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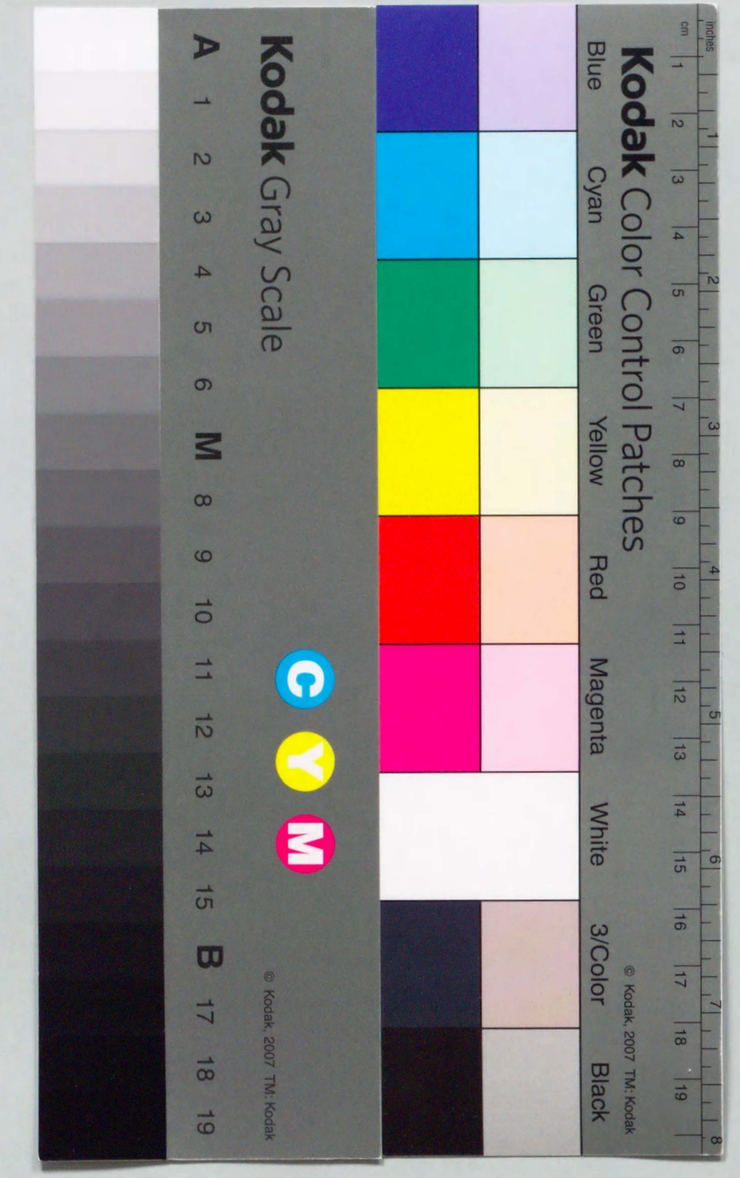


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# The Role of Neutrophil Elastase in Human Pulmonary Artery Endothelial Cell Injury

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**Key Words**
Human neutrophil
Human endothelial cell
Cell injury
Neutrophil elastase
Erythromycin

**Abstract**
Neutrophils are thought to play a key role in tissue injury. We investigated the role of human neutrophil-derived elastase in the induction of injury to human pulmonary artery endothelial cells. Incubation of endothelial cells with neutrophils increased the release of lactate dehydrogenase activity, thrombomodulin, and preloaded fura-2 from endothelial cells, indicating that neutrophils induce endothelial cell injury. Attachment alone of neutrophils to endothelial cells appeared to induce activation because elastase release and N-formyl-mentionyl-leucyl-phenylalanine (fMLP)-induced superoxide ($O_2^-$) production from neutrophils incubated with endothelial cells were greater than from neutrophils only. When endothelial cell were incubated with neutrophils stimulated by fMLP or phorbol myristate acetate, the amount of elastase in the medium and endothelial cell damage was further enhanced. However, when neutrophils were blocked from direct attachment to endothelial cells using a membrane filter, endothelial cell damage was ameliorated, while exogenous neutrophil elastase and medium containing neutrophil-released elastase did not induce endothelial cell injury. An inhibitor of neutrophil elastase, ONO-5046 Na, as well as erythromycin, which reduces neutrophil-derived elastase, dramatically inhibited neutrophil-induced endothelial cell injury. Superoxide dismutase (SOD) partially inhibited injury. Injury was completely inhibited by treatment with a combination of ONO-5046 Na and SOD. These results suggest that attachment of neutrophils to endothelial cells is important for endothelial cell damage and that neutrophil-derived elastase plays an important role in endothelial cell injury in combination with $O_2^-$. In addition, ONO-5046 Na and erythromycin may be useful in treating diseases worsened by excessive neutrophil activity.

## Introduction

Endothelial cell injury is thought to be induced predominantly by the action of neutrophils [1–3]. Many disease states, including adult respiratory distress syndrome, are manifestations of an interaction between neutrophils and endothelium [4]. Neutrophil-induced endothelial cell damage is considered to be mediated through active oxygen species [5]. We have previously reported that erythromycin, a 14-membered ring macrolide, reduces $O_2^-$ production by neutrophils [6].

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Neutrophil-induced endothelial cell damage is also caused by proteases released from neutrophils [7]. Neutrophil elastase reportedly induces an increase in the permeability of endothelial monolayers [8, 9]. However, the mechanism of the cytolytic effect of neutrophil elastase on endothelial cells is controversial [8, 10, 11].

In the present study, we confirmed that attachment of neutrophils to endothelial cells results in neutrophil activation, and attachment is a prerequisite for inducing endothelial cell injury. Next, we examined the role of neutrophil-derived elastase in endothelial cell injury. Furthermore, we investigated the effect of erythromycin on the release of elastase from neutrophils and neutrophil-induced endothelial cell injury, since erythromycin has been used for the treatment of diseases worsened by neutrophils, such as diffuse panbronchiolitis [12–14].

## Materials and Methods

*Reagents.* Erythromycin and fura-2 acetoxymethyl ester were obtained from Wako (Osaka, Japan). Human neutrophil elastase was from Elastin Products (Owensville, Mo., USA). Phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (fMLP), ferricytochrome *c*, and superoxide dismutase (SOD) were from Sigma (St. Louis, Mo., USA). ONO-5046 Na was a generous gift from Ono Pharmaceutical (Osaka, Japan). All reagents were of analytical grade.

*Preparation of Human Neutrophils.* Human neutrophils were isolated from normal human donors as previously described [15]. In brief, after elimination of erythrocytes by dextran sedimentation followed by brief hypotonic lysis, the cell suspension was centrifuged in a Ficoll-sodium iothalamate gradient to separate neutrophils from lymphocytes, monocytes, and platelets. Isolated cells were suspended in a Hepes-buffered salt solution [135 m*M* NaCl, 5 m*M* KCl, 5 m*M* glucose, 20 m*M* Hepes (pH 7.4)] and stored on ice until use.

*Assay of $O_2^-$ Production.* $O_2^-$ production was measured as previously described [15]. The assay mixture (1.0 ml) contained 50 μ*M* ferricytochrome *c*, 1 m*M* $CaCl_2$, and $1\times10^6$ cells in Hepes-buffered salt solution. Cells were incubated at 37°C, $O_2^-$ production was initiated by addition of fMLP (1 μ*M*) or PMA (10 ng/ml) as a stimulant and measured by determining the rate of SOD-inhibitable ferricytochrome *c* reduction at 550–540 nm using a dual-wavelenght spectrophotometer (Hitachi 557). $O_2^-$ release was calculated using a molar absorption coefficient of 19,100 $M^{-1}cm^{-1}$.

*Measurement of $[Ca^{2+}]_i$ with Fura-2.* $[Ca^{2+}]_i$ (intracellular calcium ion concentration) was measured as previously described [16]. Neutrophils were loaded with fura-2 by incubating a cell suspension with 2 μ*M* fura-2 acetoxymethyl ester for 30 min at 33°C. The cells were pelleted by low-speed centrifugation, washed twice, and resuspended in the Hepes buffer. Measurements were performed with a Shimadzu RF-1500 spectrofluorophotometer in a stirred plastc cuvette maintained at 37°C. The excitation and emission wavelenghts were 340 and 500 nm, respectively. Free $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F)$, where F is the fluorescence intensity of the dye in the cells, and $F_{max}$ and $F_{min}$ are the intensities at saturating and zero calcium concentrations, respectively. The dissociation constant ($K_d$) of fura-2 for $Ca^{2+}$ was assumed to be 224 n*M* at 37°C. $F_{max}$ and $F_{min}$ were determined empirically by the addition of Triton X-100 (0.05%) and EGTA (10 m*M*), respectively.

*Measurement of Neutrophil Elastase Concentration.* Neutrophil elastase concentrations in supernatants from incubation media were measured with a specific enzyme immunoassay kit (Neutrophil Elastase EIA, Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan) using a spectrophotometer (Labo Science, EAR 340 AT).

*Culture of Endothelial Cells.* Human pulmonary artery endothelial cells were obtained from Sanko Junyaku (Tokyo, Japan). The cells were passaged into 25- and 175-$cm^2$ flasks and six-well tissue culture plates (Becton Dickinson, Oxnard, Calif., USA) in modified MCDB 131 medium supplemented with 2% fetal bovine serum, gentamycin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and bovine brain extract (10 μg/ml) in a humidified 5% $CO_2$, 95% air atmosphere at 37°C. Experiments were performed with confluent endothelial monolayers between passages 7 and 12. Prior to experiments, cells were washed with modified MCDB 131 medium.

*Assay of Lactate Dehydrogenase Activity.* The supernatants from incubation media were assayed for lactate dehydrogenase (LDH) activity as previously described [6].

*Assay of Fura-2 Release.* Confluent endothelial monolayers were loaded with 2 μ*M* fura-2 acetoxymethyl ester for 30 min, incubated in fresh medium for 60 min, and then exposed to the experimental medium. The fluorescence intensity of the experimental medium was measured at 340-nm excitation and 500-nm emission wavelengths with a spectrofluorophotometer (Shimadzu RF-1500).

*Assay of Thrombomodulin Release.* Thrombomodulin concentrations in the supernatants of incubation media were measured by enzyme immunoassay (TM Panacela, Fujirebio Inc.) using a spectrophotometer (Aroka AEC-2000).

*Evaluation of Endothelial Cell Injury.* Endothelial cell injury was evaluated according to the method of Abe et al. [17]. For evaluation by LDH release, neutrophils and the indicated agents were added to confluent monolayers of endothelial cells in 25-$cm^2$ flasks, and LDH activity in the medium was assayed. Total LDH activity was measured following exposure of the cells to 0.1% Triton X-100. LDH release from neutrophils alone was assayed simultaneously, and LDH release from endothelial cells was calculated by subtracting LDH release from neutrophils from that measured in the presence of both cell types. Results were expressed as a percentage of total endothelial cell LDH, calculated as [(experimental release–spontaneous release)/(maximum release–spontaneous release)]×100%. For evaluation by fura-2 release, neutrophils and the indicated agents or vehicle were added to confluent monolayers of fura-2-loaded endothelial cells in 25-$cm^2$ flasks, and the fluorescence intensity of the medium was determined. Maximum intensities were obtained by treatment of cells with 0.1% Triton X-100. The percentage specific fura-2 release was calculated as [(experimental release–spontaneous release)/(maximum release–spontaneously release)]×100%. For evaluation by thrombomodulin release, neutrophils and the indicated agents or vehicle were added to

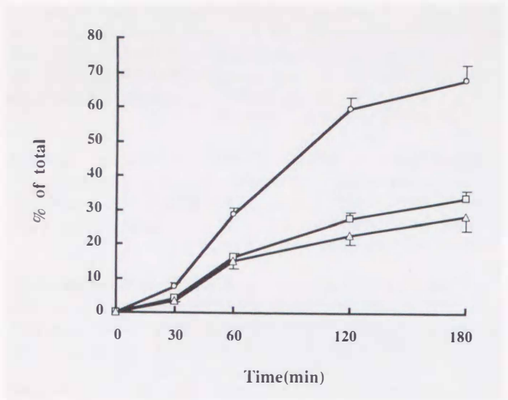



**Fig. 1.** Effect of $H_2O_2$ on the release of LDH, thrombomodulin and Fura-2 from endothelial cells. Endothelial cells ($5\times10^5$) were incubated for various times with 50 m*M* $H_2O_2$. The release of LDH (○), thrombomodulin (□) and fura-2 (△) from endothelial cells was measured as described in 'Materials and Methods'. Data are means ± SD of four independent experiments and are expressed as percentages of the total LDH, thrombomodulin or fura-2 released from the endothelial cells.

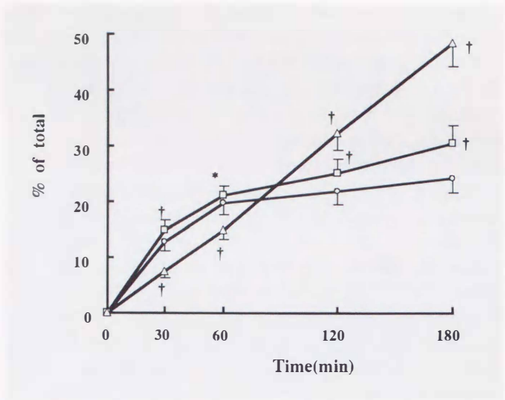



**Fig. 2.** Time course of LDH release from endothelial cells incubated with unstimullated or stimulated neutrophils. Endothelial cells ($5\times10^5$) were incubated for various times with neutrophils ($4\times10^6$) that were unstimulated (○) or stimulated with either PMA (10 ng/ml, △) or fMLP (1 μ*M*, □), after which the release of LDH from the endothelial cells was determined. Data are means ± SD of four independent experiments. * $p<0.05$, † $p<0.01$ vs. respective control (no stimulant).

confluent monolayers of endothelial cells in 175-$cm^2$ flasks, and thrombomodulin in the medium was assayed. Total thrombomodulin was measured after exposure of the cells to 0.1% Triton X-100. Results are expressed as a percentage of total endothelial cell thrombomodulin, calculated as [(experimental release–spontaneous release)/(maximum release-spontaneous release)]×100%.

*Statistical Analysis.* Data are presented as means ± SD (for the figures, SD values were not included when smaller than the symbols) and were analyzed by Student's t test or Welch's t test as appropriate. A p value of $<0.05$ was considered statistically significant.

## Results

### *Effect of Neutrophils on Pulmonary Artery Endothelial Cells*

To estimate endothelial cell injury, we measured LDH activity, and fura-2 and thrombomodulin concentration released from endothelial cells into the medium. The release of these substances appeared to represent endothelial cell injury because they were also released in parallel experiments from endothelial cells injured by treatment with $H_2O_2$ (fig. 1). To confirm the role of neutrophils in endothelial cell injury, we added neutrophils to endothelial cell monolayers and measured LDH activity in the culture medium. Experiments were performed with $4\times10^6$ neutrophils and $5\times10^5$ endothelial cells to minimize the amount of LDH released from neutrophils, which was virtually identical among independent experiments. Endothelial cells incubated alone released only trace amounts of LDH under these experimental conditions. As shown in figure 2, neutrophils induced release of LDH from endothelial cells, suggesting that neutrophils induce endothelial cell damage. Under these conditions, neutrophils were firmly attached to endothelial cells. After endothelial cells were washed out gently, neutrophils were not detached from endothelial cells (fig. 3). To further confirm induction of endothelial cell damage by neutrophils, we measured fura-2 release from preloaded endothelial cells and thrombomodulin release from endothelial cells. Both fura-2 and thrombomodulin release were increased by incubation with neutrophils (fig. 4). Thus, endothelial cells were damaged by attachment and accumulation of neutrophils on endothelial cells.

To investigate whether neutrophils were activated by attachment to endothelial cells, we measured neutrophil $O_2^-$ production and neutrophil-derived elastase in the medium. As shown in tables 1 and 2, incubation of neutrophils with endothelial cells for 120 min resulted in enhancement of fMLP-induced $O_2^-$ production and enhanced release of elastase from neutrophils attached to endothelial cells, compared with neutrophils alone.



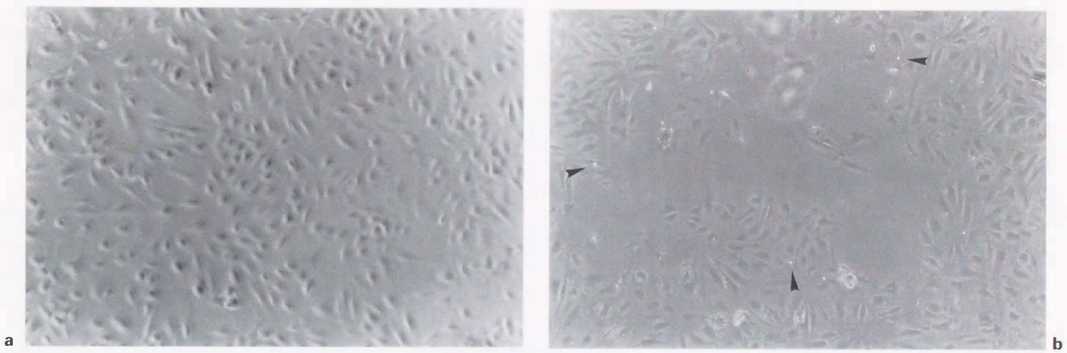



**Fig. 3.** Detachment of endothelial cells by the action of neutrophils. **a** Confluent endothelial cells. **b** Endothelial cells ($5\times10^5$) were incubated with neutrophils ($4\times10^6$) for 120 min (phase contrast microscopy, ×40). Arrows point to the firmly attached neutrophils.

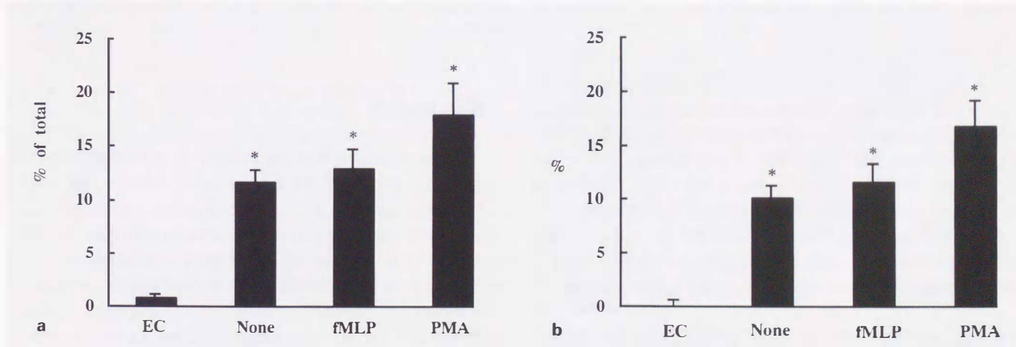



**Fig. 4.** Effect of neutrophils on the release of thrombomodulin and fura-2 from endothelial cells. Endothelial cells ($5\times10^5$) were incubated with unstimulated or stimulated neutrophils for 120 min. Fura-2-loaded endothelial cells were incubated with neutrophils for 60 min. The concentrations of thrombomodulin and fura-2 in the medium were determined as described in 'Materials and Methods'. Data are means ± SD of three independent experiments and are expressed as percentages of the total fura-2 and thrombomodulin contents of the endothelial cells. EC = Endothelial cells alone; none = endothelial cells and neutrophils (no stimulation); fMLP = endothelial cells and neutrophils (fMLP-stimulated); PMA = endothelial cells and neutrophils (PMA-stimulated). **a** Release of thrombomodulin. **b** Release of fura-2. * $p<0.01$ vs. control (no neutrophils).

When neutrophils were blocked from direct attachment to endothelial cells by a membrane filter, endothelial cell damage was ameliorated compared with the damage induced by direct attachment of neutrophils (fig. 5).

To examine the effect of neutrophil activation on endothelial cell damage, we assayed LDH activity in the medium when endothelial cells were incubated with neutrophils stimulated by fMLP and PMA. The amounts of LDH and thrombomodulin released from endothelial cells incubated with fMLP- and PMA-stimulated neutrophils exceeded those released from endothelial cells incubated with unstimulated neutrophils at 180 and 120 min, respectively, af-

**Table 1.** Enhancement of neutrophil $O_2^-$ production by attachment to endothelial cells

| | $O_2^-$ production, nmol/min/$10^6$ cells | |
|---|---|---|
| Stimulant: | fMLP | PMA |
| Neutrophils alone | 1.20±0.04 | 6.20±0.40 |
| Neutrophils + endothelial cells | 1.76±0.12* | 6.28±0.42 |
| Endothelial cells alone | 0.0 | 0.0 |

Cells were incubated for 120 min. $O_2^-$ production was measured as described in 'Materials and Methods'. Data are means ± SD of five independent experiments. *$p<0.01$, vs. respective control (neutrophils alone).

**Table 2.** Enhancement of neutrophil-derived elastase release by attachment to endothelial cells

| | Neutrophil elastase, ng/ml | | |
|---|---|---|---|
| Stimulant: | (–) | fMLP | PMA |
| Neutrophils alone | 99.8±11.5 | 149.8±13.2 | 236.4±22.2 |
| Neutrophils + endothelial cells | 153.2±31.5* | 209.6±19.9** | 338.6±62.1** |

Cells were incubated for 120 min. Neutrophil elastase concentration in the medium was measured as described in 'Materials and Methods'. Data are means ± SD of five independent experiments. *$p<0.05$, **$p<0.01$ vs. respective control (neutrophils alone).

ter stimulation (fig. 2, 4). PMA and fMLP had no significant effect on LDH and thrombomodulin release from endothelial cells alone (data not shown).

*Effect of Neutrophil Elastase on Endothelial Cell Injury*

When endothelial cells were incubated with neutrophils, endothelial cells were injured and the medium contained neutrophil elastase activity. When neutrophils were activated by fMLP and PMA, endothelial cell damage by neutrophils was enhanced and the amount of neutrophil elastase in the medium increased in parallel with the damage (fig. 2, 4, table 2). However, the medium itself did not induce endothelial cell injury. In addition, exogenously added neutrophil elastase did not induce endothelial cell damage (fig. 5).

*Effects of ONO 5046 Na and Erythromycin on Neutrophil-Induced Endothelial Cell Damage*

We examined the effect of ONO 5046 Na, an inhibitor of neutrophil elastase, on neutrophil-induced endothelial cell injury. As shown in figure 6, this drug reduced the release of LDH from endothelial cells incubated with neutrophils in a dose-dependent manner. Fura-2 and thrombomodulin release from endothelial cells incubated with neutrophils also were inhibited by addition of ONO 5046 Na (fig. 7). Furthermore, LDH, fura-2 and thrombomodulin release were inhibited almost completely by treatment with a combination of ONO 5046 Na and SOD (fig. 8); SOD alone caused about a 50% inhibition in the release of LDH from endothelial cells incubated with neutrophils (data not shown).

Next, we examined the effect of erythromycin on endothelial cell injury induced by neutrophils. Erythromycin reduced the release of elastase from neutrophils (table 3). This may be due to its effect on intracellular $Ca^{2+}$ concentration in neutrophils, because it reduced $Ca^{2+}$ influx from the extracellular medium (fig. 9). Erythromycin inhibited LDH release from endothelial cells incubated with neutrophils (table 3).

## Discussion

We have shown that incubation of human neutrophils with human endothelial cell monolayers results in endothelial cell damage even when the neutrophils are not artificially activated, and that attachment of neutrophils to endothelial cells is important for neutrophil-induced endothelial cell damage. It is unclear as to why neutrophils which were not stimulated induced endothelial cell damage. Attachment of neutrophils to endothelial cells seemed to induce activation of neutrophils and more enhance injury of endothelial cells by diffusible factors. It is unlikely that neutrophils were activated during the separation procedure because the amount of $O_2^-$ released from unstimulated neutrophils was low, though the possibility that the neutrophils were primed during processing cannot be excluded. Enhancement of neutrophil-derived elastase and fMLP-induced $O_2^-$ production by neutrophils suggests that neutrophil attachment to endothelial cells is an important factor for neutrophil activation and endothelial cell damage, though the mechanism by which attachment to endothelial cells activates neutrophils is not clear. Although neutrophils in peripheral blood do not induce endothelial cell damage, this may be because neutrophils do not attach to endothelial cells under physiological conditions. Incubation of human neutrophils with human endothelial cell monolayers may resemble abnormal conditions in which adhesion molecules are



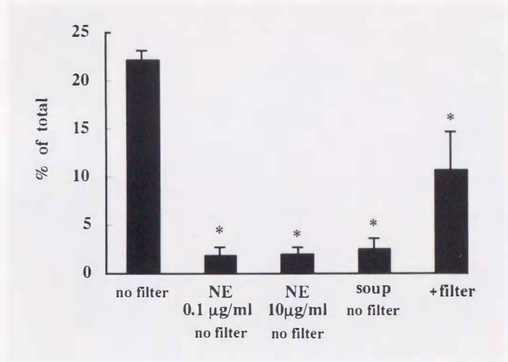



**Fig. 5.** LDH release from endothelial cells. Endothelial cells ($2\times10^5$) were incubated for 120 min with neutrophils ($1.6\times10^6$), with medium collected from a flask containing neutrophils incubated for 120 min, or with exogenous neutrophil elastase (0.1 and 10 μg/ml). LDH release from the endothelial cells was then determined. Where indicated (+filter), direct attachment of neutrophils to endothelial cells was blocked using a membrane filter (pore size 3 μm, pore density $2.0\times10^6/cm^2$, distance from membrane to the bottom of well 0.9 mm, Falcon 3501, Becton Dickinson, Oxnard, Calif., USA). Data are means ± SD of four independent experiments. *$p<0.01$ vs. respective control (with neutrophils, no filter). NE = Exogenous neutrophil elastase; soup = medium from a flask containing endothelial cells injured by neutrophils.

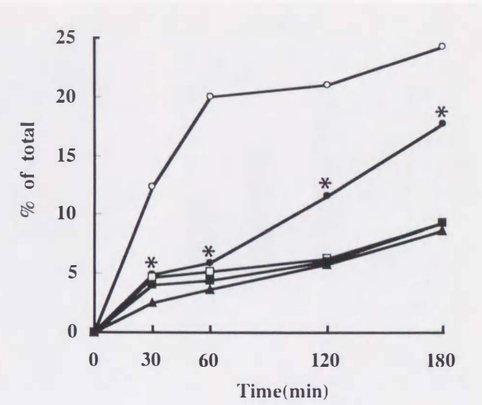



**Fig. 6.** Effect of ONO 5046 Na on neutrophil-induced LDH releases from endothelial cells. Endothelial cells were incubated with unstimulated neutrophils and various concentrations of ONO 5046 Na up to 120 min. ONO 5046 Na concentrations were 0.005 μ*M* (●), 0.05 μ*M* (□), 0.5 μ*M* (■), 5 μ*M* (▲) and 0 μ*M* (○). LDH release from endothelial cells was determined as described in 'Materials and Methods'. Data are means ± SD of three independent experiments and are expressed as a percentage of the total LDH content of the endothelial cells. *$p<0.01$ vs. respective control (no ONO 5046 Na).

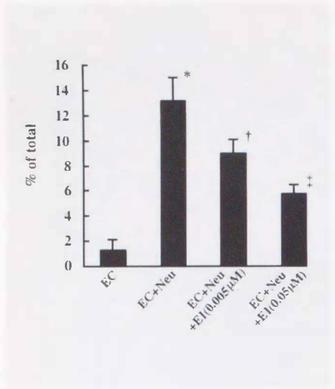
expressed, allowing neutrophil adherence to endothelial cells.

Our results suggest that neutrophil elastase does not induce endothelial cell damage by itself, which is compatible with a previous report by Varini et al. [10]. However, neutrophil elastase seems to be an important factor for neutrophil-induced endothelial cell damage because the degree of endothelial cell damage paralleled the amount of neutrophil elastase in the medium. Furthermore ONO 5046 Na, an inhibitor of neutrophil elastase, and erythromycin, which inhibits the release of elastase from neutrophils, both ameliorated endothelial cell injury. Although it has been reported that endothelial cell damage is mediated through active oxygen species produced by neutrophils [4], neutrophil elastase may be more important for neutrophil-induced endothelial cell damage because ONO 5046 Na and erythromycin inhibited neutrophil-induced endothelial cell damage more strongly than SOD. Since the combination of SOD and ONO 5046 Na completely inhibited neutrophil-induced endothelial cell damage, such damage might be predomin-

**Table 3.** Effect of erythromycin on elastase release from neutrophils and LDH release from endothelial cells

| | Elastase ng/ml | LDH % of control |
|---|---|---|
| Endothelial cells | ND | 1.2±0.6 |
| Neutrophils | 95.0±12.0 | 0.0 |
| Neutrophils + endothelial cells | 131.6±19.3 | 21.8±2.1 |
| Neutrophils + endothelial cells + erythromycin (50 μ*M*) | 84.0±10.5* | 5.2±1.7* |

Cells were incubated for 120 min. The release of LDH from endothelial cells and of neutrophil-derived elastase was determined as described in 'Materials and Methods'. Data are means ± SD of three independent experiments and are expressed as a percentage of the total LDH activity in endothelial cells or elastase content in neutrophils. *$p<0.01$ vs. respective control (neutrophils + endothelial cells). ND = Not detected.

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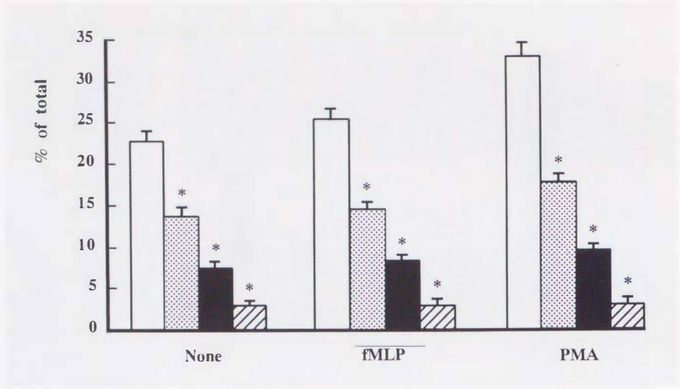


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**Fig. 7.** Effect of ONO 5046 Na on unstimulated neutrophil-induced thrombomodulin release from endothelial cells. Cells were incubated for 120 min. Thrombomodulin release from endothelial cells was determined as described in 'Materials and Methods'. Data are means ± SD of three independent experiments and are expressed as a percentage of the total thrombomodulin content of the endothelial cells. *$p<0.01$ vs. respective control (endothelial cells only). †$p<0.05$, ‡$p<0.01$ vs. respective control (neutrophils + endothelial cells). EC = Endothelial cells; Neu = neutrophils; El = elastase inhibitor (ONO 5046 Na).

**Fig. 8.** Effect of the combination of ONO 5046 Na and SOD on neutrophil-induced LDH release from endothelial cells. Endothelial cells ($5\times10^5$) were incubated with neutrophils ($4\times10^6$) that were either unstimulated or stimulated with PMA (10 ng/ml) or fMLP (1 μ*M*) in the presence of ONO 5046 Na alone (0.5 μ*M*, ■), SOD alone (10 μg/ml, □), or ONO 5046 Na (0.5 μ*M*) and SOD (10 μg/ml, ▨) for 120 min, after which the release of LDH from the endothelial cells was determined. Data are means ± SD of three independent experiments. *$p<0.01$ vs. respective control (no ONO 5046 Na, no SOD, □).

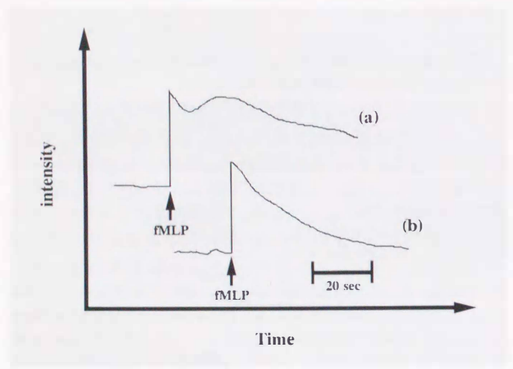


**Fig. 9.** Effect of erythromycin on $[Ca^{2+}]_i$ of neutrophils. $[Ca^{2+}]_i$ was measured as described in 'Materials and Methods'. Neutrophils were preincubated in Hepes buffer supplemented with 1 m*M* $CaCl_2$ and then stimulated with 1 μ*M* fMLP. The calculated $[Ca^{2+}]_i$ before and immediately after the addition of fMLP was about 130 and 780 n*M*, respectively. a = Control; b = neutrophils were pretreated with 25 μ*M* erythromycin for 120 min. The traces are representative of four independent experiments.

antly mediated through cooperative action between neutrophil elastase and $O_2^-$.

In conclusion, attachment of neutrophils to endothelial cells induces neutrophil activation and is necessary for neutrophil-induced endothelial cell damage. In addition, neutrophil elastase induces endothelial cell damage in combination with other mediators such as $O_2^-$. Prevention of neutrophil attachment to endothelial cells or inhibition of neutrophil elastase activity appears necessary for the treatment of diseases worsened by the action of neutrophils. ONO 5046 Na and erythromycin may be useful tools for the inhibition of disorders caused by neutrophil-induced endothelial cell damage, such as adult respiratory distress syndrome.

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