Neutrophil-mediated endothelial injury in vitro mechanisms of cell detachment.

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J Clin Invest. 1981;68(6):1394-1403. https://doi.org/10.1172/JCI110390.

Research Article

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Neutrophil-mediated Endothelial Injury In Vitro

MECHANISMS OF CELL DETACHMENT

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ABSTRACT Neutrophil-mediated endothelial injury was assessed in vitro using assays of cell lysis and cell detachment. Activation of human peripheral blood neutrophils adherent to human umbilical vein endothelial cell monolayers by serum-treated zymosan produced dose-dependent endothelial cell detachment without concomitant cell lysis. This injury was inhibited by neutral protease inhibitors, but not by catalase or superoxide dismutase. Neutrophils from a patient with chronic granulomatous disease also produced endothelial cell detachment when activated by serum-treated zymosan similar to normal neutrophils. Endothelial detachment was also produced by cell-free postsecretory media from activated neutrophils or by partially purified human neutrophil granule fraction and was inhibitable by tryptic, elastase, and serine protease inhibitors, but not by an acid protease inhibitor. Analysis of iodinated endothelial cell surface proteins that had been exposed to partially purified neutrophil granule fraction showed complete loss of proteins migrating in the region of fibronectin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This result was prevented in the presence of neutral protease inhibitors. We conclude that neutrophil-derived neutral proteases mediate endothelial cell detachment in vitro through digestion of endothelial cell surface proteins including fibronectin.

INTRODUCTION

Circulating neutrophils often adhere transiently to vascular endothelium before returning to the circulation or migrating to subadjacent tissues (1, 2). The proportion of adherent neutrophils increases with acute inflammation (3). Although the mechanisms underlying neutrophil accumulation at inflammatory sites are not fully defined, inflammatory mediators appear to play an important role (4–6). Neutrophils become activated by components of complement, as well as by circulating or bound immune complexes, endotoxin, or a number of other particulate or soluble stimuli (7). Neutrophil activation may lead to the release of potentially cytotoxic products such as granule proteases and toxic oxygen radicals (8, 9). Injury to the endothelium as an "innocent bystander" by these products of activated adherent neutrophils may enhance the local inflammatory response. Endothelial injury and detachment or altered endothelial integrity will increase permeability changes and initiate platelet thrombus formation. Endothelial denudation will also allow free access of inflammatory cells and mediators to subendothelial tissue.

Using cultured human umbilical vein endothelial cells and human peripheral blood neutrophils we have examined the mechanisms of endothelial injury by activated neutrophils in vitro. Serum-treated zymosan (STZ)[1] was used to stimulate neutrophils for study of both granule proteases and oxygen radicals as cytotoxic systems. In these studies endothelial cell lysis and detachment were measured simultaneously to distinguish lethal lytic injury from nonlytic detachment.

METHODS

Reagents. The chloromethyl ketone elastase inhibitor (CKEI), acetyl-alanyl-alanyl-prolyl-valyl CH_2Cl, was pro-

Portions of this work were presented at the 93rd Annual Meeting of American Association of Physicians, Washington D. C., May 1980.

Received for publication 9 February 1981 and in revised form 22 July 1981.

[1] *Abbreviations used in this paper*: ^{3}H-AA, labeled amino acid; BSA, bovine serum albumin; CGD, chronic granulomatous disease; CKEI, chloromethyl ketone elastase inhibitor; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; SBTI, soybean trypsin inhibitor; SOD, superoxide dismutase; STZ, serum-treated zymosan; XO, xanthine oxidase.

vided by Dr. William Arend, University of Texas at Houston, and was dissolved in dimethyl sulfoxide as a 1 M stock. Soybean trypsin inhibitor (SBTI) was obtained from Millipore Corp., Bedford, Mass. Xanthine oxidase (XO) (grade 1), catalase (beef liver, 37,000 U/mg), lactoperoxidase, *N*-ethyl-maleimide, hydrogen peroxide, dimethyl sulfoxide, phenylmethylsulfonyl fluoride (PMSF), bromphenol blue, dithiothreitol, glycerol, sodium dodecyl sulfate (SDS), and urea were obtained from Sigma Chemical Co., St. Louis, Mo. Superoxide dismutase (SOD) was obtained from Miles Laboratories Inc., Goodwood, South Africa. Xanthine (A grade) was obtained from Calbiochem-Behring Corporation, American Hoechst Corp., San Diego, Calif. Pepstatin A was obtained from U. S. Biochemical Corp., Cleveland, Ohio.

Preparation of cultured cells. Endothelial cells were obtained by collagenase (Calbiochem-Behring, B grade) treatment of human umbilical cords (10). Cells were grown to confluence in Waymouth's medium MB 752/I (Irvine Scientific, Santa Ana, Calif.) with 20% newborn calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) at 37° in humidified air with 5% CO_2 atmosphere. Endothelial cells at confluence maintained a pavement-like monolayer and contained Factor VIII antigen as detected by fluorescein-conjugated rabbit antihuman Factor VIII antibody. Only primary passage endothelial cells were used in these studies. In some experiments normal diploid human fibroblasts in 4th passage provided by Dr. Peter Byers, University of Washington, and bovine aortic endothelial cells in 8th to 12th passage, provided by Dr. Stephen Schwartz, University of Washington, were also used as target cells.

Preparation of neutrophils. Suspensions of peripheral blood neutrophils were prepared from heparinized peripheral blood of healthy donors, using standard techniques of sequential Ficoll-Hypaque gradient separation, dextran sedimentation, and hypotonic lysis of erythrocytes (11, 12). Preparations comprised >95% neutrophils by Wright's stain and >98% viable cells by trypan blue dye (Gibco Laboratories) exclusion. Purified neutrophils were suspended in Hanks' balanced salt solution (HBSS) (Gibco Laboratories) with 0.5% bovine serum albumin (BSA) (Miles Laboratories, Inc., Elkhart, Ind.) at a concentration of 2×10^4 cells/μl. In some experiments blood was also obtained from a patient with chronic granulomatous disease (CGD) through Dr. Henry Rosen, University of Washington, and neutrophils were prepared as for normal donor neutrophils.

Injury assays. Primary passage human endothelial cells were plated in Falcon Microtest II plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at 4×10^4 cells/well in 100 μl of 20% newborn calf serum in Waymouth's medium. The final plating density was $\sim 1 \times 10^5$ cells/cm^2, providing a visually confluent endothelial cell monolayer after overnight incubation. Adherent cell counts at 18 h ranged from $2.0-3.0 \times 10^4$ cells/well with <10–15% variation between replicates. ^{51}Chromium as sodium [^{51}Cr]chromate (1 mCi/ml in saline, 200–500 Ci/g, New England Nuclear, Boston, Mass.) was added at the time of plating at a concentration of 10 μCi/ml. After an 18–24 h overnight incubation and labeling, the monolayers were washed with five rapid, successive well-volume exchanges of 0.1% BSA in Dulbecco's phosphate-buffered saline (PBS) (Gibco) dispensed by pipette and removed by vacuum aspiration. Wells were first aspirated using a 1.0 ml pipette (Falcon Labware), fitted through a one-hole rubber stopper to prevent the pipette tip from touching the monolayer. Controlled suction was provided by a vacuum pump (Gast Manufacturing Corp., Benton Harbor, Mich.) maintained at 5 lb/in^2. Wash media were dispensed by hand pipette at ~250 μl per well. After the final wash, test media or cells were added to a final volume of 100 μl per well. Incubations were conducted up to 8 h. ^{51}Cr release was determined by aspiration of 50 μl of supernatant from each well with care not to disturb the monolayer. These aliquots were transferred to glass tubes and counted in a gamma spectrophotometer (Searle Diagnostics, Inc., subsidiary of G. D. Searle & Co., Des Plaines, Ill.) to a statistical error of <1%. In each experiment maximum and control (spontaneous) release were determined as the means of 6–8 replicates. Maximum release was determined from wells incubated in 0.5% Triton X-100 (New England Nuclear) that released >90% of the cell layer counts into the supernatant. Control release was determined from wells incubated in the appropriate media alone and was 10–15% of the maximum release in a 3-h incubation. Results were expressed as percent specific ^{51}Cr release as follows: % release = (cpm test − cpm control/cpm Triton − cpm control) × 100.

After aspiration of the aliquot for percent ^{51}Cr release, the monolayer was washed with five well-volume exchanges of 0.1% BSA/PBS using rapid pipetting and vacuum aspiration. Adherent cells were >95% viable by trypan blue dye exclusion and control monolayers remained visually confluent after this washing procedure. The adherent cells in each well were harvested by incubation for 30 min in 100 μl per well of 0.02% EDTA and 0.05% trypsin in isotonic saline (Irvine Scientific). The harvested cell layer was counted in a gamma spectrophotometer to a statistical error of <1%. Direct counting of adherent cells after washing and trypsin harvesting using an electronic cell counter (Coulter Model B, Coulter Electronics Inc., Hialeah, Fla.) showed a direct relationship with the cell number determined by ^{51}Cr retention (Fig. 1). After a 3-h incubation and wash procedure control wells alone retained 78.1±0.4% of total counts per minute per well (mean of 12 separate experiments±1 SE). Since spontaneous release of label was 10–15% over this period, the control monolayers remained >90% intact by determination of ^{51}Cr retention. In a representative 3 h incubation, cpm control release was 2712±136, cpm Triton release was 22,162±406, cpm retained in control was 18,255±1,463, and cpm retained after Triton

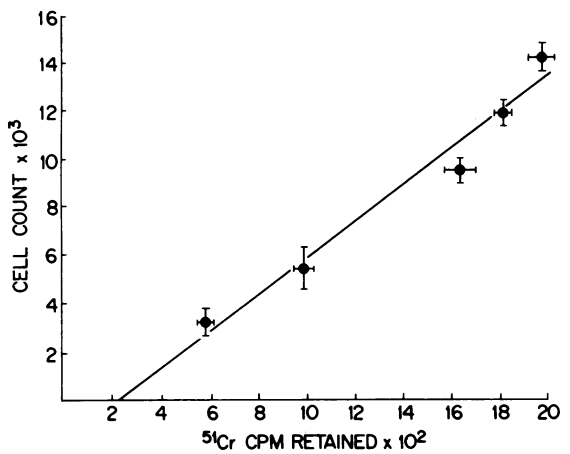

FIGURE 1 Measurement of endothelial cell detachment by ^{51}Cr retention. Endothelial cells were plated at varying cell densities and allowed to adhere for 18 h. For each plating density six replicates were labeled with ^{51}Cr and six were unlabeled. The number of adherent endothelial cells determined by retention of ^{51}Cr-label is directly proportional to cell number determined by electronic counter, and is represented mathematically by the equation $y = 7.4x - 1711$, $r = 0.98$. Each point is the mean of 6 replicates±1 SE.

was 937±123 (means of six replicates±1 SE). Counts per minute retained in control were determined as the mean of 6–8 replicates and test results were expressed as percent detachment: % detachment = (cpm retained in control − cpm retained in test/cpm retained in control) × 100. Since control monolayers remained confluent, the test results were similar whether expressed as percentage of the total activity or percent detachment. The standard error of the mean of 6–8 replicates for both assays was generally <5–10%.

Mixed ^{3}H-amino acids (^{3}H-AA) (1 mCi/ml, New England Nuclear) were substituted in some experiments for ^{51}Cr and identical results for percent specific release and percent detachment were observed (Results). The viability of the adherent cells after washing could also be demonstrated by their uptake of ^{3}H-AA (10 uCi/ml in 0.5% BSA/HBSS for 2 h then washed three times) which also permitted quantification of adherent cell number (Results).

For neutrophil experiments, 50 μl of neutrophils (2×10^{4}/μl in 0.5% BSA/HBSS) were first added and allowed to settle for 10 min before 50 μl of 0.5% BSA/HBSS with or without neutrophil-activating agent were added. Final neutrophil concentration was thus 10^{4}/μl yielding a final neutrophil total in 100 μl of 10^{6}/well.

Gel analysis. First passage human umbilical vein endothelial cells were plated at 10^{6} cells/35-mm Diam well (Flow Laboratories, subsidiary of Flow General, Inc., Hamden, Conn.) in 20% newborn calf serum in Waymouth's medium and allowed to adhere overnight forming a visually confluent monolayer. Iodination of cell surface proteins using Na^{125}I (17 Ci/mg, carrier free, New England Nuclear), lactoperoxidase, and glucose-glucose oxidase was performed according to the method of Hynes (13). Following iodination, the viable confluent endothelial cell monolayers were exposed to PBS alone or to neutrophil granule fraction with or without inhibitor in PBS. After a 30-min incubation when detachment with granule fraction was first visually apparent as rounding up and retraction of endothelial cells, the monolayers were harvested for gel electrophoresis by rubber policemen after the addition of the following inhibitors: 1 mM PMSF, 10 μM pepstatin A, 25 mM EDTA, and 10 mM N-ethylmaleimide. The cell layer was pelleted by centrifugation for 3 min in a Beckman microfuge B (Beckman Instruments, Inc., Palo Alto, Calif.) at 9,000 *g*, resuspended, and washed three times in PBS with inhibitors. Following the final wash the cell layer pellet was dissolved in gel electrophoresis sample buffer (2% SDS, 0.1 M Tris-HCl pH 6.8, 1.0 M urea, 0.002% bromphenol blue, 20% glycerol) with dithiothreitol 7.7 mg/ml and boiled for 2 min. Equal counts of each sample were displayed on 5% acrylamide SDS slab gels (35 mA for 4 h). The gel was dried and autoradiography was performed using an enhancing screen (Ilford Inc., Paramus, N. J.). Molecular weight determinations were made from radiolabeled fibroblast medium run on parallel gel slots.

Preparation of STZ. STZ (Fleishmann Laboratories, subsidiary of Standard Brands, Inc., N. Y.) was prepared as previously described (14). Zymosan was boiled and washed twice with 140 mM sodium chloride, homogenized and resuspended in PBS at 10 mg/ml and kept at −70°C. Before use an aliquot was thawed, centrifuged, and resuspended in fresh human serum at a concentration of 10 mg/ml for 30 min at 37°C. This preparation of STZ was then centrifuged, washed with PBS, and resuspended in 0.5% BSA/HBSS at appropriate concentrations.

Neutrophil granule preparations. Neutrophil postsecretory media was prepared by incubating intact neutrophils (10^{5}/μl) with STZ (5.0 mg/ml) for 3 h. Following incubation STZ-activated neutrophils were sedimented by centrifugation at 250 *g* for 5 min and the supernatant decanted and assayed immediately for endothelial cell-detaching activity. Myeloperoxidase activity of neutrophil supernatants was assayed by Dr. Henry Rosen, University of Washington (15). Partially purified neutrophil granule fractions from granule-rich cell lysates were prepared by sucrose density gradient centrifugation as previously described (16, 17). Endothelial cell-detaching activity of the granule fractions was assayed after dilution in HBSS.

RESULTS

Endothelial cell injury in cell-free systems. The capacity of this assay to distinguish lethal lytic injury from nonlytic detachment is illustrated by comparing the effects of hydrogen peroxide and trypsin. Hydrogen peroxide produced both endothelial cell lysis and detachment (Fig. 2 A). Detached cells were trypan blue positive and residual attached cells were trypan blue negative. Trypsin, on the other hand, produced detachment without lysis; both adherent and detached cells were viable by trypan blue dye exclusion (Fig. 2 B). The oxygen radical generating system of xanthine-XO (18, 19) also produced both dose-dependent endothelial cell ^{51}Cr release and detachment in a 4-h incubation (Fig. 3 A). However, at all points endothelial cell detachment was greater than ^{51}Cr release. Xanthine-XO-mediated injury was inhibited by catalase, SOD, or by boiling of the enzyme before incubation (Fig. 3 B). The time course of injury demonstrated that ^{51}Cr-release became significant after 4 h of incubation (% release: 0.5 h, 0.4±0.35; 1 h, 1.0±0.4; 2 h, 2.6±0.4; 4 h, 24.8±3.4; means of 8 replicates±1 SE).

Endothelial cell detachment without lysis. When ^{51}Cr-labeled human endothelial cells were incubated with neutrophils alone there was minimal injury to the

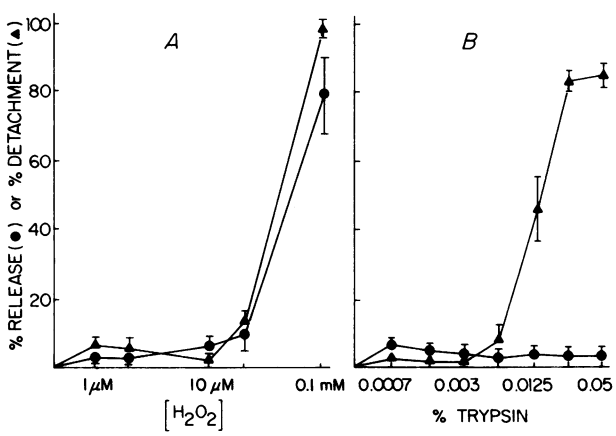

FIGURE 2 Comparison of endothelial cell detachment to ^{51}Cr release. Endothelial cells were incubated 4 h with varying concentrations of H_2O_2 or 2 h with varying concentrations of trypsin. Percent ^{51}Cr release (filled circles) and percent detachment (filled triangles) were determined. Each value is the mean of 6 replicates±1 SE.

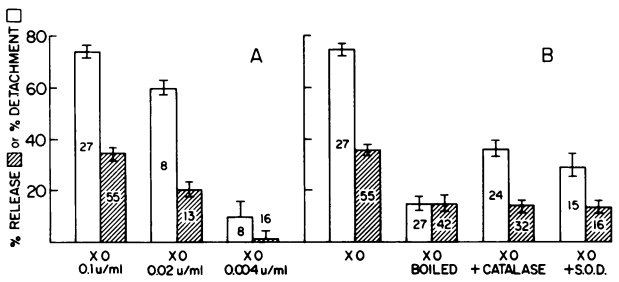

FIGURE 3 Xanthine-XO-mediated endothelial cell injury. Percent ^{51}Cr release (hatched bars) and percent detachment (open bars) were determined after 4-h endothelial cell exposure to xanthine (0.1 mM) and XO (Fig. 3 A). In Fig. 3 B the results are shown after incubation with XO (0.1 U/ml boiled for 5 min before use), catalase (100 μg/ml), and SOD (100 μg/ml). (N) represents the number of replicates±1 SE.

endothelial cell monolayers as determined by either endothelial cell ^{51}Cr release or detachment (Fig. 4). The endothelial cell monolayer remained largely intact visually before and after washing, although many neutrophils remained adherent (Fig. 5 A). Similarly, STZ alone produced no release of intracellular label or visual disruption of the monolayer. However, the addition of STZ to adherent neutrophils produced significant endothelial cell detachment at 3 h without detectable release of ^{51}Cr-label (Fig. 4). Visually, the endothelial cells exposed to STZ-activated neutrophils appeared rounded and retracted before washing (Fig. 5 B). After washing generally only a few endothelial cells remained adherent. A similar pattern of injury was also observed when bovine aortic endothelial cells were exposed to STZ-activated neutrophils. (17.0±0.8% detachment and 1.2±0.1% ^{51}Cr release following 3-h incubation with STZ 2.5 mg/ml and neutrophils $10^4/\mu$l; means of 12 replicates±1 SE).

When ^{3}H-AA were used as the intracellular label, percent release and percent detachment were equivalent to that using ^{51}Cr-labeling (Fig. 4). Similarly, when endothelial cell detachment was quantified as ^{3}H-AA uptake in a postlabeling procedure, the results were similar to prelabeling with ^{51}Cr (percent detachment: neutrophils alone 5.6±3.7%, neutrophils with STZ 66.0±4.1%; means of 12 replicates±1 SE).

To determine if lysis and release of the intracellular label would occur with time, incubations were extended to 8 h. After this period of incubation spontaneous ^{51}Cr release in 10% serum/HBSS averaged 31.2±3.5%, but specific ^{51}Cr-release by STZ-activated neutrophils remained low at 3.6±3.2%, while detachment was 67.0±5.7% (means of eight replicates±1 SE).

Detachment was observed as early as 1 h after incubation with STZ-activated neutrophils and progressively increased over 3 h (Fig. 6 A). Control neutrophils produced no significant detachment over this incubation period (Fig. 6 A). Myeloperoxidase release over this time-course in control and STZ-activated neutrophils is shown in Fig. 6 B.

STZ induced a dose-dependent detachment when the neutrophil to endothelial cell ratio was held constant (Fig. 7 A). A dose-response curve was also observed with increasing concentrations of neutrophils with effector to target ratios from 10:1 to 40:1 while maintaining the STZ concentration constant (Fig. 7 B).

This pattern of neutrophil-mediated cell detachment without significant ^{51}Cr release was not unique to endothelial cells; human fibroblasts were similarly affected by STZ-activated neutrophils in a standard 3 h incubation (neutrophils alone: percent release 9.5±0.3 and percent detachment 0.1±2.3; STZ-activated neutrophils: percent release 13.0±2.2 and percent detachment 88.0±1.8; means of eight replicates±1 SE).

Mechanisms of neutrophil-mediated endothelial cell detachment. The effect of inhibitors on endothelial cell detachment induced by intact STZ-activated neutrophils was studied (Table I). Catalase and SOD produced minimal inhibition of endothelial cell detachment. Dose-dependent inhibition of endothelial detachment by STZ-activated neutrophils was observed with SBTI and with serum. Significant inhibition of STZ-activated neutrophil-mediated endothelial cell detachment was also observed when the incubation included CKEI.

Both the cell-free postsecretory media from STZ-activated neutrophils and the partially purified neutrophil granule fraction produced endothelial cell detachment (Fig. 8). While higher concentrations of all three partially purified granule fractions produced endothelial cell detachment (data not shown), only neutrophil granule fraction B produced significant endothelial cell detachment in a 4-h incubation at a

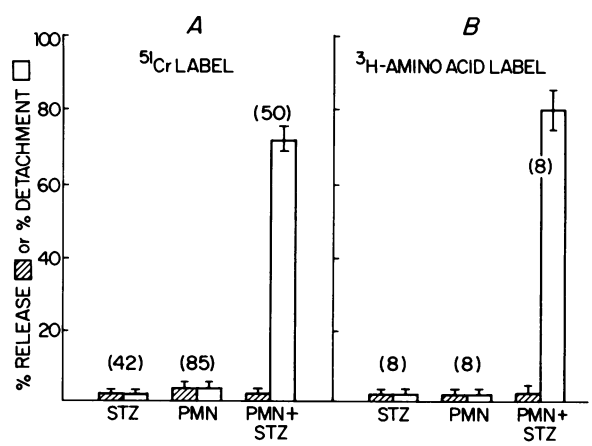

FIGURE 4 Neutrophil-mediated endothelial injury. Percent release (hatched bars) and percent detachment (open bars) are similar after 3-h exposure of endothelial cells to STZ (2.5 mg/ml) and neutrophils (PMN) ($10^4/\mu$l) in 100 μl total volume using either ^{51}Cr or ^{3}H-AA as endothelial cell label. (N) represents the number of replicates±1 SE.

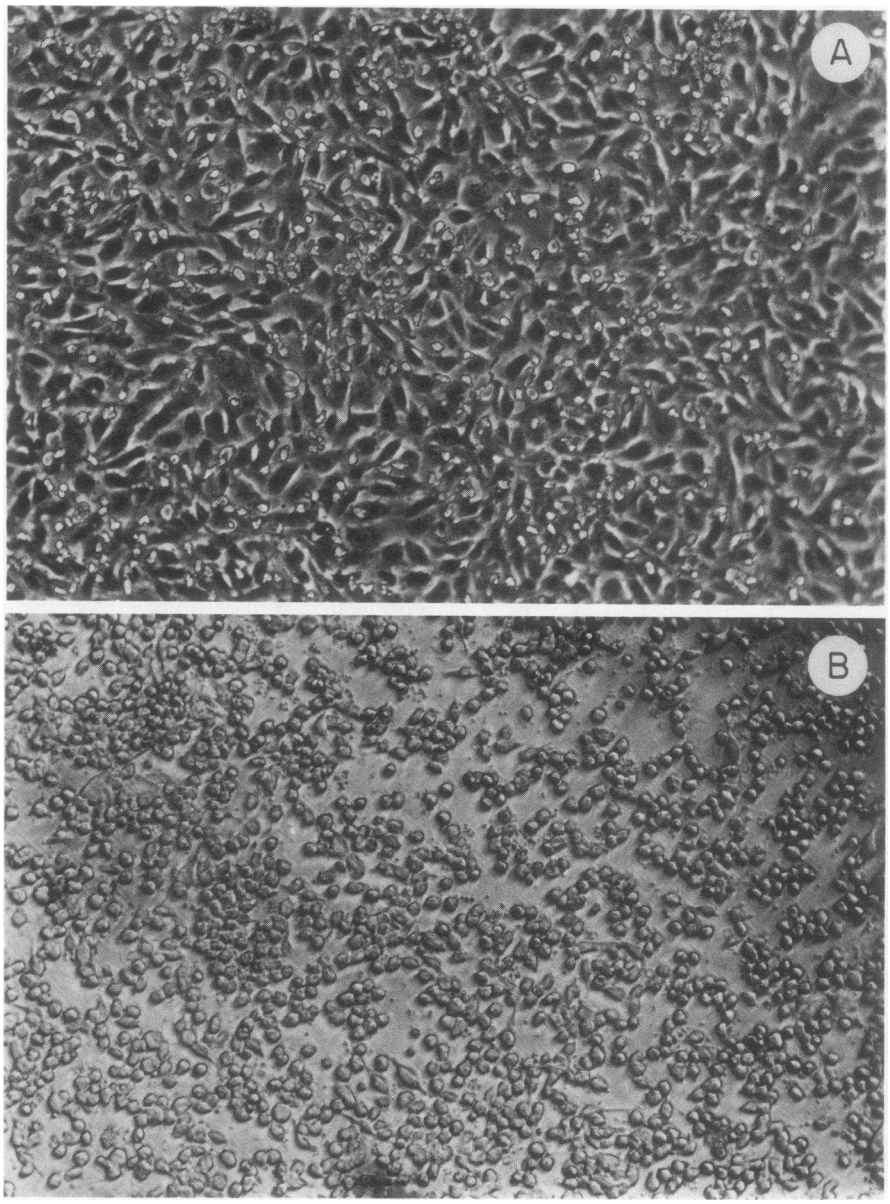

FIGURE 5 Effect of STZ-activated neutrophils on endothelial monolayer. Endothelial monolayers were incubated 3 h with neutrophils (effector to target ratio of 10:1) with and without STZ (2.5 mg/ml). Following incubation the supernatant medium was decanted and the monolayers were fixed without washing (2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate). Phase-contrast micrographs (30×) were taken of monolayers exposed to neutrophils alone (A), or STZ-activated neutrophils (B).

concentration of 5 μg/ml (Fig. 8). No significant endothelial cell ^{51}Cr release was associated with detachment in these studies. The detachment mediated by the partially purified neutrophil granule fraction was prevented by SBTI and CKEI and the serine protease inhibitor, PMSF, but not by the acid protease inhibitor pepstatin A (Fig. 9).

Since neutral protease inhibitors prevented endothelial cell detachment induced by both intact STZ-activated neutrophils and purified granule fraction, endothelial cell detachment appeared to be mediated by neutrophil-derived proteases and not by oxygen radicals. Therefore neutrophils were obtained from one patient with CGD to assess further any possible role of oxygen radicals (8). CGD-neutrophils produced endothelial cell detachment when stimulated by STZ

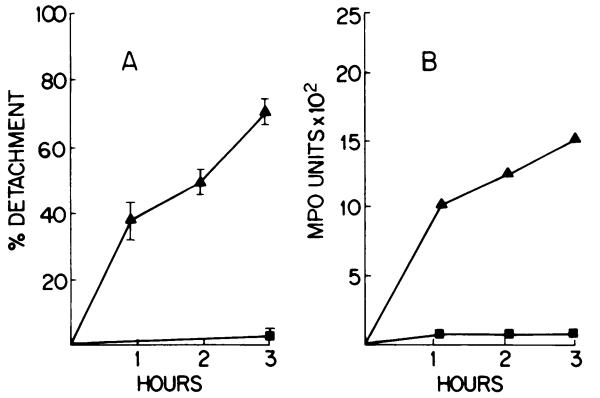

FIGURE 6 Time-course of neutrophil-mediated endothelial cell detachment. STZ-activated neutrophils (filled triangles) produce progressive endothelial cell detachment over 3 h (A). Untreated neutrophils (filled squares) produce no injury. Myeloperoxidase (MPO) release by STZ-activated neutrophils (filled triangles) is compared with untreated neutrophils (filled squares) in similar incubation conditions (B). The concentration of STZ was 2.5 mg/ml and of neutrophils was $10^4/\mu l$. Each value is the mean of 8 replicates ± 1 SE.

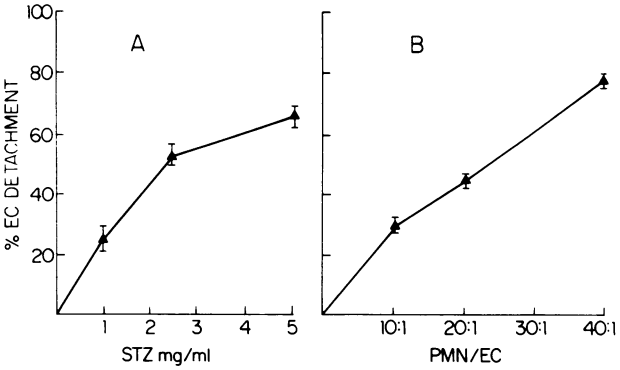

FIGURE 7 Dose-response of neutrophil-mediated endothelial cell detachment. A dose-response for endothelial cell detachment is seen in a 3-h incubation with increasing STZ concentrations at a constant neutrophil concentration of $10^4/\mu l$ (A). At a constant STZ concentration (2.5 mg/ml), endothelial cell detachment at 3 h increases with increasing neutrophil to endothelial cell (PMN/EC) ratios (B). Each value is the mean of 6 replicates ± 1 SE.

that was equivalent to normal neutrophils, indicating that the detachment was not mediated by oxygen radicals (Fig. 10).

Effect of neutrophil granule fractions on endothelial cell extracellular proteins. There was significant loss of endothelial cell surface proteins including fibronectin at the time of visual detachment following exposure to neutrophil granule fraction B (Fig. 11). Digestion of endothelial cell surface protein(s) by granule fraction B was prevented by PMSF and inhibited by SBTI and CKEI, but not by pepstatin A (Fig. 11).

DISCUSSION

Neutrophils may actively mediate tissue injury through release of granule constituents or the generation of toxic oxygen radicals. Such "innocent bystander" injury by products of activated neutrophils may aggravate local tissue injury. The endothelium is an important potential target because of its close association with neutrophils during the inflammatory process when neutrophil to endothelial cell effector to target ratios may be quite high locally and neutrophil activation could produce endothelial injury. Such endothelial injury may substantially enhance the inflammatory process by allowing increased permeability and access of circulating cells and plasma constituents to subadjacent tissue.

The assay employed in these studies simultaneously measures ^{3}H-AA or ^{51}Cr release and cell detachment and allows differentiation of a lytic from a nonlytic process producing endothelial detachment. Although both the release and detachment assays measure radiolabel lost from monolayers, the washing procedure that precedes the measurement of cell detachment also removes detached cells or those that are only loosely adherent. The detachment assay is thus a more sensitive measure of injury, since it detects both lethal, lytic injury and sublethal or nonlytic detachment.

TABLE I
Inhibition of Neutrophil-mediated Endothelial Cell Detachment

Reagent	Inhibition
	%
Catalase 100 μg/ml + SOD 20 μg/ml (36)	10.4±3.0
Catalase 500 μg/ml + SOD 100 μg/ml (16)	15.2±6.7
SBTI	
500 μg/ml (37)	34.0±4.6
1,000 μg/ml (8)	62.5±8.8
2,000 μg/ml (23)	73.6±7.3
5,000 μg/ml (8)	100 —
CKEI (Ac-Ala-Ala-Pro-Val-CH_2Cl) 1 mM (25)	28.0±2.5
Human serum	
10% (25)	11.0±3.6
40% (8)	71.3±13.9
80% (8)	73.7±9.1
100% (14)	94.0±3.4

Neutrophils ($10^4/\mu l$) and STZ (2.5 mg/ml) were used throughout in 3-h incubations at 37° in 100 μl total volume. Percent inhibition = (% detachment without inhibitor − % detachment with inhibitor)/% detachment without inhibitor × 100%. The number of replicates is given in parenthesis. The values represent means ± 1 SE.

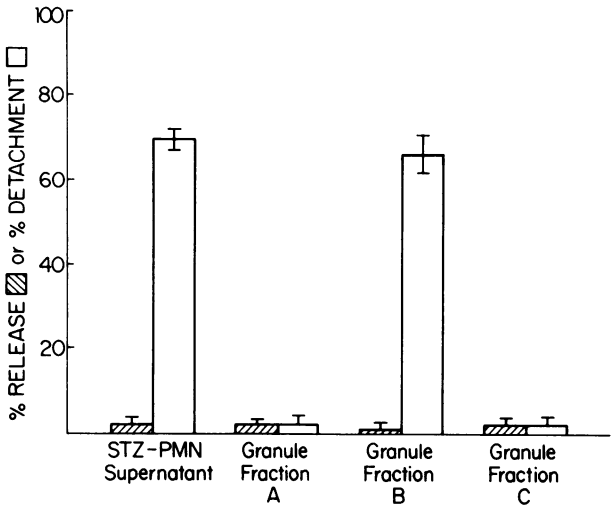

FIGURE 8 Endothelial cell detachment by postsecretory media from STZ-activated neutrophils and partially purified granule fraction. Cell-free postsecretory media from STZ (5.0 mg/ml)-activated neutrophils (PMN) ($10^5/\mu l$) produce endothelial cell detachment (open bar) without ^{51}Cr release (hatched bar) in 4-h incubation. At 5 μg/ml partially purified granule fraction B produced detachment (open bar) without ^{51}Cr release (hatched bar) at 4 h. Fractions A and C produce no detectable detachment (open bars) at 5 μg/ml. Each value is the mean of 6 replicates ± 1 SE.

This is evidenced by the detachment and ^{51}Cr release data obtained with hydrogen peroxide, trypsin, and xanthine-XO-mediated injuries (Figs. 2 and 3). Furthermore, since ^{3}H-AA or ^{51}Cr retention is directly proportional to the number of remaining adherent endothelial cells, this technique allows quantification of endothelial detachment when direct cell counting is invalid because the cell count of adherent neutrophils cannot be separated from the endothelial cell count.

Following exposure to STZ-activated neutrophils endothelial cell detachment is not associated with significant lysis measured by ^{3}H-AA or ^{51}Cr release (20, 21). It seems likely that neutrophil granule proteases induce endothelial cell detachment since both the postsecretory media from STZ-activated neutrophils and partially purified granule fraction also produced detachment. Similar nonlytic detachment by neutrophil granule preparations has been reported for HeLa cells and fibroblasts by Taubman and Cogen (22, 23). They also noted that the cell-detaching activity was inhibitable by tryptic and serine protease inhibitors as well as serum, implying neutral protease activity.

Based on the pattern of inhibition by a serine protease inhibitor (PMSF), tryptic inhibitor (SBTI), elastase inhibitor (CKEI), and serum it is likely that elastase largely mediates endothelial cell detachment observed with the granule fraction and intact STZ-activated neutrophils (24, 25). Although the CKEI is relatively specific for elastase, it also has some activity against the other major neutrophil neutral protease cathespin G (26). Thus, we cannot be certain that the cell-detaching activity is solely elastase. McDonald et al. (27) have demonstrated digestion by neutrophil granule fractions of media and cell surface fibronectin derived from human diploid fibroblasts. They have also shown that neutrophil elastase activity digests fibronectin to univalent, biologically active fragments (28). Our SDS gel electrophoresis of labeled endothelial cell surface proteins following exposure to granule fraction B also demonstrates the sensitivity of fibronectin to neutrophil-derived neutral proteases. In

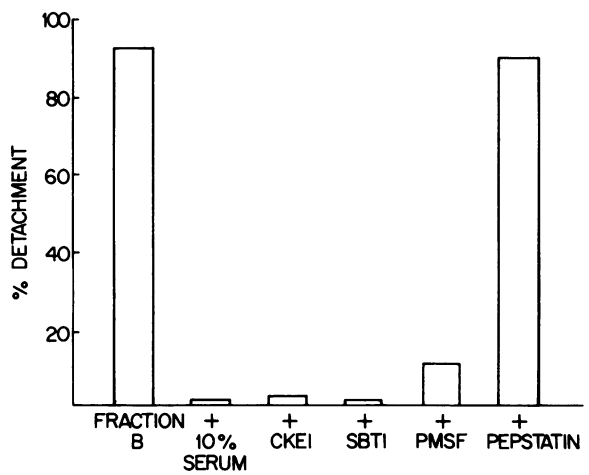

FIGURE 9 Effect of neutral protease inhibitors on endothelial cell detachment by neutrophil granule fraction. Endothelial cell detachment by partially purified granule fraction B is inhibited by 10% serum, CKEI (1 mM), SBTI (500 μg/ml), and PMSF (1 mM), but not by pepstatin A (10 μM). Inhibitors were incubated with granule fraction at room temperature for 30 min before the addition of endothelial cell monolayers. Each value is the mean of three replicates.

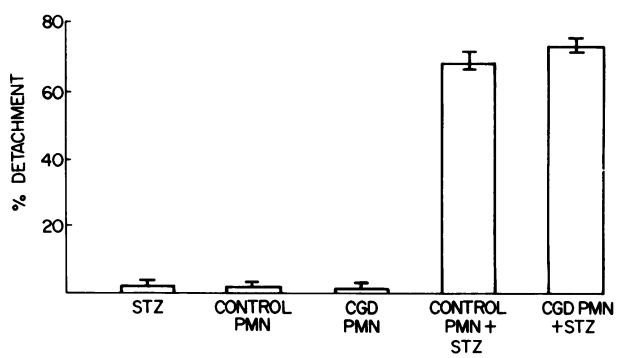

FIGURE 10 Effect of CGD neutrophils on endothelial cell detachment. CGD neutrophils produce the same endothelial cell detachment as normal neutrophils when stimulated by STZ in a standard 3-h incubation. Each value is the mean of 8 replicates ± 1 SE.

separate experiments we have also noted loss of cell surface fibronectin following exposure of [^{3}H]proline-labeled monolayers to intact STZ-activated neutrophils. However, these data do not prove that loss of fibronectin by neutrophil protease digestion is the sole cause for detachment in this system, since a number of other cell surface attachment proteins may also be digested.

In this assay system using STZ-activated neutrophils we could not demonstrate endothelial cell ^{51}Cr release or detachment mediated solely by oxygen radicals. The xanthine-XO oxygen radical generating system and reagent hydrogen peroxide produced both endothelial cell ^{51}Cr release and detachment. However, no endothelial ^{3}H-AA or ^{51}Cr release were noted after incubation with STZ-activated neutrophils and endothelial cell detachment by STZ-activated neutrophils was not inhibitable by catalase or SOD. More importantly, CGD-neutrophils produced cell detachment when activated by STZ that was comparable to the effects of control neutrophils. Sacks et al. (29) reported that oxygen radicals mediate endothelial cell toxicity by STZ- and C5a-activated neutrophils. Endothelial injury in that system, however, was minimal with specific ^{51}Cr release <10%. In our assay we were unable to demonstrate significant ^{51}Cr release by STZ-activated neutrophils even with prolonged incubation at high effector to target ratios. However, there are several important differences between the assays that might account for at least some of the differences. We employed a longer period of incubation (3–8 h vs. 1.5 h) with a higher neutrophil to endothelial cell ratio (10 to 40:1 vs. 10:1). STZ was used to activate neutrophils in our system whereas Sacks et al. (29) used primarily C5 fragment or zymosan-activated plasma. C5a-activation produces minimal granule release unless neutrophils are pretreated with cytochalasin B (7). Also their assay was performed in 20% serum whereas the present assay was performed serum-free with detachment serum-inhibitable. Finally, Sacks et al. (29) measured ^{51}Cr-release in supernatant only and did not attempt to quantify detachment. Nathan et al. (30) also described neutrophil-mediated cytotoxicity to a number of target cells with hydrogen peroxide as the apparent injury agent. Of interest in their study was the observation that several of the cell types tested were quite resistant to lysis by either reagent or neutrophil-generated hydrogen peroxide. The probable explanation for our inability to identify oxygen radical-mediated injury in our studies may be that ^{3}H-AA or ^{51}Cr release is a relatively insensitive measure of cell injury, since release of intracellular label probably reflects cell lysis (20, 21). Significant and functionally important endothelial cell injury without lysis could certainly be produced by oxygen radicals.

The subcellular location of the cell-detaching activity

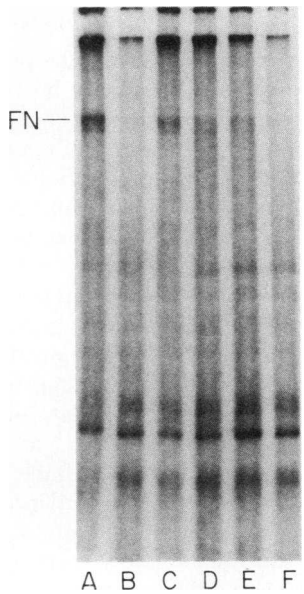

FIGURE 11 Digestion of endothelial cell surface proteins by neutrophil-granule neutral proteases. 30-min incubation of ^{125}I-labeled endothelial cell monolayers with partially purified granule fraction B (5 μg/ml) produces detachment of cells and concomitant loss of cell surface proteins including fibronectin (FN) which is inhibited by neutral protease but not acid protease inhibitors: (a) PBS, (b) granule fraction B, (c) granule fraction B with PMSF 1 mM, (d) granule fraction B with SBTI 500 μg/ml, (e) granule fraction B with CKEI 1 mM, (f) granule fraction B with pepstatin A 10 μM. Granule fraction B was incubated with inhibitors for 30 min at 4°C prior to addition to endothelial cells. Equivalent radioactivity of each sample was applied.

can be determined from the studies using the partially purified granule fractions (16, 17). Fractions A and B are comprised of the primary, peroxidase-positive azurophilic granules. Our studies demonstrate that the cell-detaching protease is a constituent of the primary granule subpopulation B. Fraction C, which demonstrated no cell-detaching activity at lower concentrations, consists of the secondary (or specific) peroxidase-negative granules. The cell-detaching activity seen with higher concentrations of fraction A and C most probably represents some unavoidable contamination with B granule contents during fractionation. The demonstration that the major cell-detaching activity is in the B fraction is consistent with the localization of neutrophil neutral proteases in the primary azurophil granule (31).

Neutrophil lysosomal enzymes have been shown to induce vascular injury in vivo and in vitro (32–35). Whether neutral proteases can induce endothelial injury in vivo in the presence of plasma inhibitors such as α_2-macroglobulin and α_1-antitrypsin is unknown. It is possible that augmented neutrophil adherence to endothelium at sites of inflammation may generate

local concentrations of proteases that exceed the capacity of the plasma inhibitors at the protected interface. Moreover, α_1-antitrypsin may be inactivated by excess elastase or oxygen radicals generated by activated neutrophils (36, 37). Finally, neutral proteases bound to substrate on the endothelial cell surface might be temporarily protected from inactivation by α_2-macroglobulin since the initial reaction of proteases with this inhibitor is competitive with respect to substrate (24). The recent demonstration of increased elastolytic activity in pulmonary lavage fluid of patients with adult respiratory-distress syndrome despite normal concentrations of α_1-antitrypsin suggests that plasma inhibitors do not necessarily prevent neutral protease activity at inflammatory sites (38).

The digestion of adhesive cell surface proteins such as fibronectin (39–43) by neutrophil proteases could produce significant alterations in endothelial cell function and affect the inflammatory response. Certainly, frank endothelial cell detachment mediated by neutrophil proteases would have profound effects on vessel permeability and thrombogenicity. However, neutrophil protease digestion of endothelial cell surface proteins without detachment could also markedly affect vessel wall integrity by altering cell-cell and cell-substrate interaction.

ACKNOWLEDGMENTS

We are indebted to Kathe Stanness for technical support.

This work was supported in part by research grants (HL-18645 and HL-11775) from the U. S. Public Health Service and R. J. Reynolds Industries, Inc. This work was performed during the tenure of a Clinician-Scientist Award from the American Heart Association with funds contributed in part by American Heart Association of Washington.

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