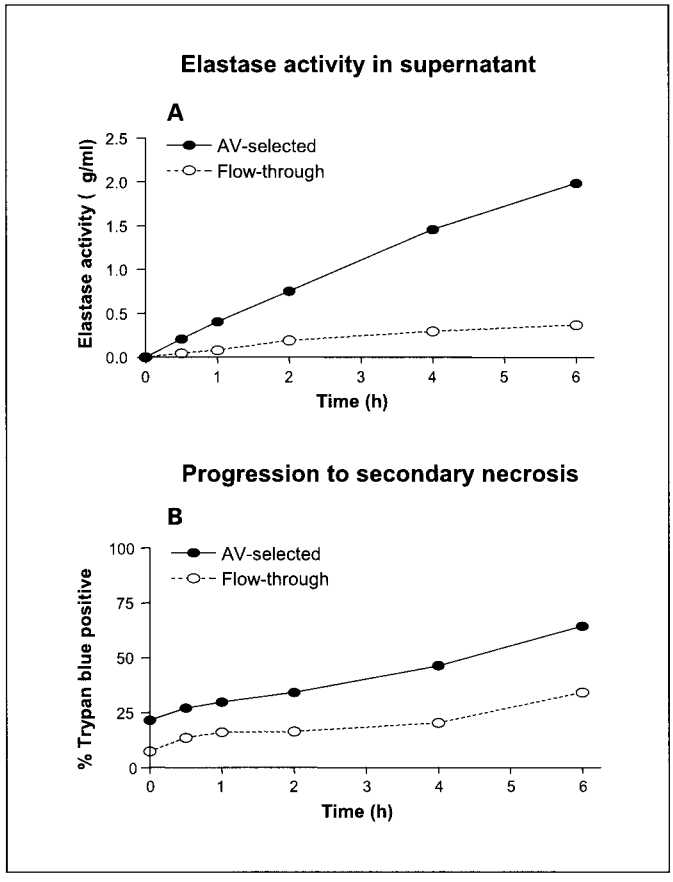


Fig. 8. Release of functional elastase activity from apoptosing neutrophils is temporally correlated with progression to secondary necrosis. Neutrophils cultured for 16 h in both the annexin V (AV)-selected and flow-through fractions were resuspended in serum-free medium at a concentration of 5×10^6/ml. Furthermore, elastase activity in the supernatant (**A**) and membrane integrity (**B**) were determined after incubation for various periods. The elastase activity of the samples was then compared with a standard curve determined using known concentrations of purified elastase. Membrane integrity was assessed by trypan blue staining. Data are expressed as the mean of triplicates, and the results displayed are representative of 4 experiments.

are able to breach the cell membrane integrity and possibly, in turn, cause cell detachment.

Discussion

Our study demonstrates that early apoptotic neutrophils induce lung epithelial cell detachment. This detachment is strictly serine proteinase-dependent and CD18-independent. It also correlates with an increase in elastase activities in the extracellular milieu and the loss of membrane integrity in adherent lung epithelial cells.

A healthy human adult turns over almost 1×10^9 neutrophils/kg of body mass daily [11]. Once recruited to an inflammatory site, neutrophil life spans were demonstrated to be extended ex vivo [14, 35]. Notwithstanding, mature neutrophils are committed to undergoing apoptosis. Apoptosis is an intracellular remodeling process with initially limited autoproteolysis and oligonucleosomal cleavage which is accompanied by downregulation of neutrophil inflammatory responses [29, 30, 59]. This process eventually leads to cellular disintegration, with the breach in cellular membrane integrity (secondary necrosis). Although histotoxic neutrophilic granular contents have been widely stated to be released under this scenario, an exhaustive literature search yielded surprisingly few peer-reviewed original studies that characterized the release of functional proteinase activities from these shrinking, autoproteolysing neutrophils. Utilizing annexin V-conjugated paramagnetic beads that selectively bind to exposed cell surface phosphatidylserine residues present on apoptotic but not live neutrophils [22, 31, 55], this study revealed more accelerated release of functional elastase activity from predominantly early apoptotic neutrophils, compared to that from predominantly live, senescent, resting neutrophils. Furthermore, the timing of this release correlates with the progression of early apoptotic neutrophils to secondary necrosis and lung epithelial cell detachment in vitro. Thus, this study clearly confirms that resting apoptosing neutrophils release functional elastase activity in vitro.

Early apoptotic neutrophils differ from live, selectively activated neutrophils in that despite increased cell surface CD18, they do not require the participation of the β2 integrin, CD18, for the adhesion and release of granule contents [16]. On the other hand, SLPI, one of the endogenous serine proteinase-specific antiproteinase defenses, abrogates both live, activated neutrophil- and apoptosing neutrophil-induced lung epithelial cell detachment. Importantly, exogenous SLPI does not protect the membrane integrity of apoptosing neutrophils.

There are relatively few investigations which have quantified the occurrence of apoptotic neutrophils in the course of acute inflammatory lung diseases in vivo. Thus, the role of dying neutrophils in lung injury is unclear. In patients with ARDS, apoptotic neutrophils in bronchoalveolar lavage fluid are not abundantly identifiable morphologically but are present during the first few days of the initial onset of disease [35]. This phenomenon may be consistent with the prevailing concept that apoptotic neu-

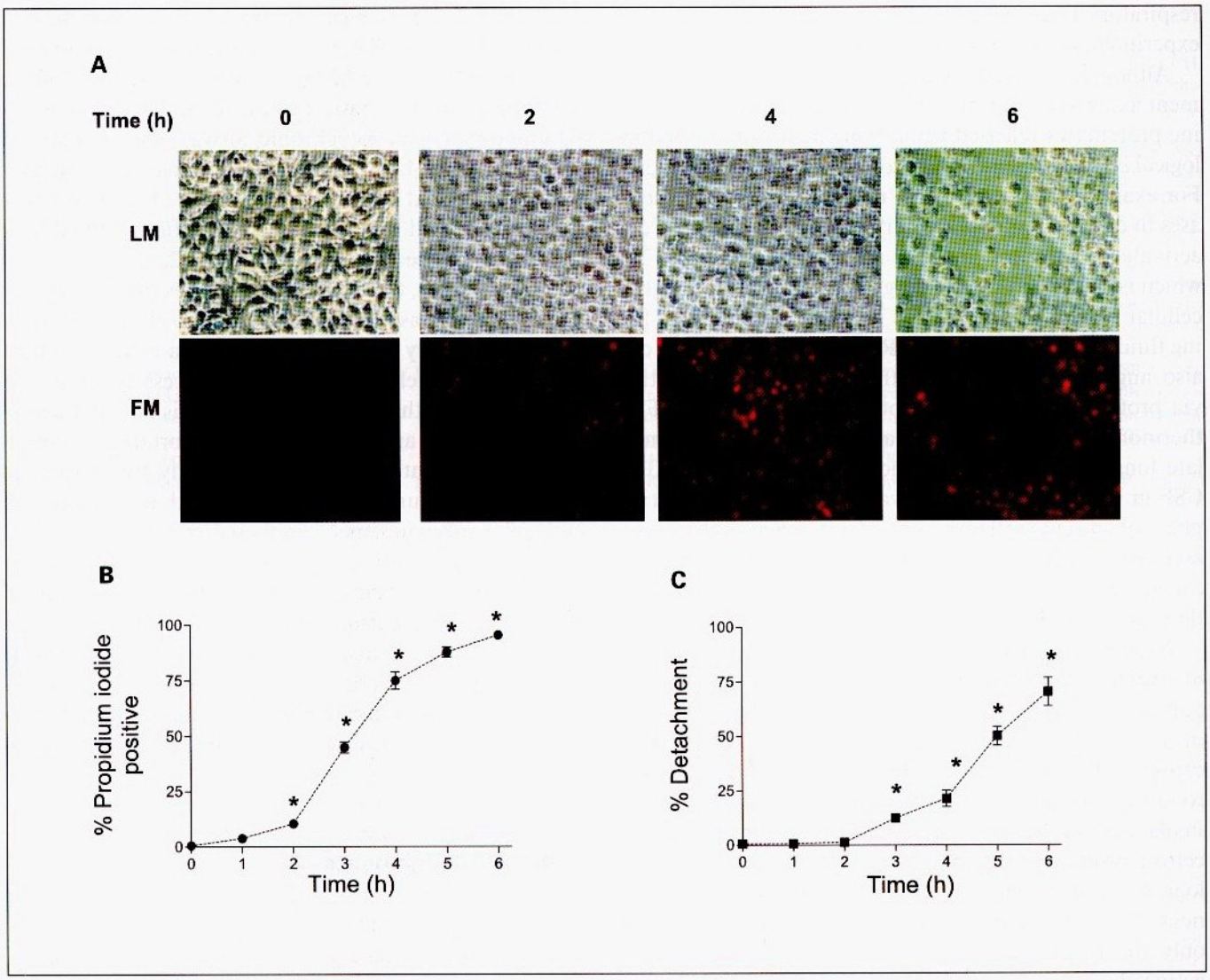


Fig. 9. Apoptosing neutrophils damaged the cell membrane integrity of A549 cells before inducing detachment. The annexin V-selected fraction of neutrophils cultured for 16 h (1×10^6/well in 200 µl of culture medium) was coincubated with A549 cells for 6 h. **A** Cells were observed using fluorescence microscopy. LM = Light microscope; FM = fluorescence microscope. Original magnification ×400. **B**, **C** The percentage of propidium iodide-stained adherent A549 cells (**B**) and cell detachment (**C**) were determined hourly for 6 h. Data are expressed as the mean ± SEM of 4 experiments performed in triplicate. * $p < 0.05$ compared with the data 1 h earlier.

trophils are rapidly recognized and ingested by professional and semiprofessional phagocytic cells [49, 50]. Alternatively, the rapid transition from early apoptosis to apoptotic body formation, secondary necrosis and eventual total cellular disintegration may lead to a certain degree of underestimation. Furthermore, current histochemical techniques and in vitro phagocytosis assays do not distinguish whether the clearance of dying neutrophils occurs before or after the breach of membrane integrity. One recent study observed that numerous, persistent apoptotic neutrophils are present in lung abscesses formed in experimental pneumonia in vivo [28]. This important observation implies that the balance between neutrophil apoptosis and their phagocytic clearance can be disturbed. Indeed, this possibility is supported by two further ex vivo studies that specifically verified the functional impairment of alveolar macrophages, both in clearing apoptotic neutrophils in children suffering from recurrent

respiratory tract infections, and in an animal model of experimental smoke inhalation injury [26, 43].

Although this study used a lung epithelial cell detachment assay as an in vitro bioassay to characterize the serine proteinases released from dying neutrophils, the biological effects of neutrophil proteinases are wide-ranging. For example, besides a direct role of neutrophil proteinases in experimental lung injury [23, 38, 45], elastase can activate neutrophil matrix metalloproteinase-9 [21], which is capable of solubilizing the human alveolar extracellular matrix [15, 42] and is increased in epithelial lining fluid from patients with ARDS [38]. Cathepsin G can also augment inflammation through platelet activation via proteinase-activated receptor-4 activation [47]. Furthermore, neutrophil elastase and α-defensins can stimulate lung epithelial cell production of IL-8 and/or GM-CSF in vitro [39, 54]. Conceivably, during the resolution phase of an acute inflammatory insult, functional proteinases released from apoptosing neutrophils that have escaped timely phagocytic clearance may perpetuate inflammation and contribute to tissue injury.

Numerous in vitro investigations have suggested that neutrophils contribute not only to host defenses against pathogenic organisms but also to defense against tissue injury. Neutrophil proteinases are among the essential causes of these dual effects. Moreover, the realization that robust, endogenous antiproteinase systems are generally in place further leads to the conclusion that maintaining a certain proteinase/antiproteinase balance in vivo is a biological necessity. The current paradigm of a self-limiting, neutrophil-mediated inflammatory process entails not only the timely recruitment of neutrophils capable of active phagocytosis and degranulation, but also their subsequent efficient removal from inflammatory sites [25]. The appreciation that multiple recognition mechanisms exist, whereby effete granulocytes are eliminated by both professional and semiprofessional phagocytes, further indicates the critical importance of this process [12, 20, 48, 56]. However, confirmatory in vivo experiments that directly test the importance of neutrophil apoptosis in the resolution phase of this paradigm are not yet available.

Our data showed that the cell membrane integrity of A549 cells was significantly disrupted in advance of detachment. The molecular mechanisms of detachment of A549 cells or airway epithelial cells are complex and not completely known. The disruption of cell-matrix adhesion and cell-cell tight junctions, and the induction of apoptosis-necrosis have been observed on or in A549 cells [3, 9, 10, 27, 32, 33]. The mechanisms are inducer-specific in part. The disruption and desquamation of airway and lung epithelial cells resulting from cellular damage can increase the permeability of the lung parenchyma to fluid and cells [2, 4, 58]. Many biological cascades follow, resulting in acute airway narrowing and pulmonary inflammatory edema or chronic airway remodeling and lung fibrosis [2, 4, 58]. Related symptoms, including dyspnea, cough and wheezing, can occur. The pulmonary function test will reveal airway obstruction, limited lung expansion and decreased gas diffusion [2, 4, 48].

In conclusion, this study demonstrates that apoptosing neutrophils release, at least, functional elastase activity during secondary necrosis and induce a cytotoxic effect on airway epithelial cells. As this process is rapid, our results indicate that neutrophil apoptosis may not necessarily be benign, and emphasize the importance of removal of these disintegrating cells promptly by phagocytic cells. Clearly, further study is needed to explore the molecular mechanisms of epithelial cell detachment induced by apoptotic neutrophils and to document the occurrence of neutrophil apoptosis and the kinetics of apoptotic neutrophil removal in various disease states. Finally, our results suggest that a therapeutic strategy for inducing granulocyte apoptosis en masse so as to attenuate active lung inflammation should consider the simultaneous enhancement of clearance mechanisms for apoptosing granulocytes.

Acknowledgements

The authors would like to thank the National Science Council of the Republic of China (NSC 90-2314-B-182A-074 and 91-2314-B-182A-060) and the Chang-Gung Medical Research Project (CMRP-1323) for financially supporting this research.

References

1 Amitani R, Wilson R, Rutman A, Read R, Ward C, Burnett D, Stockley RA, Cole PJ. Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. Am J Respir Cell Mol Biol 4:26–32;1991.

2 Ayars GH, Altman LC, O'Neil CE, Butcher BT, Chi EY. Cotton dust-mediated lung epithelial injury. J Clin Invest 78:1579–1588;1986.

3 Ayars GH, Altman LC, Rosen H, Doyle T. The injurious effect of neutrophils on pneumocytes in vitro. Am Rev Respir Dis 129:964–973; 1984.

4 Bargout R, Jankov A, Dincer E, Wang R, Komodromos T, Ibarra-Sunga O, Filippatos G, Uhal BD. Amiodarone induces apoptosis of human and rat alveolar epithelial cells in vitro. Am J Physiol Lung Cell Mol Physiol 278: L1039–L1044;2000.

5 Barnett CC Jr, Moore EE, Mierau GW, Partrick DA, Biffl WL, Elzi DJ, Silliman CC. ICAM-1-CD18 interaction mediates neutrophil cytotoxicity through protease release. Am J Physiol 274:C1634–C1644;1998.

6 Beatty PG, Ledbetter JA, Martin PJ, Price TH, Hansen JA. Definition of a common leukocyte cell-surface antigen (Lp95–150) associated with diverse cell-mediated immune functions. J Immunol 131:2913–2918;1983.

7 Belaaouaj A, McCarthy R, Baumann M, Gao Z, Ley TJ, Abraham SN, Shapiro SD. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. Nat Med 4:615–618;1998.

8 Burnett D, Chamba A, Hill SL, Stockley RA. Neutrophils from subjects with chronic obstructive lung disease show enhanced chemotaxis and extracellular proteolysis. Lancet ii: 1043–1046;1987.

9 Cardella JA, Keshavjee S, Mourgeon E, Cassivi SD, Fischer S, Isowa N, Slutsky A, Liu M. A novel cell culture model for studying ischemia-reperfusion injury in lung transplantation. J Appl Physiol 89:1553–1560;2000.

10 Cox G, Crossley J, Xing Z. Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. Am J Respir Cell Mol Biol 12: 232–237;1995.

11 Dancey JT, Deubelbeiss KA, Harker LA, Finch CA. Neutrophil kinetics in man. J Clin Invest 58:705–715;1976.

12 Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, Gregory CD. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature 392:505–509;1998.

13 Dewald B, Baggiolini M. Evaluation of PAF antagonists using human neutrophils in a microtiter plate assay. Biochem Pharmacol 36: 2505–2510;1987.

14 Dibbert B, Weber M, Nikolaizik WH, Vogt P, Schoni MH, Blaser K, Simon HU. Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: A general mechanism to accumulate effector cells in inflammation. Proc Natl Acad Sci USA 96:13330–13335;1999.

15 Donaldson K, Miller BG, Sara E, Slight J, Brown RC. Asbestos fibre length-dependent detachment injury to alveolar epithelial cells in vitro: Role of a fibronectin-binding receptor. Int J Exp Pathol 74:243–250;1993.

16 Dransfield I, Stocks SC, Haslett C. Regulation of cell adhesion molecule expression and function associated with neutrophil apoptosis. Blood 85:3264–3273;1995.

17 English D, Andersen BR. Single-step separation of red blood cells. Granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J Immunol Methods 5:249–252;1974.

18 Erwig LP, Gordon S, Walsh GM, Rees AJ. Previous uptake of apoptotic neutrophils or ligation of integrin receptors downmodulates the ability of macrophages to ingest apoptotic neutrophils. Blood 93:1406–1412;1999.

19 Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest 101:890–898;1998.

20 Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 405:85–90;2000.

21 Ferry G, Lonchampt M, Pennel L, de Nanteuil G, Canet E, Tucker GC. Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury. FEBS Lett 402:111–115; 1997.

22 Fesus L, Thomazy V, Autuori F, Ceru MP, Tarcsa E, Piacentini M. Apoptotic hepatocytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action. FEBS Lett 245:150–154;1989.

23 Fletcher DS, Osinga DG, Hand KM, Dellea PS, Ashe BM, Mumford RA, Davies P, Hagmann W, Finke PE, Doherty JB, et al. A comparison of alpha 1-proteinase inhibitor methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone and specific beta-lactam inhibitors in an acute model of human polymorphonuclear leukocyte elastase-induced lung hemorrhage in the hamster. Am Rev Respir Dis 141:672–677; 1990.

24 Hart SP, Ross JA, Ross K, Haslett C, Dransfield I. Molecular characterization of the surface of apoptotic neutrophils: Implications for functional downregulation and recognition by phagocytes. Cell Death Differ 7:493–503; 2000.

25 Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. Am J Respir Crit Care Med 160:S5–S11; 1999.

26 Herlihy JP, Vermeulen MW, Joseph PM, Hales CA. Impaired alveolar macrophage function in smoke inhalation injury. J Cell Physiol 163:1–8;1995.

27 Huang CD, Huang KH, Lin HC, Wang CH, Kuo HP. Neutrophil adherence to lung epithelial cells induce interleukin-8 release. Changgeng Yi Xue Za Zhi 22:392–399;1999.

28 Kazzaz JA, Horowitz S, Xu J, Khullar P, Niederman MS, Fein AM, Zakeri Z, Lin L, Rhodes GC. Differential patterns of apoptosis in resolving and nonresolving bacterial pneumonia. Am J Respir Crit Care Med 161:2043–2050;2000.

29 Khwaja A, Tatton L. Caspase-mediated proteolysis and activation of protein kinase Cdelta plays a central role in neutrophil apoptosis. Blood 94:291–301;1999.

30 Knepper-Nicolai B, Savill J, Brown SB. Constitutive apoptosis in human neutrophils requires synergy between calpains and the proteasome downstream of caspases. J Biol Chem 273: 30530–30536;1998.

31 Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84:1415–1420; 1994.

32 Kuo HP, Lin HC, Hwang KH, Wang CH, Lu LC. Lipopolysaccharide enhances substance P-mediated neutrophil adherence to epithelial cells and cytokine release. Am J Respir Crit Care Med 162:1891–1897;2000.

33 Lin HC, Wang CH, Yu CT, Hwang KS, Kuo HP. Endogenous nitric oxide inhibits neutrophil adherence to lung epithelial cells to modulate interleukin-8 release. Life Sci 69:1333–1344;2001.

34 Mainardi CL, Dixit SN, Kang AH. Degradation of type IV (basement membrane) collagen by a proteinase isolated from human polymorphonuclear leukocyte granules. J Biol Chem 255:5435–5441;1980.

35 Matute-Bello G, Liles WC, Radella F 2nd, Steinberg KP, Ruzinski JT, Jonas M, Chi EY, Hudson LD, Martin TR. Neutrophil apoptosis in the acute respiratory distress syndrome. Am J Respir Crit Care Med 156:1969–1977;1997.

36 McClure CD, Schiller NL. Effects of *Pseudomonas aeruginosa* rhamnolipids on human monocyte-derived macrophages. J Leukoc Biol 51:97–102;1992.

37 McClure CD, Schiller NL. Inhibition of macrophage phagocytosis by *Pseudomonas aeruginosa* rhamnolipids in vitro and in vivo. Curr Microbiol 33:109–117;1996.

38 Mulligan MS, Desrochers PE, Chinnaiyan AM, Gibbs DF, Varani J, Johnson KJ, Weiss SJ. In vivo suppression of immune complex-induced alveolitis by secretory leukoproteinase inhibitor and tissue inhibitor of metalloproteinases 2. Proc Natl Acad Sci USA 90:11523–11527; 1993.

39 Nakamura H, Yoshimura K, McElvaney NG, Crystal RG. Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. J Clin Invest 89:1478–1484;1992.

40 Okrent DG, Lichtenstein AK, Ganz T. Direct cytotoxicity of polymorphonuclear leukocyte granule proteins to human lung-derived cells and endothelial cells. Am Rev Respir Dis 141: 179–185;1990.
41 Owen CA, Campbell EJ. The cell biology of leukocyte-mediated proteolysis. J Leukoc Biol 65: 137–150;1999.
42 Palmgren MS, deShazo RD, Carter RM, Zimny ML, Shah SV. Mechanisms of neutrophil damage to human alveolar extracellular matrix: The role of serine and metalloproteases. J Allergy Clin Immunol 89:905–915;1992.
43 Pryjma J, Kaszuba-Zwoinska J, Pawlik J, Pogorzelski A, Lis G, Baran J, Zebrak J. Alveolar macrophages of children suffering from recurrent infections of respiratory tract are less efficient in eliminating apoptotic neutrophils. Pediatr Pulmonol 27:167–173;1999.
44 Ramamoorthy C, Sasaki SS, Su DL, Sharar SR, Harlan JM, Winn RK. CD18 adhesion blockade decreases bacterial clearance and neutrophil recruitment after intrapulmonary *E. coli*, but not after *S. aureus*. J Leukoc Biol 61:167–72;1997.
45 Rees DD, Brain JD. Effects of cystic fibrosis airway secretions on rat lung: Role of neutrophil elastase. Am J Physiol 269:L195–L202; 1995.
46 Ren Y, Savill J. Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. J Immunol 154:2366–2374;1995.
47 Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. J Biol Chem 275:6819–6823;2000.
48 Savill J, Dransfield I, Hogg N, Haslett C. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature 343:170–173;1990.
49 Savill J, Smith J, Sarraf C, Ren Y, Abbott F, Rees A. Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis. Kidney Int 42:924–936; 1992.
50 Savill JS, Henson PM, Haslett C. Phagocytosis of aged human neutrophils by macrophages is mediated by a novel 'charge-sensitive' recognition mechanism. J Clin Invest 84:1518–1527; 1989.
51 Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest 83:865–875;1989.
52 Squier MK, Schnert AJ, Sellins KS, Malkinson AM, Takano E, Cohen JJ. Calpain and calpastatin regulate neutrophil apoptosis. J Cell Physiol 178:311–319;1999.
53 Tkalcevic J, Novelli M, Phylactides M, Iredale JP, Segal AW, Roes J. Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G. Immunity 12:201–210;2000.
54 Van Wetering S, Mannesse-Lazeroms SP, Dijkman JH, Hiemstra PS. Effect of neutrophil serine proteinases and defensins on lung epithelial cells: Modulation of cytotoxicity and IL-8 production. J Leukoc Biol 62:217–226;1997.
55 Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184:39–51;1995.
56 Walsh GM, Sexton DW, Blaylock MG, Convery CM. Resting and cytokine-stimulated human small airway epithelial cells recognize and engulf apoptotic eosinophils. Blood 94:2827–2835;1999.
57 Walzog B, Seifert R, Zakrzewicz A, Gaehtgens P, Ley K. Cross-linking of CD18 in human neutrophils induces an increase of intracellular free Ca^{2+}, exocytosis of azurophilic granules, quantitative up-regulation of CD18, shedding of L-selectin, and actin polymerization. J Leukoc Biol 56:625–635;1994.
58 Wang SZ, Xu H, Wraith A, Bowden JJ, Alpers JH, Forsyth KD. Neutrophils induce damage to respiratory epithelial cells infected with respiratory syncytial virus. Eur Respir J 12:612–618;1998.
59 Whyte MK, Meagher LC, MacDermot J, Haslett C. Impairment of function in aging neutrophils is associated with apoptosis. J Immunol 150:5124–5134;1993.