Yonago Acta medica 1999;42:21–29

Alveolar Epithelial Cell Line, A549 Cells Inhibit Neutrophil Apoptosis

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Though cellular localization at the site of an inflammatory challenge is the critical first step in a neutrophil response, little has been known about the effects of alveolar epithelial cells on the dynamics of neutrophils. To investigate these effects, we used human purified neutrophils incubated with monolayers of human alveolar epithelial cells (A549), quiescent or preactivated with recombinant human tumor necrosis factor (TNF)-α for 24 h. Laser scanning cytometry (LSC) was employed to detect nuclear morphological changes and quantitate DNA strand breaks in neutrophils. The experiments revealed that A549 cells inhibited neutrophil apoptosis, and that this inhibitory effect was enhanced when the A549 cells were preactivated with TNF-α for 24 h. These results suggest that alveolar epithelial cells may be potentially able to contribute to promote inflammatory mechanisms by delaying neutrophil apoptosis.

Key words: apoptosis; A549; laser scanning cytometry; terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling method

In many lung diseases, neutrophils contribute to the pathogenesis of these diseases (Diaz et al., 1989; Sibille and Reynolds, 1990). An important factor determining whether inflammation resolves or progresses to a chronic inflammatory state is the process by which recruited neutrophils and their potentially noxious contents are removed from inflamed tissues (Haslett et al., 1989). Normally, these terminally differentiated phagocytes undergo spontaneous apoptosis and then lead to macrophage ingestion (Cox et al., 1995). This may represent a neutrophil removal mechanism that is important both in the control of inflammatory tissue injury and for the normal resolution process of inflammation. Even though neutrophil recruitment from the bloodstream to respiratory tissue is the initial step of alveolar inflammation, the effects of alveolar epithelial cells on the dynamics of neutrophils has not been previously studied. So the hypothesis has been proposed that alveolar epithelial cells might modulate neutrophil apoptosis. To test this hypothesis, we studied neutrophil nuclear morphological changes and DNA strand breaks incubated with monolayers of A549 cells, quiescent or preactivated with recombinant human tumor necrosis factor (TNF)-α for 24 h. TNF-α is an important cytokine, active in the inflammation in the lung, whose sources are mainly macrophages. Because alveolar macrophages are in the front line of pulmonary defense and a rich source of cytokines, they are uniquely capable of stimulating the alveolar epithelium by secretion of TNF-α. The experiment was performed by laser scanning cytometry (LSC), which combines the advantages of both flow and image cytometry (Kamentsky and Kamentsky, 1991).

Subjects and Methods

Monolayer preparation of A549 cells

The human A549 alveolar epithelial cell line (ATCC, Rockville, MD) shows similar features

Abbreviations: D-MEM/F-12, Dulbecco's modified Eagle medium/nutrient mixture F-12; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule-1; LSC, laser scanning cytometry; PBS, phosphate-buffered saline; PI, propidium iodide; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

as type II alveolar epithelial cells. The cells were grown as monolayers in 35 mm tissue culture dishes incubated in 100% humidity and 5% CO_2 at 37˚C. Dulbecco's modified Eagle medium/nutrient mixture F-12 (D-MEM/F-12) media containing 365 mg/L L-glutamine (Life Technologies, Inc., Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used as the growth media. The cells that form the monolayers were harvested with trypsin (0.25%) and EDTA (0.1%) in Mg^{2+}- and Ca^{2+}-free phosphate buffered saline (PBS) (Research Institute for Medical Disease, Osaka) centrifuged at low speed ($250 \times g$, 5 min), resuspended in fresh medium and plated at a concentration of 2×10^5 cells/dish. The cells were grown to confluence on tissue culture dishes for 3 to 4 days. Monolayer integrity was ensured by phase contrast microscopy.

Isolation of neutrophils

Neutrophils were isolated from the peripheral blood of healthy volunteers, as previously described by Ferrante and Thong (1980), employing Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden). Only neutrophil preparations with a purity greater than 95% (as determined by microscopic examination of smears of the cell preparation) and viability greater than 95% (as assessed by using trypan blue exclusion) were used for these studies. Neutrophils were finally resuspended in D-MEM/F-12 at a concentration 2.5×10^6 cells/mL.

Incubation of neutrophils with A549 cells in a quiescent state

After A549 cells were washed carefully twice with culture medium, neutrophils were plated 1 mL onto a tissue culture dish coated with confluent monolayers of A549 cells, and were allowed to be incubated for an indicated length of time.

Incubation of neutrophils with A549 cells in a preactivated state with TNF-α

After A549 cells were pretreated with 100 ng/mL TNF-α (Genzyme, Cambridge, MA) for 24 h and then washed carefully twice with culture medium, neutrophils were plated 1 mL onto tissue culture dish coated with confluent monolayers of activated A549 cells, and were allowed to be incubated for an indicated length of time. Neutrophils incubated in medium alone served as the control.

Slide staining and LSC scanning protocol

After incubation at 6 and 24 h, the supernatant which contained the neutrophils were collected and fixed with 4% formalin (3×10^6 cells/mL) for 10 min, then pipetted 50 μL onto glass microscope slides, and thereafter allowed to air dry. In order to detect morphological changes and DNA strand breaks, the samples were processed using the TdT kit (Apop Tag Direct; ONCOR, Inc., Gaithersburg, MD) employing the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, according to the manufacturers instructions. TUNEL is a method of choice for rapid identification and quantification of DNA fragmentation (Gavrieli et al., 1992). DNA strand breaks were directly labeled with fluorescein isothiocyanate (FITC) conjugated deoxynucleotides in a single step reaction. The cells were counterstained with the DNA intercalating fluorochrome propidium iodide (PI; 10 μg/mL, Sigma Chemical Co., St. Louis, MO) solution containing 0.1% RNase (Sigma). After staining, coverslips were immediately placed on the slides and sealed with nail polish.

LSC measurement

We used the LSC of Kamentsky and Kamentsky (1991), build up by Olympus Optics Co. (LSC 101; Tokyo, Japan). The cells were illuminated with a 488 nm wavelength light from an argon ion laser and their emission was separated by a 570 nm dichroic mirror. Green FITC (530 ± 20 nm) and red PI (> 610 nm) fluorescences were

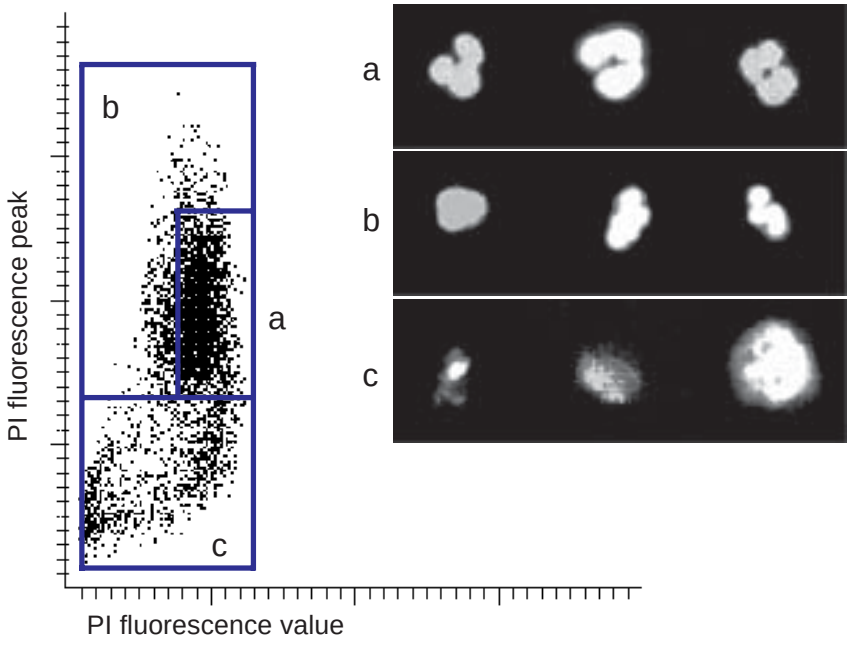


Fig. 1. A 2-parameter (PI fluorescence value versus PI fluorescence peak) cytogram of suspension neutrophils at 24 h. The cells producing the data points in each of the 3 regions labelled **a** to **c** were automatically relocated, and a gallery of digital photographs for each of the regions were created.

measured by separate photomultipliers. In most cases, a number greater than 5,000 cells were examined for approximately 10 min. Overlapping nuclei were automatically excluded from the counting by special statistical filters. Three parameters were used for expressing nuclear features. Among the features caluculated were the peak, corresponding to the highest pixel value within the segmenting contour, the area, corresponding to the total number of pixels within the segmenting contour, and the value, which is the sum of all pixel values within the segmenting contour. Data were expressed using 2-parameter dots of possible permutations of these 3 parameters.

Gating region in the cytograms

We defined 3 gating regions in a cytogram of PI fluorescence value versus PI fluorescence peak (Fig. 1). The cluster of cells that were not found at the beginning of the present experiment appeared at incubation time (Fig. 2). Region a contains all the neutrophils at 0 h. Region b, which is above and left of region a, was created to cover the cluster of cells that appeared mainly at 6 h. Region c, which is lower to region a, was created to cover the cluster of cells that appeared mainly at 24 h. The cells in each indicated region were automatically counted and expressed as a percentage of the total number. In the experiment using 2 fluorochromes, the FITC positive cells were separated by a rectangular gating region (Fig. 3A) in a cytogram showing PI fluorescence value and FITC fluorescence value. These gated FITC positive cells were automatically relocated in a cytogram showing PI fluorescence value versus PI fluorescence peak (Fig. 3B). TUNEL positive cells were counted and also expressed as a percentage of the total cell number.

Inspection of nucleus

In every experiment, the cells producing the data points were automatically relocated and observed by microscope or digital photos.

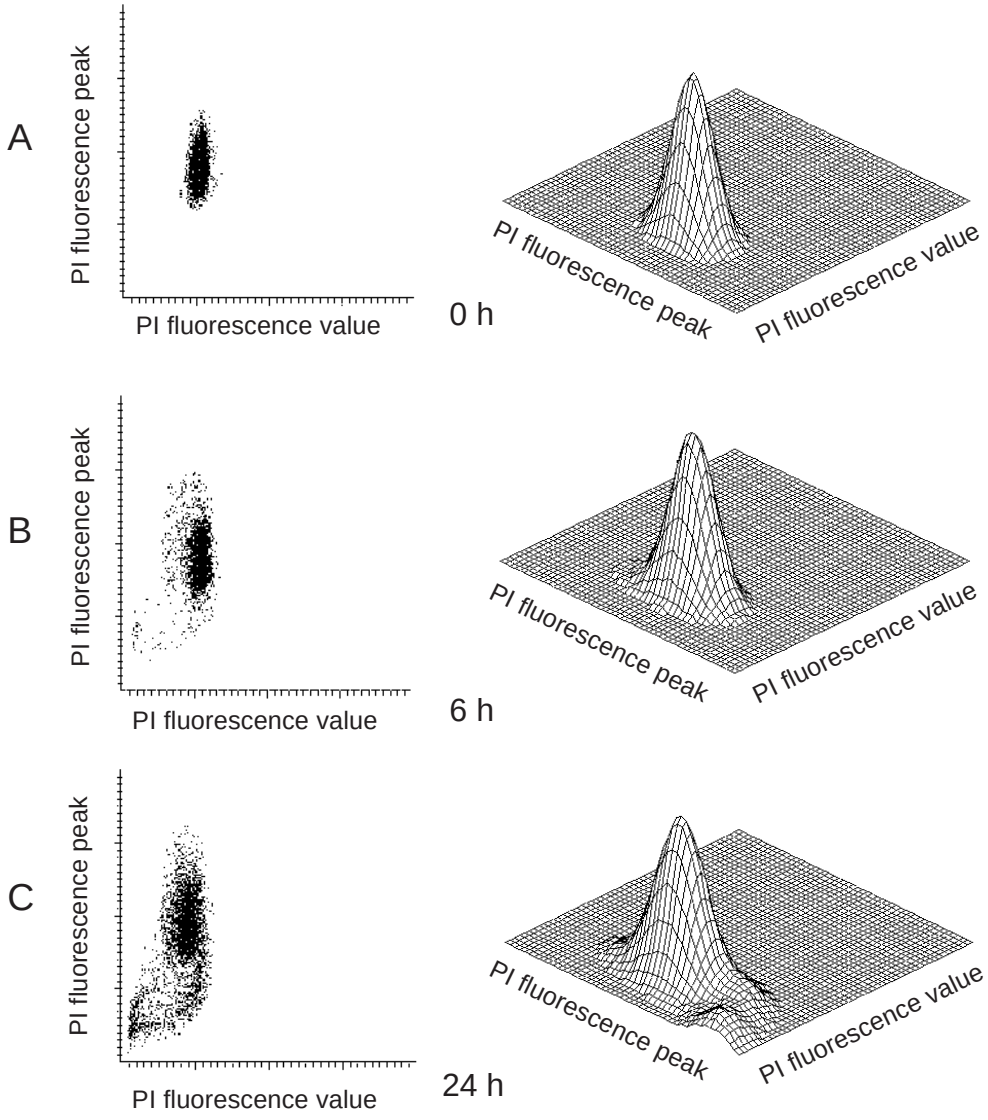
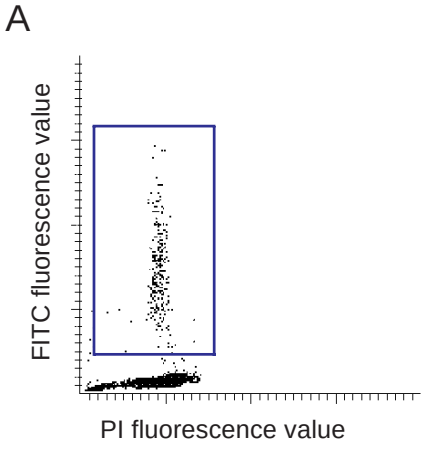
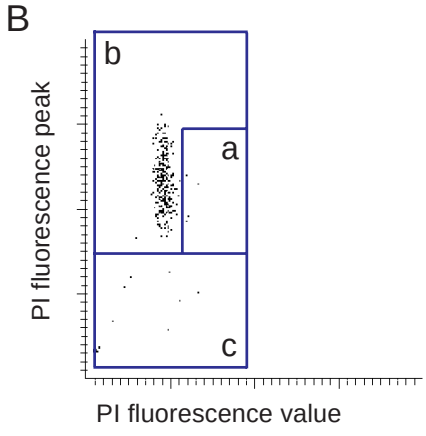


Fig. 2. A PI fluorescence value versus PI fluorescence peak cytogram and 2-parameter histogram of suspension neutrophils were made at each indicated time point. **A**: newly purified neutrophils, **B**: after 6 h incubation, **C**: after 24 h incubation. The cluster of cells that were not found in the control cytogram (**A**) appeared at incubation time (**B** and **C**). 3D histograms show a smooth peak at 0 h, and then another smaller peak appearing through 6 and 24 h.

Statistics

All the data were presented as mean ± SD. The Kruskal-Wallis and Mann-Whitney *U* tests were used for statistical comparisons of results between different groups, and *P* values less than 0.05 were considered to be statistically significant. These statistical analyses were performed with the Stat View 4.11 statistics software (Abacus Concepts, Berkeley, CA).





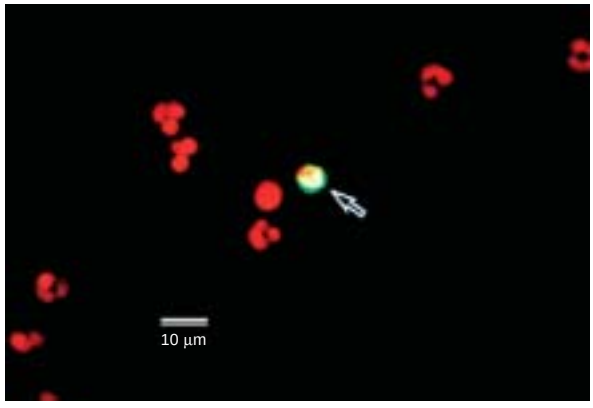


Fig. 3A. A cytogram of PI fluorescence value versus FITC fluorescence value represents suspension neutrophils at 24 h using the TUNEL method. The rectangular region contains neutrophils that incorporated FITC labeled deoxynucleotide in the reaction catalyzed by TdT.

Fig. 3B. TUNEL positive cells in rectangular region in Fig. 3A were relocated in a cytogram of PI fluorescence value versus PI fluorescence peak.

Fig. 3C. Direct inspection showed small nuclei with hypercondensation of chromatin labeled with fluorochrome-conjugated deoxynucleotide.

Results

Inspection of neutrophil nuclei in each indicated region

In every experiment, the cells contained in region a exhibited lobulated nuclei. The cells in region b had bright and small nuclei compared with the cells in region a. The cells in region c were all in various sizes of nuclei with the least dense chromatin of any of the cell groups (Fig. 1).

The differential percentage of neutrophils incubated with A549 cells in a quiescent state

After 6 h of incubation time, the percentage of each indicated region was not significantly different compared with that of suspension neutrophil cultures. After 24 h, the percentage of region a was higher (P = 0.01), while the percentage of both region b and c were lower (P = 0.02 and P = 0.01, respectively) than those of suspension neutrophils (Fig. 4). Representative cytograms are shown in Fig. 5A.

The differential percentage of neutrophils incubated with A549 cells in a preactivated state with TNF-α

After 6 h, the percentage of region a was higher (P = 0.01), while the percentage of region c was lower (P = 0.01) than those of suspension neutrophils. After 24 h, the percentage of region a was higher (P = 0.01) and the percentage of both region b and c were lower (P = 0.01, both)

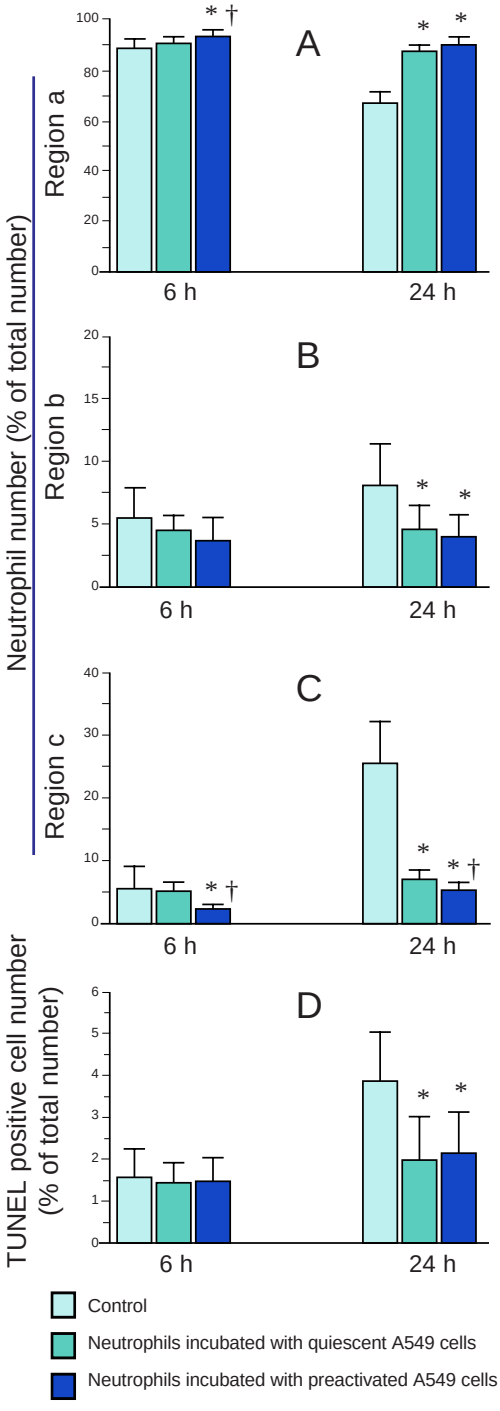


Fig. 4. Differential percentages of neutrophils in each indicated region (**a**, **b** and **c**) and percentages of TUNEL positive cells. The data are expressed as mean ± SD from 8 independent experiments. **P* < 0.05 compared with control neutrophils. †*P* < 0.05 compared with neutrophils incubated with quiescent A549 cells.

than those of suspension neutrophils. Furthermore, the percentage of region a at 6 h was higher and the percentage of region c at both 6 and 24 h were lower ($P = 0.01$ and $P = 0.03$ for 6 h and 24 h, respectively) than those of neutrophils incubated with quiescent A549 cells (Fig. 4). Representative cytograms are shown in Fig. 5B.

TUNEL positive cells

FITC positive cells showing small nuclei with hypercondensation of chromatin labeled with FITC-conjugated deoxynucleotides (Fig. 3C) were almost all located in region b, some extending to region c (Fig. 3B). At 6 h, these FITC positive cell percentages were not significantly different, but at 24 h, the percentage was higher in suspension neutrophils than in neutrophils in the other 2 groups (Fig. 4D).

Discussion

Apoptosis or programmed cell death is a mechanism of cellular death believed to play a pivotal role in a wide variety of physiological conditions (Thompson, 1995), and its deregulation has been proposed to contribute to the pathogenesis of many diseases (Newell et al., 1990). The morphological event characterizing apoptosis is nuclear pyknosis associated with chromatin condensation and the biological "hallmark" is internucleosomal chromatin fragmentation (Wyllie et al., 1980). The apoptosis process does not elicit an inflammatory response, nor does it damage adjacent cells (Fesus et al., 1991) because apoptotic neutrophils are rapidly ingested by alveolar macrophages (Cox et al., 1995).

In our experiment, TUNEL positive cells were almost all located in region b, some extending to region c. Taking into account that TUNEL is a method of choice for rapid identification and quantification of DNA fragmentation, the cells in region b which were strongly TUNEL positive with small condensed nuclei were thought to be apoptotic cells when the nuclear chromatin had begun to condense. Be-

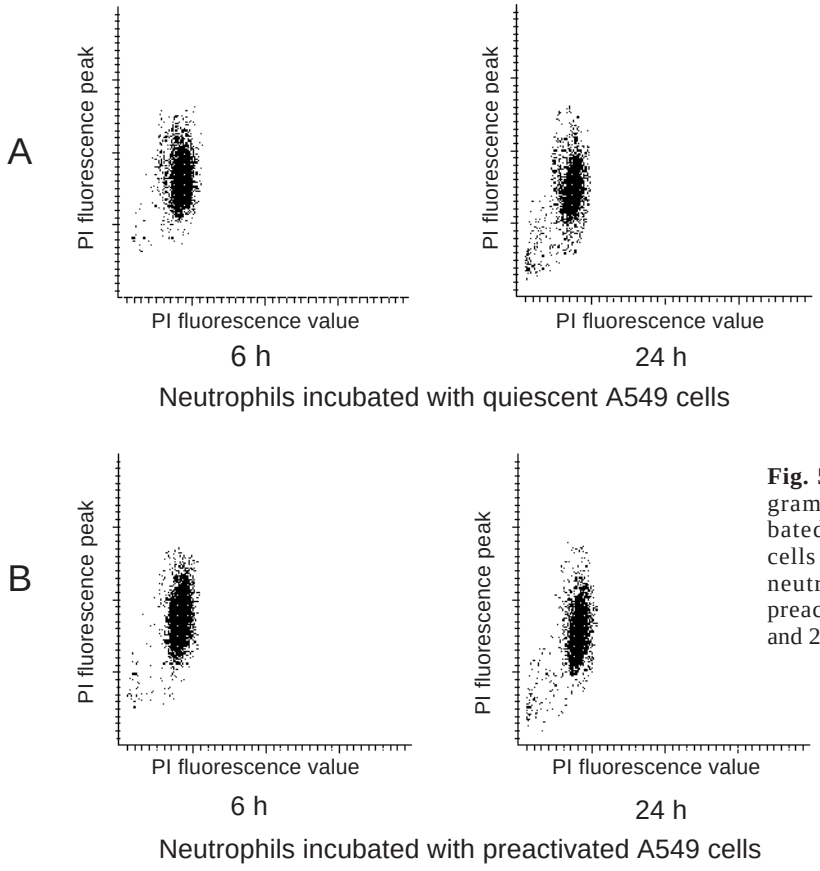


Fig. 5. Representative cytograms of neutrophils incubated with quiescent A549 cells at 6 and 24 h **(A),** and neutrophils incubated with preactivated A549 cells at 6 and 24 h (**B**).

sides, the cells in region c were supposed to be apoptotic bodies which were all in various sizes of nuclei with the least dense chromatin of any of the cell groups. In examining the images, it became clear that the cells progressed from purified control cells in region a to apoptotic cells in region b, and then to possible apoptotic bodies in region c.

Comparison of neutrophils incubated with quiescent alveolar epithelial cells to those of suspension neutrophil cultures demonstrated that quiescent alveolar epithelial cells delayed neutrophil apoptosis, and this inhibitory effect against neutrophil apoptosis was enhanced when A549 cells had been treated with TNF-α for 24 h prior to their use for neutrophil plating. These data suggest that alveolar type II cells may be potentially able to contribute in promoting inflammation by delaying neutrophil apoptosis. Estimates of neutrophil apoptosis represent the static measurement of a dynamic process and reflect the equilibrium between the development of apoptosis and phagocytosis of apoptotic neutrophils by macrophages. Theoretically, inhibition of neutrophil apoptosis could result in increased numbers of viable neutrophils. This may prolong the neutrophil alveolitis and contribute to acute lung injury. But from the stand point of host defense, this should be desirable because the active life of neutrophils may be prolonged in order to fight invading microbes and to allow time for recruited monocytes, which are unable to take up apoptotic neutrophils, to differentiate into phagocytically competent macrophages.

When the lung is in an inflammatory state, neutrophils can survive longer than those in a physiological state, thus possibly preparing against external noxious factor which can cause TNF-α secretion from alveolar macrophages.

Taken together, we supposed that alveolar epithelial cells are not only serving simply as a passive barrier but also play an active role in self defense by prolonging neutrophil survival and progress inflammatory response for the host in both physiological and pathological states.

As previously reported, some cytokines affect the rate of neutrophil apoptosis (Brach et al., 1992; Lee et al., 1993). Indeed, TNF-α treatment of alveolar epithelial cells could affect the rate of neutrophil apoptosis by causing the production of interleukin-6 which can delay neutrophil apoptosis (Biffl et al., 1996); our experimental conditions, in which the epithelial cells were washed twice to remove TNF-α and conditioned medium containing cytokines before plating of neutrophils, make this possibility unlikely.

On the other hand, Ginis and Faller (1997) reported that upregulation of adhesion molecules on the surface of the vascular endothelium delayed neutrophil apoptosis. Due to their low baseline expression of adhesion molecules (Rothlein et al., 1988), A549 cells were selected as a source of alveolar epithelial cells for the present study. But TNF-α stimulation specifically upregulates intercellular adhesion molecule-1 (ICAM-1) expression on A549 cells (Arnold and König, 1996). Signals derived from these adhesion molecules on alveolar epithelial cells might affect neutrophil apoptosis. More detailed research should be undertaken to determine the cause of this effect.

One advantage of LSC is the possibility of cell staining on slides which eliminates cell loss which inevitably occurs due to repeated centrifugations during sample preparation for flow cytometry. Another advantage stems from the possibility of localization of particular cells on a slide for visual microscopic inspection, after the initial measurement and automatic relocation (Kamentsky and Kamentsky, 1991). Because the specimen is spatially fixed on a glass microscope slide, individual specimens or cells can be analysed repeatedly if desired. The instrument, thus, combines advantages of both flow and image cytometry. Li and coworkers (1996) used LSC for the detection of DNA strand breaks labeled with fluorochrome in apoptotic cells and proposed its advantages. The methods for the detection of apoptosis are usually laborious and time consuming. In addition, it can be difficult to get quantitative information on the number of apoptotic cells. However, LSC can identify features of cells from fluorescent measurements and provide us with rapid quantitative information in a convenient way because its preparation requirements are minimal.

In summary, the experiments revealed that incubation of neutrophils with A549 cells inhibited neutrophil apoptosis, and that this inhibitory effect was enhanced when the A549 cells were preactivated with TNF-α for 24 h. A greater understanding of alveolar epithelial cell and neutrophil interaction would provide new insights into the pathophysiology of lung diseases, which in turn may promote the development of novel approaches to their treatment.

Ackowledgments: We express our appreciation to Prof. Takao Sasaki, Third Dept. of Internal Medicine, Prof. Nobuaki Kaibara, First Dept. of Surgery, and Prof. Tadashi Terada, Second Dept. of Pathology, Faculty of Medicine, Tottori University, for their kind advice.

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(Received December 2, Accepted December 8, 1998)