# Shear stress-induced apoptosis of adherent neutrophils: A mechanism for persistence of cardiovascular device infections

**Matthew S. Shive*, Mariah L. Salloum*, and James M. Anderson*†‡**

*Department of Biomedical Engineering and †Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106

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**The mechanisms underlying problematic cardiovascular device-associated infections are not understood. Because the outcome of the acute response to infection is largely dependent on the function of neutrophils, the persistence of these infections suggests that neutrophil function may be compromised because of cellular responses to shear stress. A rotating disk system was used to generate physiologically relevant shear stress levels (0–18 dynes/cm$^2$; 1 dyne = 10 $\mu$N) at the surface of a polyetherurethane urea film. We demonstrate that shear stress diminishes phagocytic ability in neutrophils adherent to a cardiovascular device material, and causes morphological and biochemical alterations that are consistent with those described for apoptosis. Complete neutrophil apoptosis occurred at shear stress levels above 6 dynes/cm$^2$ after only 1 h. Morphologically, these cells displayed irreversible cytoplasmic and nuclear condensation while maintaining intact membranes. Analysis of neutrophil area and filamentous actin content demonstrated concomitant decreases in both cell area and actin content with increasing levels of shear stress. Neutrophil phagocytosis of adherent bacteria decreased with increasing shear stress. Biochemical alterations included membrane phosphatidylserine exposure and DNA fragmentation, as evaluated by *in situ* annexin V and terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL) assays, respectively. The potency of the shear-stress effect was emphasized by comparative inductive studies with adherent neutrophils under static conditions. The combination of tumor necrosis factor-$\alpha$ and cycloheximide was ineffective in inducing >21% apoptosis after 3 h. These findings suggest a mechanism through which shear stress plays an important role in the development of bacterial infections at the sites of cardiovascular device implantation.**

The use of clinical cardiovascular devices in the treatment of cardiovascular disorders has substantially increased worldwide in recent years. For example, each year more than 60,000 vascular grafts and 175,000 prosthetic heart valves are implanted in humans, along with the use of over 200 million intravascular catheters (1–3). Unfortunately, serious and often catastrophic flow-associated device infection occurs at a rate ≈3% and poses a significant medical challenge, with most cases requiring surgical intervention. Therefore, considerable numbers of patients are affected, and the associated 20–40% mortality rate is staggering.

The mechanisms underlying implant-associated infections are not understood, but it is known that the presence of an implanted artificial material reduces the effectiveness of host defense systems and decreases the number of organisms required to initiate infection (4–6). The host's acute response to infection largely depends on the function of neutrophils, which sequester and destroy foreign microorganisms through phagocytosis and the generation of reactive oxygen species. In this study, we hypothesized that, within the complex vascular device environment of dynamic flow and a foreign material, adherent neutrophils are unable to fulfill their bactericidal role as a consequence of the cellular response to shear stress.

Shear stress, which notably inhibits apoptosis in endothelial cells (7), also is known to modulate endothelial cell morphology, function, and the expression of cell surface molecules (8–11). At present, however, equivalent hemodynamic studies with neutrophils are lacking and limited to neutrophil adhesion and morphological observations (12–14). Shear stress causes neutrophil pseudopod retraction and preferentially rounded morphology in macrophages (13, 15), but its role in the regulation of adherent neutrophil function has not been explored. We have shown that neutrophil adhesion to polyurethane is highly shear stress-dependent and modulated by adsorbed serum proteins (14, 16), and that transient exposure to polyurethane under shear stress does not affect the ability of nonadherent neutrophils to generate reactive oxygen species (14). The present studies focused on neutrophils adherent to a polyurethane being investigated for use in cardiovascular devices and examined cellular and apoptotic changes resulting from adhesion under shear stress.

Apoptosis is a highly regulated process that results in the orderly breakdown and death of a cell. Morphologically, it is characterized by cell shrinkage and nuclear condensation, during which cells preserve membrane integrity, and membrane budding, which progresses to the formation of membrane-bound apoptotic bodies. Apoptotic cells also undergo biochemical alterations, including an early-stage loss of normal cell membrane asymmetry with the translocation of phosphatidylserine (PS) to the outer leaflet of the membrane phospholipid bilayer, as well as late-stage endonuclease activity resulting in DNA fragmentation (17, 18). Various agents influence apoptosis in neutrophils, e.g., tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and FAS ligand induce apoptosis (19, 20), whereas lipopolysaccharide, granulocyte–macrophage colony-stimulating factor, and other inflammatory mediators inhibit apoptosis (21, 22). Furthermore, apoptosis progresses at different rates in adherent neutrophils, according to the activating signals triggered by particular adhesion substrates (23, 24).

In this paper, we demonstrate that physiological levels of shear stress cause morphological and biochemical alterations in neutrophils adherent to a cardiovascular device material that are consistent with those described for apoptosis. This effect of shear stress is potent, resulting in complete apoptosis of adherent neutrophils after 60 min at physiologically relevant shear levels >6 dynes/cm$^2$ (1 dyne = 10 $\mu$N). Consequently, neutrophils exposed to these shear levels also exhibited diminished phago-

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Abbreviations: RDS, rotating disk system; CHX, cycloheximide; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; TNF-$\alpha$, tumor necrosis factor-$\alpha$; SEM, scanning electron microscope.

†To whom reprint requests should be addressed at: Case Western Reserve University, Institute of Pathology, Room 306, 2085 Adelbert Road, Cleveland, OH 44106.

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cytic ability. These findings suggest a mechanism through which shear stress plays an important role in the persistence of bacterial infections at sites of cardiovascular device implantation under flow conditions.

## Materials and Methods

**Adhesion Substrate.** The substrate was a 200-$\mu$m thick polyetherurethane urea film, which currently is being evaluated for cardiovascular device applications. The polyetherurethane urea was a segmented block copolymer (number-average molecular weight, $M_n$ = 50,000) of methylene di(*p*-phenyl isocyanate), chain-extended with ethylene diamine and poly(tetramethyleneglycol) ($M_n$ = 2,000). Using stainless steel dies, solution-cast films were cut into 15- and 17-mm diameter disks for use in static and rotating disk system (RDS) experiments, respectively. Film samples were sonicated in both mild detergent and methanol for 10 min each, rinsed with distilled, deionized water (>18.2 M$\Omega$; Millipore), and air-dried.

**Neutrophil Isolation.** Human neutrophils were isolated from citrated blood according to a published method (25). Briefly, cells were subjected to density gradient centrifugation in Ficoll/Paque (Amersham Pharmacia) and sedimentation in dextran T500 (pH 7.4; Amersham Pharmacia) followed by hypotonic lysis of residual erythrocytes. The resulting cell population was >98% viable, as verified by trypan blue exclusion, and contained 95.2 ± 0.9% neutrophils ($n$ = 3), as determined by staining with modified Wright's stain (Sigma). For experiments, isolated neutrophils were suspended in RPMI medium 1640 (GIBCO) supplemented with 20% autologous human serum.

**Neutrophil Adhesion Under Shear Stress.** An RDS was used to generate well defined and reproducible dynamic flow conditions, the details of which have been previously described (26). Briefly, shear stress at the surface of the rotating disk is directly proportional to the radial distance from the center of the disk, where shear is zero. However, the flux of suspended neutrophils to the surface is independent of radial distance and is uniform at all points, allowing adhesion to be directly correlated to shear stress. Each component of the RDS was autoclaved before use. Film disks then were attached to support disks with Spot-O-Glue adhesives (Avery Dennison, Azusa, CA), mounted on the rotator arm of the RDS, and lowered into a 50-ml polytetrafluoroethylene beaker containing 20% serum medium at 37°C in a constant-temperature water bath. A rotation speed of 350 rpm was used to generate the physiologically relevant shear stress range of 0–18 dynes/cm$^2$ across the surface of the film disk. Neutrophils were pipetted into the test suspension subsequent to the start of rotation at a concentration of $3 \times 10^6$ cells per ml for all experiments. After 60 min, the test suspension was removed immediately by fluid exchange (to prevent loss of adherent cells caused by air/water interface) with 150 ml of fresh PBS (Sigma), followed by an exchange of 150 ml of 1% paraformaldehyde (Sigma) for cytoskeletal analyses, prompt removal of the film sample for *in situ* membrane viability and apoptosis assays, or continued static incubation for 30 or 60 min after shear removal, followed by fixation for reversibility experiments.

**Static Neutrophil Adhesion.** Sample film disks were secured in 24-well tissue-culture polystyrene plates (Falcon 3047; Becton Dickinson) with silicone rubber rings (Cole–Palmer) and incubated with $3 \times 10^5$ neutrophils in a volume of 1 ml. For cytoskeletal analyses, neutrophils that were otherwise untreated or were treated with $10^{-7}$ M chemoattractant C5a (Sigma) were allowed to adhere for 60 min at 37°C. This concentration of C5a has been shown to optimally up-regulate CD11b/CD18 surface expression in neutrophils (27) and was used here as a positive control for actin polymerization. Samples then were rinsed three times with PBS and fixed with 1% paraformaldehyde. For biochemical apoptosis studies, neutrophils were allowed to adhere for 60 min at 37°C, after which the nonadherent cell suspension was replaced with fresh 20% human serum in RPMI medium 1640, with or without TNF-$\alpha$ (1 $\mu$g/ml; Sigma) and cycloheximide (CHX) (10 $\mu$g/ml; Sigma). This combination has been demonstrated to induce >85% apoptosis in suspended neutrophils (19). After further incubation periods of 0, 1, 2, or 3 h, the sample disks were rinsed three times with PBS.

**Morphological Membrane and Nuclear Studies.** After submersion of film disks in 10% trypan blue in PBS for 30 sec, *in situ* membrane integrity of adherent cells was immediately assessed by using light microscopy. Nuclear morphology of fixed cells was observed following May-Grünwald/Giemsa (Sigma) staining. Qualitative analysis of neutrophil membrane integrity and nuclear condensation were performed for adherent neutrophils at incremental distances from the center of the disks, representing areas of increasing shear stress.

**Actin Content and Cell Area Distribution.** Filamentous actin was labeled using rhodamine phalloidin (Molecular Probes) as described elsewhere (28). Neutrophils were visualized with a ×60 oil immersion objective on a confocal scanning laser microscope (MRC-600; Bio-Rad), and actin-labeling intensity was quantified with confocal-dedicated software (COMOS; Bio-Rad). Series of images along the $z$ axis using 1.5-$\mu$m incremental slices were obtained with a motorized stage. Individual neutrophils were analyzed by confocal software after defining each cell area by hand, resulting in volumetric pixel information directly proportional to F-actin content and cell area (mm$^2$).

**Scanning Electron Microscopic (SEM) Analysis.** Selected film disks from shear stress experiments were processed for SEM analysis by using 5-min serial ethanol dehydration rinses and two 15-min hexamethyldisilazane treatments, and then they were coated with 200 Å of gold palladium. Morphological observations of adherent neutrophils at increasing shear levels were made, including cell shape (spread or round), membrane structures (pseudopodia, ruffling, blebbing, or smooth), and qualitative neutrophil area.

**Functional Studies.** A clinical isolate of bacterial strain *Staphylococcus epidermidis* RP62A was preseeded onto the film disks by incubating the disks with 2 ml of PBS containing $10^8$ colony-forming units for 45 min at 37°C. The preseeded disks then were exposed to shear stress (0–18 dynes/cm$^2$) for 15 min on the RDS, which served to rinse the disk of non- or lightly adherent bacteria. The cell-free RPMI medium 1640 then was replaced with 20% human serum in RPMI medium 1640 by using fluid transfer, and freshly isolated neutrophils were added as above and allowed to adhere under shear for 60 min. Film samples were removed, processed, and observed under SEM. The extent to which adherent neutrophils were able to locate and phagocytose preseeded bacteria was assessed and correlated to shear stress levels.

***In Situ* Determination of Apoptosis Using Annexin V.** The extent of neutrophil apoptosis was determined by using binding of annexin V to membrane PS (29). After static and shear stress adhesion experiments, rinsed substrate samples were covered with 100 $\mu$l of annexin V-FITC conjugate (R & D Systems) at a concentration of 0.025 $\mu$g/ml and incubated in the dark for 15 min at room temperature. Prior counterstaining of all isolated neutrophils with 8 $\mu$M Syto17 (Molecular Probes) at 37°C for 30 min allowed an apoptotic/total adherent cell ratio to be calculated by using a dual-filter (488/568 nm) confocal scanning laser microscopic configuration. Because shear stress increases with distance from

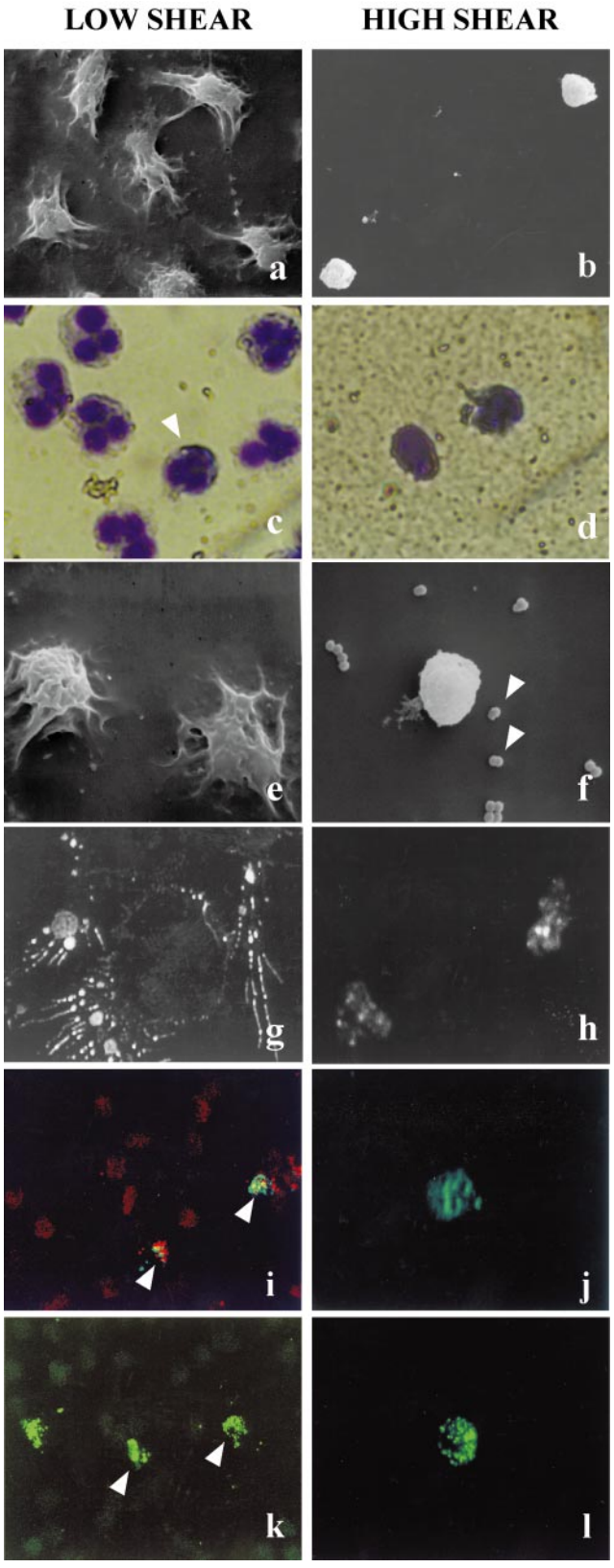


**Fig. 1.** Effects of 60-min exposure to low (0–2 dynes/cm$^2$) and high (>6 dynes/cm$^2$) shear stress on neutrophils adherent to polyetherurethane urea. (*a* and *b*) Shear stress causes major morphological changes in neutrophils. Representative scanning electron micrographs demonstrate that adherent neutrophils exposed to low shear were primarily well spread, in contrast to those under high shear levels, which showed condensed and irregular morphologies. (×1,000.) (*c* and *d*) May-Grünwald/Giemsa staining revealed that neutrophils under low shear exhibited characteristic multilobed nuclei, with an occasional condensed nucleus (arrowhead in *c*). Nuclear condensation was observed in all cells exposed to high shear levels. (×100.) (*e* and *f*) Shear stress affects the phagocytic ability of adherent neutrophils. Representative scanning electron micrographs show that neutrophils exposed to low shear effectively located and phagocytosed preseeded *S. epidermidis*. Neutrophils under high shear apparently were incapable of interacting with the bacteria. Arrowheads in *f* show numerous bacteria in close proximity to a condensed neutrophil under high shear. (*g* and *h)* Neutrophil F-actin distribution is influenced by shear stress. F-actin was labeled with rhodamine phalloidin and visualized by using confocal microscopy. (×3,300.) Spread neutrophils in areas of low shear localized actin in pseudopodia, whereas neutrophils exposed to high shear exhibited compact actin distribution and low-intensity staining. (*g*, ×60, and *h*, ×3, computerized zoom.) (*i* and *j*) Membrane PS is exposed on neutrophils under shear stress. Syto17-counterstained (red) adherent neutrophils exposing membrane PS were identified with annexin V-FITC (green). Areas of low shear contained few annexin V-positive neutrophils, whereas high-shear areas contained sparse but exclusively annexin V-positive neutrophils. (*i*, ×60, and *j*, ×3, computerized zoom.) (*k* and *l*) DNA fragmentation also was identified after shear stress exposure. By using TUNEL-fluorescein labeling, low numbers of neutrophils with fragmentation were observed under low shear, whereas a high percentage of the sparse cells under high shear exhibited fragmentation (*k*, ×60, and *l*, ×3, computerized zoom.)

the disk center, confocal stage micrometers were used to determine coordinates of counting fields, which were then correlated to shear stress. Similarly, spontaneous and TNF-$\alpha$- and CHX-induced apoptosis were visualized on static control films, and apoptotic ratios were calculated after 200 total adherent cells were counted.

***In Situ* Determination of Apoptosis Using Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling (TUNEL).** A TUNEL (30) assay was used to detect late-stage apoptotic neutrophils. After static and shear stress adhesion experiments, substrate films with adherent neutrophils were incubated for 60 min with terminal deoxynucleotidyltransferase and dUTP-fluorescein conjugate (R & D Systems) for 60 min, rinsed, and visualized with a confocal scanning laser microscope. Apoptotic ratios were calculated as for the annexin V assay. Counterstaining was excluded because uniform cytoplasmic background fluorescence was sufficient for total adherent cell quantification.

**Statistical Analysis.** Statistical analysis consisted of unpaired Student's *t* tests completed with STATVIEW (Version 4.1; Abacus Concepts, Berkeley, CA). Averages were calculated from at least three different donors for each experiment, and differences were considered significant only for $P < 0.05$.

## Results

**Morphological and Functional Observations.** Initially, we asked how shear stress influenced adherent neutrophil morphology and their ability to phagocytose adherent bacteria. SEM analysis demonstrated a larger and more spread morphology in cells under low shear (0–2 dynes/cm$^2$), whereas high-shear (>6 dynes/cm$^2$) areas contained compact cells with irregular shape and budding (Fig. 1 *a* and *b*). The convoluted cell membranes may indicate the formation of membrane-bound apoptotic bodies of varying size caused by high shear forces. Furthermore, SEM evaluation of adherent neutrophils after 30 or 60 min of static incubation, after adhesion under shear, confirmed the irreversibility of the morphological alterations. The removal of fluid shear had no effect on neutrophil morphology (data not shown). Fig. 1*c* shows the multilobed characteristic of neutrophil nuclei in an area of low shear. Those neutrophils exposed to higher shear, although more sparse, exhibited condensed nuclei, as seen in Fig. 1*d*. We also observed consistent exclusion of

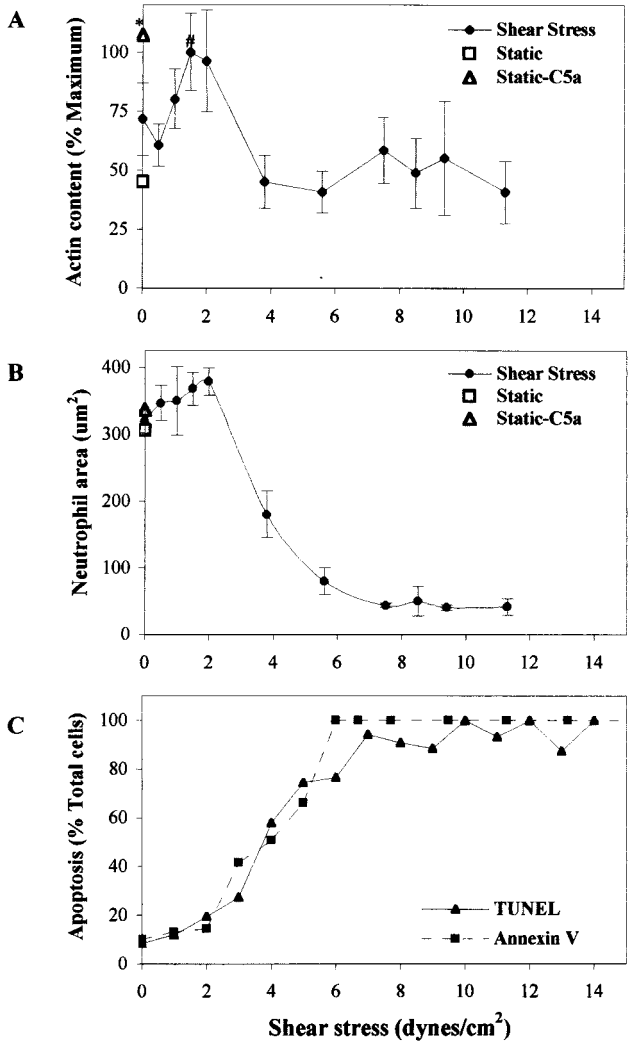


**Fig. 2.** Effects of 60-min exposure to shear stress on neutrophils adherent to polyetherurethane urea. (*A*) F-actin content is modulated by shear stress. The maximum actin content detected through confocal analysis at 1.5 dynes/cm$^2$ was significantly greater than were actin levels at 3.8, 5.6, and 11.3 dynes/cm$^2$. *, $P < 0.04$. Additionally, static controls incubated with C5a demonstrated significant increases in F-actin content. *, $P < 0.05$ as compared with static controls without C5a or 3.8 and 5.6 dynes/cm$^2$ shear levels. (*B*) Neutrophil area decreased with increasing shear stress. When compared with areas at all shear levels of 3.8 dynes/cm$^2$ or greater, direct measurements produced significant differences in neutrophil area for static controls with and without C5a ($P < 0.05$) and shear levels of 0, 1, and 2 dynes/cm$^2$ ($P < 0.02$). (*C*) Shear stress produced a rapid and synchronous progression from PS exposure to DNA fragmentation, as determined by *in situ* annexin V and TUNEL assays, respectively. Annexin V binding increased significantly for all shear levels at and above 3 dynes/cm$^2$ as compared with 0, 1, and 2 dynes/cm$^2$ ($P < 0.05$). Similarly, TUNEL-positive apoptosis was significantly greater at all shear levels of 4 dynes/cm$^2$ or above when compared with 0, 1, 2, and 3 dynes/cm$^2$ ($P < 0.007$). Data represent means ± SEM in *A* and *B*. Multiple experiments were completed separately for actin/area (9), annexin V (5), and TUNEL (7) studies, using neutrophils from at least three different blood donors.

trypan blue at all shear levels (data not shown), confirming a distinctive and vital characteristic of apoptosis: the preservation of membrane integrity, and ruling out the possibility of necrotic cell death.

When adherent to the polyurethane with preseeded bacteria, neutrophils in areas of low shear were efficient in clearing adherent bacteria, but those in areas of high shear seemed

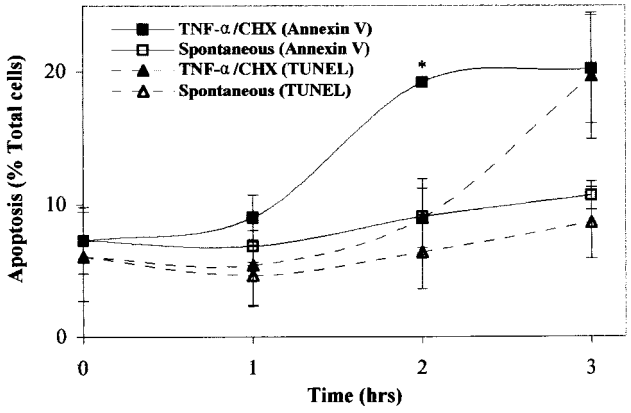


**Fig. 3.** Chemical induction of apoptosis in neutrophils adherent to static polyetherurethane urea. The combination of TNF-α and CHX induced asynchronous exposure of PS and DNA fragmentation, but was unable to produce apoptotic proportions over 20.2% even after 3 h. *, $P < 0.04$ as compared with spontaneous apoptosis at 1, 2, and 3 h. Data represent means ± SEM. Experiments were completed in duplicate with three different blood donors.

incapable of interacting with the bacteria, even when the bacteria were in close proximity to the neutrophils. Scanning electron micrographs in Fig. 1 *e* and *f* show low-shear areas containing highly spread cells and the notable absence of bacteria, in contrast to the areas of high shear, which contained small, condensed cells surrounded by bacteria, suggesting diminished phagocytic ability.

**F-actin Content and Cell Area Are Shear-Stress-Dependent.** Cytoskeletal rearrangements that occur in adherent neutrophils after exposure to shear stress were investigated. Confocal analysis of total F-actin content showed that polymerization of actin increased with increasing shear stress in areas of low shear, 0–1.5 dynes/cm$^2$ (Fig. 2*A*). F-actin content was maximum at 1.5 dynes/cm$^2$ and then steadily decreased until a threshold shear of 7.5 dynes/cm$^2$, above which F-actin remained constant regardless of shear level. Fig. 1*g* demonstrates that F-actin in adherent neutrophils under low shear was primarily accumulated within pseudopodia. In contrast, high-shear stress caused a retraction of pseudopods and a condensed actin distribution (Fig. 1*h*). Static control experiments resulted in equivalent F-actin content as compared with zero shear levels on the RDS. The addition of the chemoattractant C5a to the static controls cells produced significantly increased F-actin polymerization when compared with zero shear and static controls without C5a (Fig. 2*A*), a response similar to activation with chemotactic peptide fMet-Leu-Phe (31). Neutrophils activated by C5a assembled equivalent amounts of F-actin as did those exposed to shear stress levels of 1.5 dynes/cm$^2$, the F-actin maximum in this study.

The area distribution of adherent neutrophils with increasing shear stress was even more striking. Fig. 2*B* illustrates that while maintaining uniform area under low shear (0–1.9 dynes/cm$^2$), neutrophils adherent in areas of >6 dynes/cm$^2$ showed a significant decrease in area. Both C5a-activated and nonactivated static neutrophils demonstrated similar cell areas as compared to adherent neutrophils exposed to 0–1.5 dynes/cm$^2$ shear, regardless of F-actin content. This observation demonstrates that cell area does not necessarily increase proportionately with actin polymerization and that neutrophils reach a spreading maximum at 1.5 dynes/cm$^2$, which does not increase with activation or shear stress.

**Membrane PS Is Exposed on Adherent Cells Under Shear Stress.** The relationship between the level of shear stress and the degree of

apoptosis of adherent neutrophils was explored by using annexin V binding to PS and is demonstrated in Fig. 2*C*. Above 2 dynes/cm$^2$, apoptosis increased as a function of increasing shear stress. Shear levels of >6 dynes/cm$^2$, however, induced complete (100%) apoptosis of adherent neutrophils after 60 min. PS exposure, which facilitates apoptotic cell recognition and removal by macrophages (32), was detected on small proportions of neutrophils at low shear (Fig. 1*i*) and was distributed uniformly on the surfaces of all of the small and irregularly shaped neutrophil membranes under high-shear levels (Fig. 1*j*). The potency of the inductive effect of shear stress is further demonstrated by comparison of these data with those from static control experiments. Fig. 3 shows that spontaneous apoptosis of neutrophils adherent to the polyetherurethane urea in static controls reached levels of 11% over 3 h, whereas induction using a combination of TNF-$\alpha$ and CHX augmented apoptosis to only 20% after 3 h.

**DNA Fragmentation Occurs in Adherent Cells Under Shear Stress.** Labeling of DNA fragments in shear-exposed neutrophils not only confirmed our annexin V results, but offered temporal information on the extent of apoptosis in these cells. We were surprised to observe that the proportion of cells exhibiting DNA fragmentation, a late-stage event, was essentially indistinguishable from those with exposed PS, a marker for early apoptosis (Figs. 1 *k* and *l* and 2*C*). Within a cell population, apoptosis is characteristically an asynchronous event. Our results, however, indicate that high shear produces a rapid and synchronous progression of neutrophils through the sequence of events leading to cell death, an effect similar to UV irradiation (33). Even under low-shear conditions, the same proportion of neutrophils exposing PS also had undergone some level of DNA fragmentation. In contrast, static controls induced with TNF-$\alpha$ and CHX exposed PS after only 1 h, which was followed by a slower occurrence of DNA fragmentation at 2 h, reaching levels similar to PS exposure after 3 h (Fig. 3). This asynchronicity in the static cells was predictable (21, 22) and serves to confirm the potency of the shear stress effect.

## Discussion

We report in this paper that neutrophils adherent to a cardiovascular biomaterial under physiological levels of shear stress undergo cellular alterations leading to decreases in F-actin content and cell area, as well as diminished phagocytic function. These cells also display morphological (cell shape and nuclear condensation) and biochemical (PS exposure and DNA fragmentation) characteristics of cells undergoing apoptosis. These findings suggest a mechanism in which shear stress plays an important role in regulating neutrophil function and which may underlie the development and persistence of cardiovascular implant-associated bacterial infections.

A striking aspect of our observed shear stress-induced apoptosis is the rapidity with which it occurred. Shear stress-induced changes in endothelial cells occur after 6–12 h, and smooth muscle cells require a 24-h exposure to shear stress to exhibit alterations (11, 34). The high percentages of apoptosis that we observed in neutrophils after only 60 min of exposure to shear stress (Fig. 2*C*) indicate that the signal for apoptosis is probably received during the initial stages of adhesion (23). Indeed, even with attempts to accelerate cell death with TNF-$\alpha$ and CHX, adhesion to the same polyurethane substrate under static conditions served only to rescue neutrophils from cell death as compared with suspended cells (19). Furthermore, although the average residence time of the adherent neutrophils we examined is unknown, it may have been <60 min if an active exchange of cells at the polyurethane surface occurred that involved detachment and further adhesion of new cells. This scenario has important implications relative to how quickly this apoptosis is induced and would imply that apoptosis of adherent neutrophils actually develops more rapidly than we conclude here.

Our results also suggest that adhesion to polyurethane under shear stress triggers the signal for apoptosis, which is delivered as a function of shear level. Precisely what levels of shear stress are necessary to induce cellular alterations seem to be dependent on the *in vitro* system used, whether cells are adherent or in suspension, and on the experimental time frame (12, 13, 15). Mechanical trauma through viscometer shearing of nonadherent neutrophils was shown to negatively alter neutrophil morphology and function at much higher levels of shear stress, >150 dynes/cm$^2$ (35, 36). Because carotid and femoral artery shear levels reach only 8–11 dynes/cm$^2$ (37), we used a shear range of 0–18 dynes/cm$^2$ to encompass physiological shear ranges encountered in many cardiovascular devices. Although our RDS lacks pulsatile flow encountered *in vivo*, our present finding that 6 dynes/cm$^2$ is significant in neutrophil responses is consistent with studies using endothelial and smooth muscle cells, in which substantial differences in cellular responses at shear levels of 5 dynes/cm$^2$ or less compared with levels at 15 dynes/cm$^2$ or more were reported (38, 39).

It generally is acknowledged that apoptosis is modulated through adhesion to different substrates (23, 24). For example, adhesion to a quiescent endothelial cell monolayer increases neutrophil apoptosis as compared with suspended neutrophils after 6 h. This effect is in contrast to IL-1-activated endothelium, which inhibited neutrophil apoptosis by 2-fold. Likewise, interactions with substrates may serve to prime the cell for secondary apoptotic signals. When adhesion is mimicked by crosslinking of integrins (CD11b/CD18), neutrophils are significantly more susceptible to TNF-$\alpha$-induced apoptosis than either crosslinking or TNF-$\alpha$ treatment alone (40). Similarly, neutrophil adhesion to fibronectin accelerated subsequent TNF-$\alpha$-induced apoptosis (24), a response probably resulting from interactions with the $\alpha_5\beta_1$ integrin (fibronectin receptor) (41). Our studies suggest that the apoptotic signal received via shear stress may be the result of similar priming on neutrophil adhesion to polyurethane. In this case, integrins may act as mechano-receptors, i.e., they respond to fluid flow by aggregating in focal adhesion-like sites and by transducing mechanical stress into chemical signals through cytoskeletal structures (42). This generalized hypothesis recently was corroborated when $\alpha_v\beta_3$ integrin was implicated as a shear stress mechano-receptor in modulating migration in neutrophils after interactions with specific surface-anchored ligands (43). Although protein adsorption to our polyurethane substrate recently was characterized (44), it was our earlier studies that indicated adsorbed C3 and fibronectin modulate neutrophil adhesion under similar shear stress conditions (16). Fibronectin has been implicated in neutrophil apoptosis and may play a role here (41).

Shear stress may exert its effects through perturbations in intracellular second-messenger levels. Elevated intracellular $Ca^{2+}$ or reactive oxygen species each have been implicated as activators of signaling for apoptosis (45, 46). Oxidant-dependent activation of caspases (47) could lead to the organized disassembly of the cell, including actin cleavage (48) and nuclear fragmentation through endonuclease activation (18).

Regulation of F-actin polymerization and depolymerization is essential to neutrophil function (49). Low-shear levels promoted the increased assembly and accumulation of F-actin in newly formed pseudopods in adherent neutrophils (Fig. 1*g*), an event that may be closely related to the clustering of adhesion receptors in focal adhesions (50). Functionally, cytoskeletal reorganization at these low-shear levels could serve to prepare the neutrophil for bactericidal activities, including chemotaxis, phagocytosis, and reactive oxygen species production. Our observed decreases in detectable F-actin at higher shear levels are consistent with decreased functional ability, as well as with the

apoptotic process (51). The latter may involve caspase-regulated cleavage of actin filaments (48, 52), which is believed to be responsible for the characteristic cell shrinkage and membrane blebbing in this process (18).

Furthermore, the observed effects of shear stress might restrict neutrophil adhesive capabilities because apoptosis-associated cytoskeletal cleavage could alter adhesion molecule association with the cytoskeleton (53). Neutrophil populations with high proportions of apoptotic cells display deficiencies in cytoskeletal function, chemotaxis, and phagocytosis (54), which may explain the observed inability of neutrophils adherent in areas under high shear to interact with preseeded bacteria (Fig. 1*f*) despite the reported ability of bacterial products such as lipopolysaccharide (21) to delay apoptosis and enhance functional longevity.

Implantation of a foreign material compromises inflammatory cell function through poorly understood mechanisms (4–6). Leukocytes exhibit depressed chemotactic, phagocytic, and oxidative capacities after exposure to artificial materials, and shear stress may serve to exacerbate these effects. Indeed, vascular grafts using artificial materials that are implanted in dogs are significantly more susceptible to infection than are grafts made from biological materials, both autografts or allografts (55, 56). Although the mechanisms through which a cardiovascular device permits the establishment of infection are unclear, evidence suggests that artificial materials produce distinct interactions that have deleterious effects on inflammatory cell function. We propose that shear stress-induced apoptosis of adherent phagocytic cells is a mechanism through which an implanted material under flow conditions compromises host bactericidal abilities. Accordingly, our findings have important implications for the clinical success of implanted cardiovascular devices. The morbidity and mortality associated with infections of these devices is considerable (7) and may be attributed to, in part, the failure of apoptotic neutrophils to fulfill their phagocytic role.

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