Neutrophil survival on biomaterials is determined by surface topography

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Purpose: **Cardiovascular device-centered infections are a major cause of hospital morbidity, mortality, and expense. Caused by opportunistic bacteria, this phenomenon is thought to arise because of a defect in neutrophil bacterial killing. We have shown that neutrophils that adhere to polystyrene remain viable, whereas neutrophils that adhere to the vascular biomaterials expanded polytetrafluoroethylene (ePTFE) and Dacron undergo a rapid nonapoptotic death. This study was designed to test the hypothesis that surface topography is a determinant of the nonapoptotic death response of neutrophils to biomaterials.**

Methods: **We took advantage of the ease with which a polystyrene surface can be manipulated to examine the effect of surface topography on neutrophil viability. Neutrophils were exposed to smooth or roughened polystyrene surfaces both in vivo and in vitro. Changes in cell membrane permeability and production of reactive oxygen species by individual cells were monitored with fluorescent dyes.**

Results: **Host cells and isolated human neutrophils died rapidly after adhesion to roughened polystyrene. Neutrophils adherent to roughened surfaces produced more reactive oxygen intermediates than those adherent to smooth surfaces and were first to die. The cell death response precipitated by expanded polytetrafluoroethylene, Dacron, or the roughened surfaces was significantly reduced with treatment of the neutrophils with catalase, diphenylene iodonium, or the *src* kinase inhibitor PP2 before adhesion.**

Conclusions: **Neutrophil adhesion to roughened materials triggers rapid production of reactive oxygen species and precipitates a nonapoptotic cell death. Understanding the material properties that trigger these responses is essential to development of the next generation of implantable biomaterials. (J Vasc Surg 2003;37:1082-90.)**

Cardiovascular device-centered infections are devastating complications that occur in 2% to 12% of implanted vascular biomaterial procedures. The cause of vascular prosthetic infection is an elusive and puzzling aspect of biomaterials research. The foreign body, certain bacteria, and abnormal host defenses conspire in some way to make this clinically devastating outcome a continuing menace to patients. A defect in neutrophil bacterial killing was described by Zimmerli et al,[1] but the precise biologic basis of the aberration has only been explained indirectly. We reported that neutrophil adhesion to uncoated or plasma-coated expanded polytetrafluoroethylene (ePTFE)[1] and Dacron, but not to uncoated or protein coated polystyrene surfaces, triggered within hours nonapoptotic cell death in vitro and in vivo.[2] The changes observed included increased membrane permeability and cytoplasmic degeneration in the absence of DNA fragmentation. Since the cell death response induced by biomaterials is distinct from apoptosis, we coined the term "synxenatosis," because the cell death (than*ato, death*) was precipitated by neutrophil attachment (*syn*desi) to a foreign (*xeno*) material.[2] The relative propensity of Dacron and ePTFE to become infected has never been resolved, though most clinicians favor the view that ePTFE is less infection prone. It is not clear whether these differences will be borne out over the long term or in different applications. Clearly neutrophil death is only one of several factors that influence development of clinically relevant infections, not the least of which are the plasma proteins adsorbed, bacterial adherence differences, and bacterial load.

β_2-Integrin receptors mediate neutrophil adhesion to fibrinogen-coated and fetal bovine serum (FBS)-coated surfaces.[3,4] Neutrophil spreading on the coated surfaces and the production of reactive oxygen intermediates (ROI) by adherent cells depend on treatment of the neutrophils with a physiologic soluble agonist such as tumor necrosis factor-α or fMet-Leu-Phe.[5-7] The receptors that mediate neutrophil adhesion to ePTFE, Dacron, glass, or polystyrene are unknown, inasmuch as neither β_2-integrins nor Fc receptor inhibitory antibodies prevent neutrophil adhesion to these uncoated surfaces.[8-10] Neutrophil adhesion to polystyrene or glass surfaces triggers cell spreading, ROI production, and changes in intracellular pH in the absence of exogenous agonists.[10 11] These data imply that certain surfaces trigger activation of signaling pathways in neutrophils that normally are codependent on both integrin receptor occupancy and a physiologic agonist.

Most of our knowledge of how neutrophils die is derived from in vitro studies of suspended neutrophils. Neutrophils have a short half-life of 1 to 2 days.[12] In vitro, suspended neutrophils undergo an aging process associated with changes characteristic of apoptosis, including membrane blebbing, chromatin condensation, and DNA frag-

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Competition of interest: none.

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0741-5214/2003/$30.00 + 0
doi:10.1067/mva.2003.160

mentation.[13-15] The apoptotic death of neutrophils is pivotal for elimination of aging neutrophils and resolution of infection without inflammation. It is less clear whether in vivo all neutrophils removed from circulation die by apoptosis, because both disrupted neutrophils and apoptotic neutrophils are found in old inflammatory lesions.[16] Neutrophil destruction and subsequent total release of its content could provide an efficient bacterial kill mechanism at the expense of self-destruction. Consistent with this possibility, neutrophils challenged with excess *Escherichia coli* (neutrophils-bacteria ratio 1:10) die within hours by a nonapoptotic mechanism.[17] ROI produced by neutrophils stimulated with phorbol ester similarly precipitate a nonapoptotic cell death response.[18,19]

Because neutrophils undergo nonapoptotic cell death after adhesion to ePTFE and Dacron, yet remain viable after adhesion to polystyrene, we considered the possibility that the surface topography of ePTFE and Dacron is a determinant of the cell death response to these materials. Our data are consistent with this possibility and demonstrate that roughening of a polystyrene surface can convert an inoffensive smooth surface into a surface that is toxic to neutrophils both in vivo and in vitro. Using the membrane permeable ROI-sensitive dye dihydrorhodamine 123 (DHR 123; Molecular Probes, Eugene, Ore), we show that cells that produce ROI are first to die. Furthermore, the neutrophil death response to ePTFE, Dacron, and roughened polystyrene was significantly reduced with pretreatment of the neutrophils with diphenylene iodonium (DPI),[20] an inhibitor of the NADPH oxidase complex, and the hydrogen peroxide metabolizing enzyme catalase. Consistent with the role of *src* kinases as upstream mediators of ROI production in neutrophils,[21,22] the nonapoptotic death response was also ameliorated in a dose-dependent manner with pretreatment of the neutrophils with the *src* kinase inhibitor PP2. On the basis of these data, we propose that neutrophil adhesion to roughened surfaces activates a signaling pathway that leads to hydrogen peroxide production, resulting in self-killing. These data suggest that the design of new implantable biomaterials should take into account the effect of biomaterial properties such as surface topography on host cells.

MATERIALS AND METHODS

Neutrophil isolation. Neutrophils were isolated from healthy volunteer donors. Whole blood was collected into EDTA-containing Vacutainer tubes and isolated with single-step neutrophil isolation medium (Cardinal Associates Inc, Santa Fe, NM), as described.[2,23] The neutrophils were resuspended in Hanks' balanced salt solution (HBBS) with Ca^{++} and Mg^{++} (Gibco, Grand Island, NY). The isolated neutrophils were 97% viable.

Tray and surface preparation. Neutrophils were added to 6-well or 24-well flat-bottom polystyrene plates (tissue culture grade). When indicated, neutrophils were adhered to untreated, suspension polystyrene culture dishes. To examine neutrophil responses to ePTFE and Dacron, the materials were custom fitted to line the bottom of 24-well plates. ePTFE and Dacron (kindly provided by Impra Inc, Tempe, Ariz, and Meadox Medicals Inc, Oakland, NJ, respectively) were gas sterilized before use. Polystyrene surfaces were either untreated or manually roughened by scratching the surface with forceps.

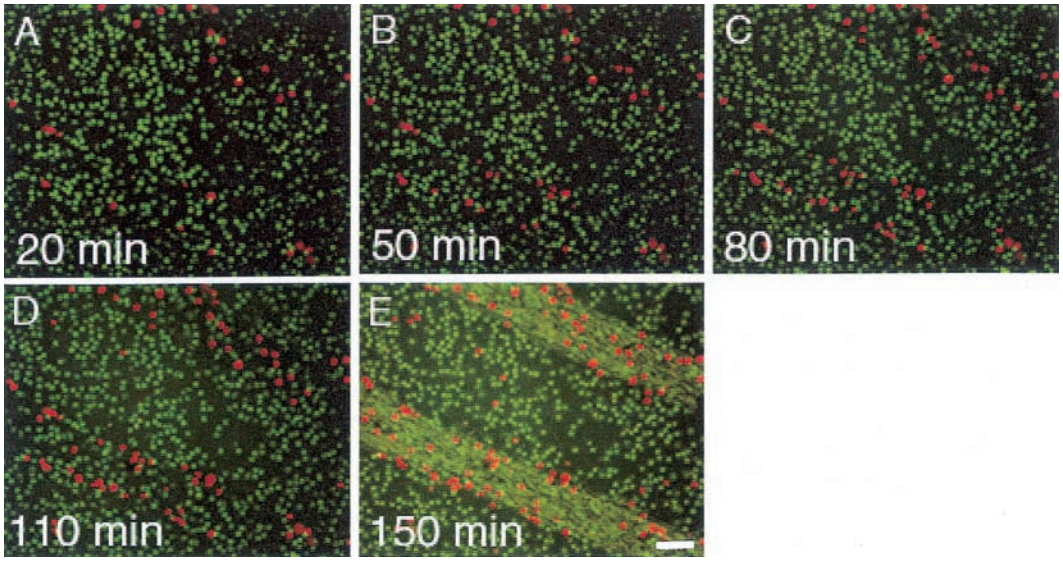
Neutrophil adhesion assays. Neutrophils (2×10^6/mL) were untreated or pretreated for 1 hour with DPI (25 μmol/L; Sigma, Chicago, Ill) or catalase (250 U/mL; Sigma) or for 10 minutes with PP2 (Calbiochem, La Jolla, Calif) at the indicated concentration.[24] The neutrophils were then added to surfaces prepared as described above. Neutrophil viability was examined at 1, 3, and 6 hours after adhesion. Changes in membrane permeability were monitored with the fluorescent dyes propidium iodide (PI; 30 μmol/L) and SYTO 9 (5 μmol/L) (Molecular Probes) as described.[2] Images of green and red fluorescent cells were acquired from three random microscopic fields for each sample. The number of green and red fluorescent cells in each field was determined with the National Institutes of Health image program. The percentage of PI-positive cells was determined for each field as a fraction of the total number of cells per field (usually 1000-2000 cells/field). Data were compiled from three to six independent experiments, with triplicates for each data point. All data are expressed as mean ± SEM of at least three experiments. Data were analyzed with the unpaired Student *t* test on the StatView program (Abacus Concepts, Berkeley, Calif). Results were accepted as significant at $P < .05$.

In vivo studies. Smooth and roughened polystyrene (1 × 1 cm) were implanted in subcutaneous pouches made in the back of three adult Sprague-Dawley rats, as described.[2,25,26] The materials were retrieved at 6 hours, inverted, and placed individually in polystyrene wells containing HBSS and the fluorescent dyes PI and SYTO 9. Images of fluorescent cells were acquired immediately.

ROI production and cell viability. To examine the temporal correlation between ROI production and increase in cell membrane permeability, neutrophils were loaded for 15 minutes with DHR 123 (2 μmol/L) before neutrophil adhesion to smooth and roughened surfaces.[27] SYTOX (1 μmol/L) (Molecular Probes), a membrane impermeable green fluorescing nucleic acid dye, was used in combination with DHR 123 to monitor changes in neutrophil membrane permeability.

RESULTS

Neutrophils adherent to roughened polystyrene surfaces either in vivo or in vitro exhibit a time-dependent decrease in cell viability. Dacron, ePTFE, and polystyrene differ in chemistry and surface topography. Whereas neutrophils adherent to ePTFE and Dacron undergo rapid, nonapoptotic death both in vivo and in vitro, neutrophils adherent to a polystyrene surface remain viable.[2] To ascertain whether surface topography is a determinant of the cell death response, polystyrene surfaces were roughened with manual scratching. Time-dependent changes in neutrophil viability after adhesion to roughened as compared with smooth areas of the polystyrene surface were monitored in



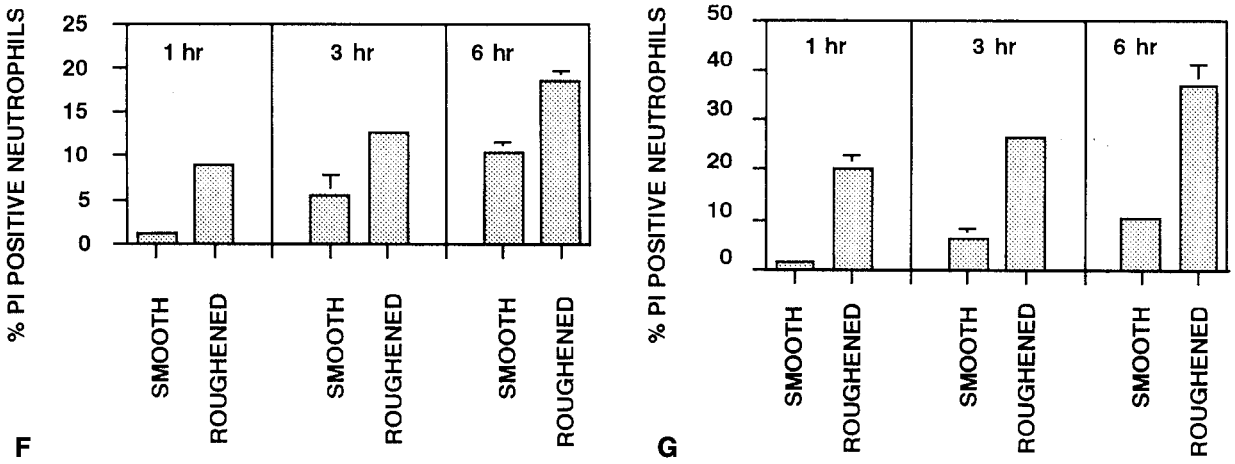


Fig 1. A-E, Neutrophil adhesion to roughened polystyrene triggers time-dependent changes in cell membrane permeability. Neutrophils were plated onto a roughened polystyrene surface in HBSS containing SYTO 9 and PI. Images were acquired within 20 minutes of adhesion at 30-minute intervals. Increase in neutrophil membrane permeability to PI was predominantly seen along the edges and within the boundaries of the roughened regions. Green lines in **E** reflect scratched area. This view was obtained by turning on bright field illumination while taking the fluorescent image. *Bar,* 40 μm. **F,** Percent of PI-positive neutrophils in random microscopic fields relative to total adherent neutrophil population. **G,** Percent of PI-positive neutrophils adherent to roughened compared with smooth surface areas. Data in **F** and **G** were obtained by adding PI and SYTO 9 to neutrophils plated onto roughened surfaces for 1, 3, or 6 hours.

vitro over 6 hours with the fluorescent vital dye combination PI and SYTO 9 (Fig 1). PI is a membrane-impermeable red fluorescing nucleic acid dye. SYTO 9 and PI compete for nucleic acid binding sites when both are present in damaged cells. Hence live cells fluoresce green, whereas dead cells fluoresce red. The percentage of PI-positive neutrophils adherent to roughened polystyrene surfaces reached 18.5% ± 1% (n = 6) by 6 hours (Fig 1, *F*). Inasmuch as the roughened areas represent a fraction of the total surface area of the well, we also evaluated the percentage of PI-positive cells relative to the neutrophil population within roughened areas. By 6 hours, 36.9% ± 4% (n = 6) of neutrophils adherent to the roughened areas were PI-positive, compared with 10.3% ± 1% (n = 6) of neutrophils adherent to smooth surfaces (Fig 1, *G*). At each time examined, more than 75% (n = 6) of PI-positive neutrophils were adherent to and localized within roughened areas of the surface. In one set of experiments, roughening of the polystyrene surfaces was accomplished with polishing with an SiC 220-grit paper, as described.[28] By 6 hours,

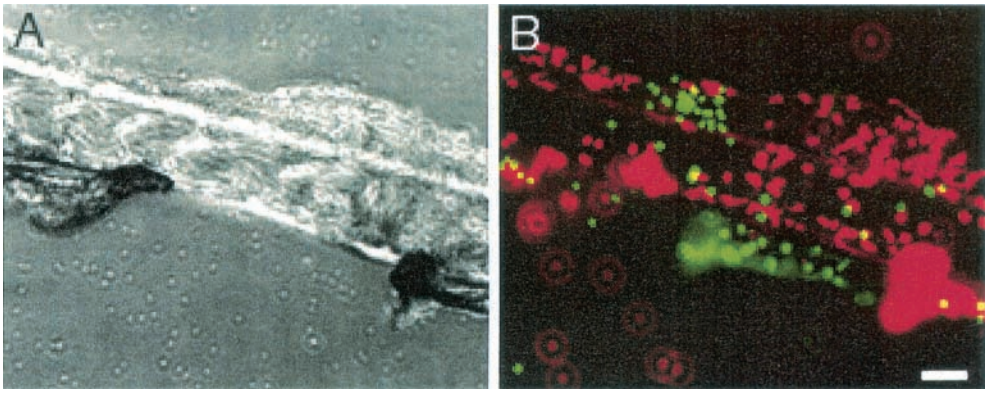


Fig 2. Host cells adherent to implanted roughened polystyrene surfaces exhibit time-dependent increase in cell membrane permeability to PI. Smooth and roughened polystyrene pieces were implanted in subcutaneous pouches made on the backs of rats. Materials were retrieved at 6 hours and stained with PI and SYTO 9. Adherent cells were examined with phase optics **(A)** and fluorescence optics **(B)**. Multiple PI-positive host cells populated roughened but not adjacent smooth areas of the implanted surface. *Bar,* 30 μm.

74.8% ± 4% of the neutrophils adherent to the polished surface were PI-positive (data not shown). Severe optical distortion caused by the polishing procedure prevented use of similarly treated surfaces in subsequent studies. An increase in cell membrane permeability is a reliable indicator of a nonapoptotic or necrotic cell death. Other markers previously used to define apoptosis that include phosphatidylserine exposure or DNA fragmentation were noted in studies in which cells died by either necrosis or apoptosis.[29,30] Nadzam et al[2] reported that the rapid increase in cell membrane permeability to PI, noted after adherence of neutrophils to ePTFE or Dacron, was accompanied by a twofold to threefold increase in lactate dehydrogenase release. The change in cell membrane permeability occurred in the absence of DNA fragmentation. Building on this body of data, we conclude that neutrophil adhesion to roughened polystyrene triggers a nonapoptotic cell death.

We next examined the effects of roughened polystyrene surfaces on host cell viability in vivo. For these studies, 1 × 1 cm pieces of smooth polystyrene and polystyrene roughened with manual scratching were implanted into subcutaneous pouches made in the backs of three rats. The materials were retrieved and stained with SYTO 9 and PI. Numerous PI-positive adherent host cells were preferentially localized along the edges and within roughened regions of the surface (Fig 2). Many of these cells were identified as neutrophils on the basis of multilobular organization of their nuclei seen at higher magnification (data not shown). These data indicated that attachment of neutrophils to roughened surfaces either in vivo or in vitro resulted in rapid increase in cell membrane permeability.

Hydrogen peroxide produced by neutrophils adherent to roughened surfaces affects neutrophil viability. Tsan[18] and Takei et al[19] reported that neutrophils exposed to phorbol ester die rapidly by a nonapoptotic mechanism. The effect of phorbol ester on neutrophil viability was inhibited by catalase, a hydrogen peroxide metabolizing enzyme, but not by superoxide dismutase. To ascertain whether the material-induced death response was linked to ROI production in general, and to hydrogen peroxide in particular, neutrophils were treated with (DPI), an inhibitor of NADPH oxidase,[20] or with catalase before exposure to surfaces. By 6 hours, pretreatment of neutrophils with DPI reduced the number of PI-positive neutrophils adherent to ePTFE, Dacron, or the roughened polystyrene surface, respectively, by 50%, 62%, and 37%, as compared with untreated control neutrophils (Fig 3). Catalase reduced the number of PI-positive neutrophils adherent to ePTFE, Dacron, or the roughened polystyrene surface, respectively, by 65%, 66%, and 75%. These data suggest that hydrogen peroxide released by neutrophils adherent to roughened surfaces is linked to the nonapoptotic death response. It is noteworthy that the antioxidant *N*-acetyl-L-cysteine and the inhibitor of the mitochondrial mega channel cyclosporin A similarly rescued ePTFE-adherent and Dacron-adherent neutrophils from death,[2] underscoring the role of ROI in cell death precipitated by either ePTFE, Dacron, or roughened polystyrene surface.

Neutrophils adherent to roughened surfaces produce more ROI than neighboring cells do, and they are first to die. We next examined the temporal and spatial correlation between the appearance of membrane-permeable neutrophils and ROI production by adherent neutrophils. For these studies, neutrophils were loaded with DHR 123 for 10 minutes and then adhered to roughened polystyrene in the presence of the green fluorescent vital dye SYTOX (Fig 4). We found that staining of ePTFE-adherent and Dacron-adherent neutrophils with either PI or SYTOX yielded indistinguishable results (data not shown). DHR 123, a cell-permeant nonfluorescent substrate, is converted by oxidation into the brightly fluorescent lipophilic cation Rhd 123.[27] Jankowski and Grinstein[27] demonstrated that the increase in Rhd 123 fluorescence in activated neutrophils depends on activation of NADPH oxidase.[27] Neutro-

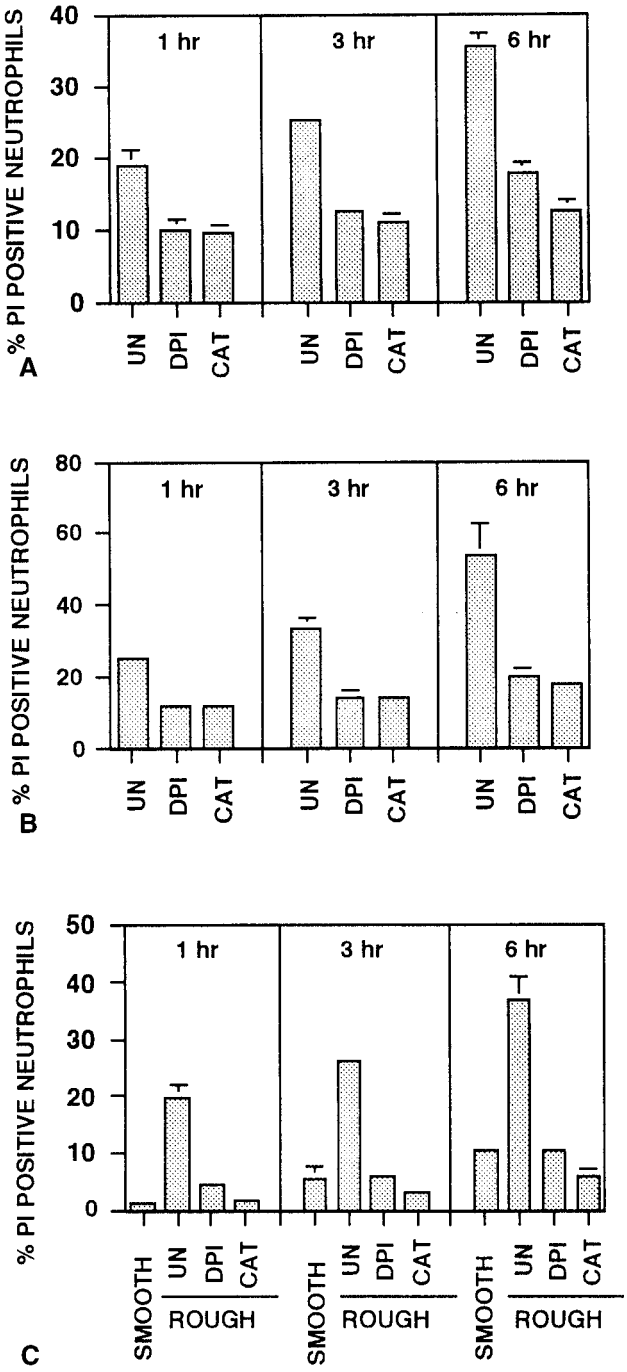


Fig 3. Effect of catalase and DPI on adherent neutrophil viability. Neutrophils were either untreated *(UN)* or pretreated for 10 minutes with catalase (*CAT;* 250 U/mL) or for 1 hour with DPI (25 μmol/L) before plating onto ePTFE **(A)**, Dacron **(B)**, and smooth or roughened polystyrene **(C)**. Shown is percent of PI-positive neutrophils adherent to surfaces for 1, 3, and 6 hours. Data represent mean ± SEM of six experiments.

phils were monitored within 10 minutes of adhesion, and images were obtained at 10-minute intervals over 2 hours. The images revealed that neutrophils adherent to roughened surfaces produce more ROI than neutrophils adherent to adjacent smooth areas of the surface. Furthermore, a direct correlation was observed between ROI production by individual cells and the change in cell membrane permeability to SYTOX. By 100 minutes, 45% to 50% of the

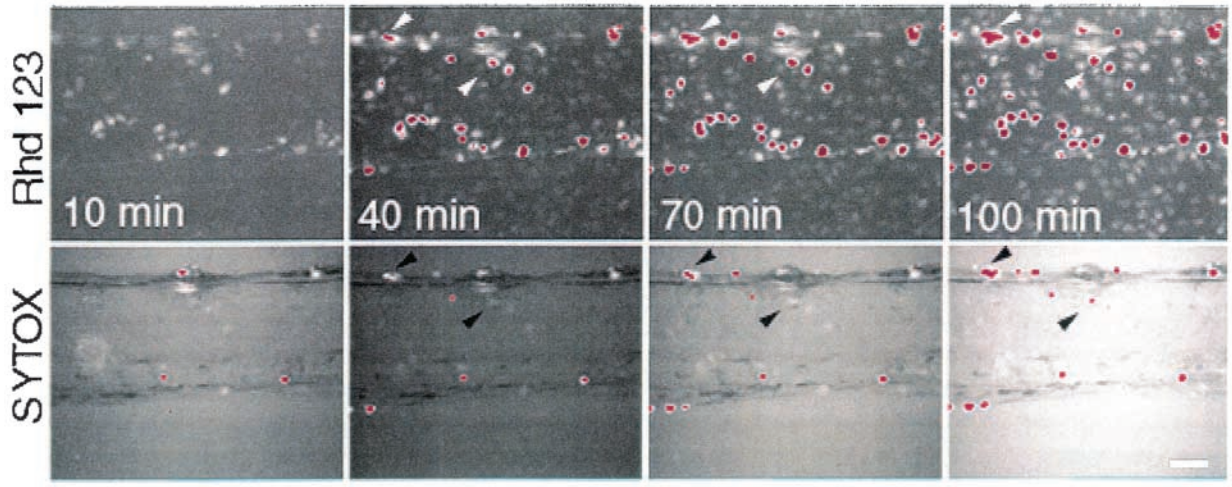


Fig 4. Neutrophils adherent to roughened surfaces produce more ROI than neutrophils adherent to smooth surfaces and are first to die. Neutrophils loaded with DHR 123 were plated onto roughened polystyrene surfaces in the presence of the green fluorescing vital dye SYTOX. Images were acquired over 120 minutes. Black and white images were processed with IPLab Scientific Imaging software. Images display saturated pixels, with the highest fluorescence values pseudocolored red. Appearance of bright Rhd 123 fluorescing cells was restricted to roughened areas of surface. *Arrowheads,* two representative cells in which ROI production clearly preceded increase in cell membrane permeability to SYTOX. *Bar,* 30 μm.

neutrophils adherent to roughened areas of the surface were Rhd 123-positive. In the same fields, 12 ± 0.3 of the neutrophils were PI-positive; more than 90% of PI-positive neutrophils were Rhd 123-positive. Together with the data obtained with DPI and catalase, these results suggest that ROI produced by cells adherent to the roughened surface contribute to cell death, change in cell membrane permeability to PI is preceded by ROI production, and ROI-induced cell death is self-inflicted.

An inhibitor of *src* kinase family members substantially ameliorates the effect of roughened surfaces on neutrophil viability. *src* kinase family members mediate integrin-dependent ROI production and neutrophil spreading.[21,22] To ascertain whether *src* kinase family members regulate the nonapoptotic death response of neutrophils to roughened surfaces, neutrophils were pretreated with PP2, an inhibitor of *src* kinase family members.[24] Pretreatment of neutrophils with PP2 reduced the number of PI-positive neutrophils adherent to ePTFE and Dacron for 6 hours, respectively, by 61% and 55% (Fig 5). PP2 also reduced the percentage of PI-positive neutrophils adherent to roughened polystyrene by 67%. The effect of PP2 on neutrophil viability was dose-dependent with an inhibitory concentration of 50% (IC_{50}) of about 5 μmol/L (Fig 5, *D*). This value is consistent with the PP2 dose necessary to inhibit T-cell tyrosine phosphorylation and proliferation.[24] These data indicate that neutrophil adhesion to roughened materials (ePTFE, Dacron, roughened polystyrene) triggers activation of a common signaling pathway that involves *src* kinase family members and that these kinases regulate the neutrophil nonapoptotic death response to roughened materials.

DISCUSSION

Prostheses made of ePTFE or Dacron have widespread clinical use. Although inert to many cell types, particularly in the absence of serum proteins, both materials are highly conducive to neutrophil and monocyte or macrophage adhesion and activation. Nadzam et al[2] uncovered a previously unappreciated lethal effect of ePTFE and Dacron on host neutrophils. Our data suggest that surface topography is an important determinant of the neutrophil nonapoptotic death response to biomaterials involving hydrogen peroxide production. The increase in neutrophil cell membrane permeability to PI after adhesion to roughened surfaces was restricted to areas of the surface that were rough. Although neutrophils adhered equally well to smooth and roughened areas of the surface, 75% of all dead cells were localized within the roughened areas of the surface. Earlier studies alluded to the possibility that roughened surfaces may trigger aberrant host responses. Salthouse[31] noted that, although implanted abraded Teflon and Dacron contained numerous macrophages and foreign body giant cells, few cells were found on a smooth polypropylene surface even after 1 month of implantation. To our knowledge, however, our data are the first to suggest that topographic features on the micron scale may determine neutrophil fate and activate a nonapoptotic cell death response.

Three lines of evidence indicate that ROI in general and hydrogen peroxide in particular are important mediators of the neutrophil nonapoptotic death response. First, catalase and DPI effectively inhibit the nonapoptotic death response of neutrophils to roughened materials. Second,

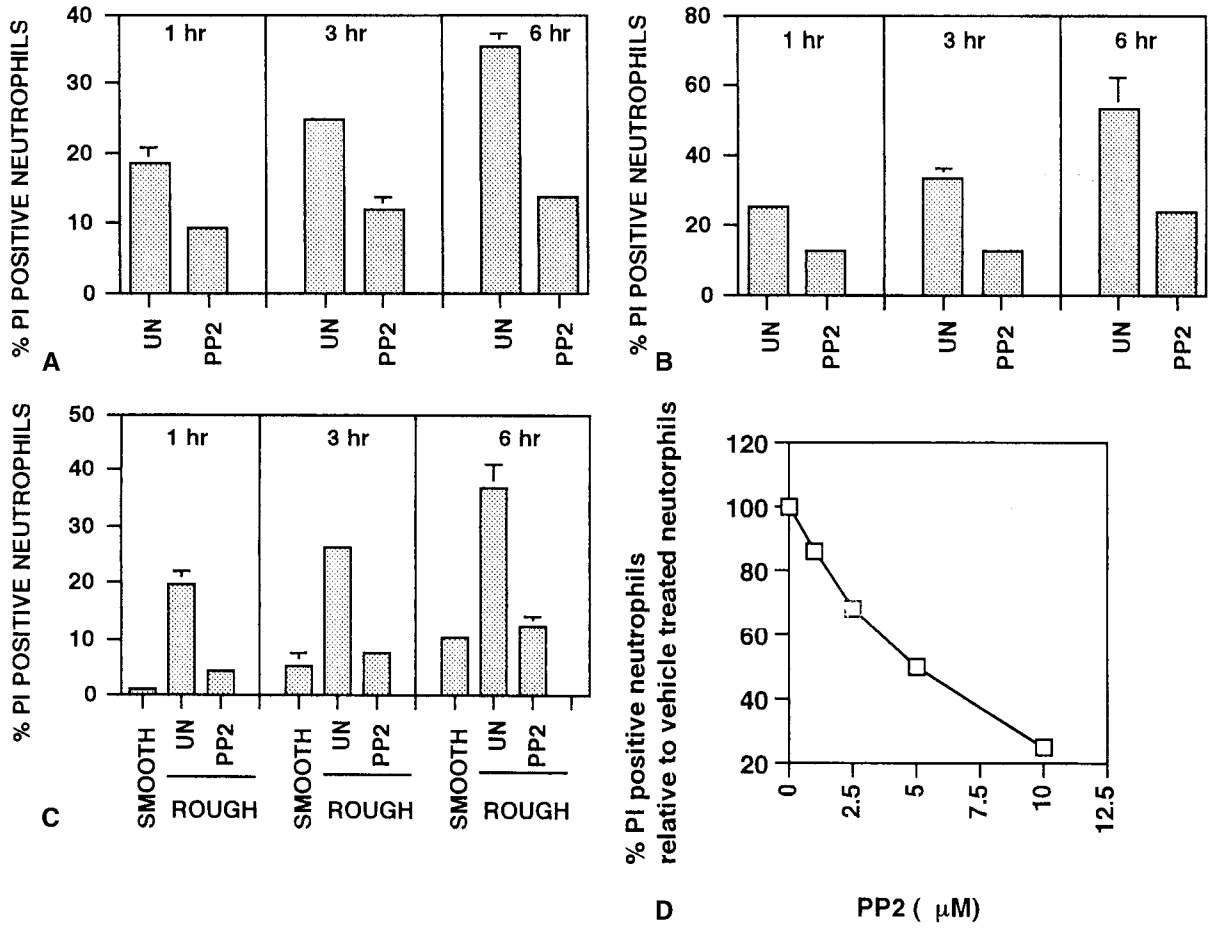


Fig 5. Effect of PP2 on adherent neutrophil viability. Neutrophils were either untreated *(UN)* or pretreated for 10 minutes with PP2 (10 μmol/L) before plating onto ePTFE **(A)**, Dacron **(B)**, and smooth or roughened polystyrene **(C)**. Shown is percent of PI-positive neutrophils adherent to surfaces for 1, 3, and 6 hours. Data represent mean ± SD of 3 experiments. In **D**, neutrophils were incubated with indicated concentration of PP2 or dimethylsulfoxide vehicle before plating onto roughened polystyrene surfaces. Data are expressed as percent of PI-positive neutrophils at 6 hours relative to dimethylsulfoxide-treated neutrophils.

neutrophils adherent to roughened areas produce more ROI than neighboring neutrophils that remain viable. Third, the increase in cell membrane permeability to vital dyes is preceded by an increase in ROI production by the very same cells. *src* kinase family members regulate the production of ROI in neutrophils.[21,22,32-34] The ability of PP2, an inhibitor of *src* kinases, to reverse the lethal effect of the materials on neutrophil viability not only supports the notion that ROI are closely linked to the cell death response triggered by the materials but also highlights the possibility that the cell death response is not an accident but reflects activation of a signaling pathway, or a nonapoptotic cell death program. The coexistence of an apoptotic and a nonapoptotic cell death program in cells is rapidly becoming an accepted concept built on solid experimental foundations (for recent review see Leist and Jaattela[35] and Denecker et al[36]). While the participants in the nonapoptotic cell death program remain to be determined, inactivation or downregulation of caspases by ROI may be a common theme.[37] Indeed, inhibition of caspases by ROI produced by activated neutrophils was reported by Fedeel et al,[38] leading these investigators to propose that "neutrophils possess two different modes of cell death." Further studies are necessary to determine the initial signaling event activated by the roughened materials that leads to the nonapoptotic death response of adherent neutrophils.

While the biologic responses documented in this study are likely to apply to some materials that are as different from each other as ePTFE, Dacron, and polystyrene, caution should be practiced in applying the same principles to all materials. A study by Eriksson et al[39] highlights this point. These authors observed that neutrophils adherent to a titanium surface, whether smooth or rough, failed to produce ROI spontaneously and remained viable. In con-

trast, we and others have shown that neutrophils adherent to ePTFE, Dacron, or glass mount a substential burst of ROI production.[8,11,23] The reason neutrophils are activated by materials such ePTFE and Dacron but not by titanium is not clear. It is also important to note that a nonapoptotic death response triggered by materials is not limited to phagocytes. Ertel et al[40] reported that several cell types, including bovine endothelial cells, NIH 3T3 cells, and mouse peritoneal macrophages, died rapidly, as demonstrated by increased membrane permeability to trypan blue, after adhesion to poly(dimethyl siloxane), polyethylene, or poly(methyl metacrylate). Serum proteins, albumin, and immunoglobulin efficiently masked the toxic effect of the materials. The authors proposed that the cell death response reflected an intrinsic physical toxic property of certain materials. The reason why these particular materials are toxic to cells remained unanswered. Shive et al[41] reported that neutrophils adherent to a polyetherurethane urea film undergo a rapid apoptotic death when exposed to physiologically relevant shear stress levels in vitro. It is thus possible that, in addition to the nonapoptotic cell death response we identified, the ability of neutrophils to phagocytose bacteria in the graft milieu is also compromised by a shear-dependent apoptotic death response on the luminal surface of grafts. In a prospective clinical study, Olofsson et al[42] recovered and analyzed 32 infected and 29 uninfected prosthetic vascular grafts. Bacteria were always localized on the outside and never on the luminal surface of the grafts.[42] These data suggest that the encounter between bacteria and phagocytes likely occurs on the outer surface of the graft in a static environment.

One of the challenges we now face is to create materials that will not interfere with the host ability to kill bacteria. Better understanding of material properties that are toxic and those that are permissive to host cells is essential for development of the next generation of implantable biomaterials.

This study was generously funded through a Focused Giving Award from Johnson & Johnson (RGS, BH) and a grant from the American Heart Association (BH, an Established Investigator of the American Heart Association).

We thank Drs Samuel C. Silverstein and Mike Jaffe for helpful discussions; Impra Inc for providing ePTFE; and Meadox Inc for providing Dacron.

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Submitted, May 23, 2002; accepted Sep 19, 2002.

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