Absence of Lipopolysaccharide-Induced Inhibition of Neutrophil Apoptosis in Patients With Diabetes

Steven D. Tennenberg, MD; Raphaela Finkenauer; Amit Dwivedi, MD

Background: Patients with diabetes mellitus are considered to be at increased risk for infections and often experience more serious and prolonged infections. Defects in neutrophil microbicidal function have been identified in diabetic patients and are believed to contribute to this problem. Neutrophil apoptosis or programmed cell death regulates functional longevity, and is an integral component of inflammation and its resolution. It remains unknown whether neutrophils from diabetic patients demonstrate defects in apoptosis.

Hypothesis: Neutrophils from diabetic patients have defects in spontaneous and/or endotoxin (lipopolysaccharide [LPS])–induced apoptosis.

Design: Peripheral venous blood samples were collected from healthy volunteers and insulin-dependent diabetic patients to harvest neutrophils for in vitro study.

Interventions and Main Outcome Measures: Neutrophils were cultured for 0 and 24 hours in the absence and presence of LPS. Normal neutrophils were also cultured under conditions of normal or high glucose concentration. Percentage of apoptotic cells was determined by flow cytometry.

Results: Normal neutrophils undergo significant apoptosis after 24 hours, and the percentage of apoptotic cells is reduced in the presence of LPS. Diabetic neutrophils undergo normal spontaneous apoptosis, but do not demonstrate LPS-induced inhibition of apoptosis. The LPS-induced inhibition of apoptosis in normal neutrophils is prevented under high-glucose conditions.

Conclusions: Neutrophils from diabetic patients demonstrated defects in LPS-induced apoptosis. High-glucose environment may mediate these findings. The inability of diabetic neutrophils to reduce apoptosis following LPS exposure resulted in relatively increased apoptosis. This would cause decreased functional longevity of neutrophils and increased neutrophil clearance from infectious sites, possibly contributing to the increased susceptibility and severity of infections in diabetic patients.

Arch Surg. 1999;134:1229-1234

Infections and infectious complications are well described in patients with diabetes mellitus and represent a serious clinical problem. Clinical data and experience suggest that patients with diabetes mellitus are at increased risk for infections and often experience more serious and prolonged infections.1-3 Numerous in vitro studies4-6 in diabetic patients have documented varied defects in neutrophil microbicidal function, which are believed to be contributory. These defects include decreases in neutrophil adherence, chemotaxis, phagocytosis, bactericidal activity, and oxygen radical production.

Neutrophil apoptosis, or programmed cell death, is a physiologic process whereby neutrophils are rendered senescent and are removed or cleared from sites of inflammation by macrophages. Neutrophil apoptosis is believed to regulate neutrophil functional longevity and is therefore, now considered an integral component of inflammation and its resolution.7,8 Although considerable research has explored neutrophil apoptosis and the effects of inflammatory mediators on apoptosis in normal neutrophils, it remains unknown whether neutrophils from diabetic patients demonstrate defects in apoptosis.

We hypothesized that diabetic neutrophils have defects in their apoptotic responses, and speculated that the resulting alterations in neutrophil functional longevity may contribute to the increased susceptibility and severity of infections in diabetic patients. We specifically sought to determine whether neutrophils from diabetic patients differ from normal neutrophils in terms of spontaneous apoptosis over time and in their apoptotic response to endotoxin (lipopolysaccharide [LPS]). In addition, we examined whether any identified defects could be attributed to the increased glucose concentration that diabetic neutrophils are exposed to in vivo.
SUBJECTS AND METHODS

SUBJECTS

Healthy subjects consisted of volunteers who denied having a history of diabetes and were taking no significant medications. Diabetic subjects were patients with insulin-dependent diabetes who presented as outpatients to clinics at the John D. Dingell (Detroit) Veterans Affairs Medical Center, Detroit, Mich. The subjects were free of active infections by history and had a recent documented glycosylated hemoglobin level of 7% to 12%. All subjects provided informed consent, and the study was approved by the Human Research Committee of Wayne State University School of Medicine, Detroit.

NEUTROPHIL ISOLATION

Peripheral venous blood was obtained by routine venipuncture and collected into the anticoagulant ethylenediamine tetraacetate. Neutrophils were isolated as previously described. Briefly, this involved dextran sedimentation, Ficoll-sodium diatrizoate (Histopaque 1077; Sigma-Aldrich Corp, St Louis, Mo), density gradient centrifugation, and brief hypotonic lysis of contaminating red blood cells. Neutrophil preparations were 96% or more pure by Wright-Giemsa staining and 98% or more viable by trypan blue exclusion. Mononuclear cells were obtained from the mononuclear band, resulting from density gradient centrifugation. After washing, they were added back to the purified neutrophil suspension at a ratio of 5% where specified.

NEUTROPHIL APOPTOSIS

Neutrophil apoptosis was measured using 2 flow cytometric assays. The first assay, designated HT, used dual-laser flow cytometry with the vital DNA-binding bisbenzimidazole dye Hoechst 33342 (HT; Sigma-Aldrich Corp) and the DNA-intercalating dye propidium iodide (PI; Sigma-Aldrich Corp). After experimentation, HT was added at a concentration of 1 µg/mL and incubated for 10 minutes at 37°C. Neutrophils were then cooled to 4°C, centrifuged, and resuspended in cold phosphate-buffered saline. The PI, 5 µg/mL, was added immediately before flow cytometric analysis. Samples were analyzed on a FACS Vantage flow cytometer equipped with a CONSORT32 workstation running LYSIS II software (all from Becton Dickinson Immunocytometry Systems [BDIS], San Jose, Calif). The PI was excited with 40 mW of 488-nm light from an argon ion laser (ILT 5000A; Ion Laser Technology, Salt Lake City, Utah), which also produced forward and side scatter signals. The HT was excited with 50 mW of all-lines UV light (351-365 nm) from an argon ion laser (Innova 90-5; Coherent, Santa Clara, Calif), which was spatially separated from the ILT laser. The PI and HT fluorescences were collected with 585/42- and 460/40-nm bandpass filters, respectively. A total of 20 000 events of list mode data were saved and analyzed with Becton Dickinson software (BDIS) run on a Power Macintosh computer (Apple Computer, Cupertino, Calif). The flow cytometer was aligned daily with SPHERO Multiparameter Fluorophore Particles (pink and yellow for PI and UV and light yellow for HT) and quality controlled with SPHERO Rainbow Calibration Particles (Spherotech, Libertyville, Ill).

The HT assay recognizes 4 distinct cell populations, based on the presence of low or high fluorescence of both HT and PI. Normal neutrophils demonstrate low fluorescence for both, and necrotic neutrophils show high fluorescence for both. Apoptotic neutrophils can be classified as either early phase (high HT, low PI) or late phase (low HT, high PI). For the purpose of this study, early- and late-phase apoptotic populations were combined as the total apoptotic population.

The second assay, designated TUNEL (TdT–mediated dUTP nick end labeling), identifies DNA fragmentation, a characteristic of apoptosis. Data from the initial set of experiments using the HT assay (healthy and diabetic subjects) are presented in Figures 1, 2, and 3. In the absence of LPS, normal neutrophils undergo marked spontaneous apoptosis by 24 hours (Figure 1). However, in the presence of LPS, there is a statistically significant relative decrease (25%) in the percentage of apoptotic cells at 24 hours (65.6% ± 3.6% vs 49.2% ± 3.1% for normal and without LPS vs normal and with LPS, respectively). The apoptotic response of normal neutrophils in the absence of LPS is unchanged in the presence of a high-glucose (25 mmol/L) environment. However, the finding that a high-glucose environment completely prevented the decreased apoptosis observed in the presence of LPS is notable.

Diabetic neutrophils attain similar levels of spontaneous apoptosis at 24 hours compared with normal neutrophils in the absence of LPS (Figures 2 and 3). Most notable is that LPS did not decrease the apoptotic response of diabetic neutrophils at 24 hours (54.6% ± 5.5% vs 57.7% ± 6.8% for diabetic and with LPS, respectively). This is in contrast to the response of normal neutrophils, which demonstrated LPS-induced inhibition of apoptosis (Figure 3). Diabetic neutrophils following LPS exposure demonstrated significantly increased apoptosis at 24 hours (17% relative increase) compared with LPS-exposed normal neutrophils (57.7% ± 6.8% vs 49.2% ± 3.1% for diabetic and with LPS vs normal and with LPS, respectively).

Data from the final set of experiments using the TUNEL assay (additional healthy and diabetic subjects) are presented in their entirety in the Table. Although the overall percentages of apoptotic cells at 24 hours are slightly decreased with this assay compared with the HT assay, the identical findings and relationships between groups are seen. The significance of the higher percentages of apoptotic neutrophils in diabetic patients compared with healthy patients at 0 hour, as seen with the TUNEL assay compared with the HT assay, is not known.

COMMENT

Neutrophil apoptosis appears to be an important mechanism that regulates both the functional longevity of neutrophils and thereby the clearance of neutrophils from...
hallmark of the apoptotic process. A commercially available flow cytometric kit was used (In Situ Cell Death Detection Kit, Fluorescein; Boehringer Mannheim, Indianapolis, Ind). This assay allows labeling of apoptosis-induced DNA strand breaks with dUTP, facilitated by the enzyme TdT. After experimentation, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and the TUNEL reaction mixture was added for 1 hour at 37°C. Samples were washed and analyzed on a FACScan flow cytometer equipped with the same software as described herein. Fluorescence excitation and light scattering were achieved with an argon ion laser tuned to 15 mW at 488 nm. Fluorescein isothiocyanate fluorescence was collected in the FL1 detector using a 530/30-nm band-pass filter. A total of 20,000 events of list mode data were saved and analyzed with PC-LYSIS software (BDIS) run on a 4DX2-66V computer (Gateway 2000, Sioux City, SD). Routine quality control of the FACScan was performed using AutoCOMP software and CaliBRITE beads (BDIS). Normal cells demonstrate a uniform peak of low fluorescence intensity, and apoptotic cells show a shift to a high-fluorescent peak.

**EXPERIMENTAL PROTOCOL**

Neutrophils were suspended in minimum essential medium (Sigma-Aldrich Corp) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), penicillin sodium, streptomycin sulfate, and glutamine. Experiments were performed with all variables (ie, with and without LPS, with and without high glucose concentrations) simultaneously. High-glucose environment was achieved by raising the media glucose concentration from its baseline of 5.5 mmol/L (100 mg/dL) to 25 mmol/L (corresponding to a glucose concentration of 5.5 mmol/L [100 mg/dL] to 25 mmol/L (corresponding to a glucose concentration of 25.0 mmol/L [450 mg/dL]). Glucose, 25 mmol/L, is a standard in vitro concentration used to simulate diabetic conditions. Cell suspensions were added into multiwell tissue culture plates and maintained in a humidified 5% carbon dioxide incubator at 37°C for the designated incubation period (30 minutes for 0 hour samples and 24 hours). At the end of the incubation period, neutrophils were thoroughly aspirated from the wells and prepared for flow cytometry analysis of apoptosis as outlined herein.

In the initial set of experiments, protocol details included the addition of 5% mononuclear cells to the neutrophil suspension and the use of LPS (Escherichia coli 0111: B4; Sigma-Aldrich Corp) at a concentration of 10 µg/mL. Mononuclear cells were added to better simulate in vivo conditions and to optimize the LPS effect via LPS-induced monocyte stimulation and cytokine production. The LPS concentration was based on dose-response curves under these conditions, which demonstrated a maximal effect at 10 µg/mL. Neutrophil incubations were in 24-well plates at a cell concentration of 1 × 10⁶ cells per milliliter. Neutrophil apoptosis was measured in duplicate with the HT assay.

In the final set of experiments, additional healthy and diabetic subjects were studied. Experimental protocol details included the absence of 5% mononuclear cells in the neutrophil suspension (to eliminate any monocyte contribution to the effect) and an LPS concentration of 1 µg/mL (optimal in dose-response curves under these conditions). Neutrophil incubations were in 96-well plates at a cell concentration of 1 × 10⁶ cells per milliliter. Neutrophil apoptosis was measured with the TUNEL assay. Duplicate samples were not routinely run, since there was minimal variability with this assay.

**STATISTICAL ANALYSIS**

Data are expressed as mean ± SEM. Comparison between group means was by mixed-design analysis of variance (2 × [2]) (group × [treatment]), and data were analyzed using an SPSS statistical package, version 8.0 (SPSS Inc, Chicago, Ill). P < .05 was accepted as statistically significant.
We chose to study the apoptotic responses of circulating neutrophils, since they are readily available and serve as the precursor of exudative neutrophils. The processes of endothelial transmigration and exudation have been shown to markedly decrease neutrophil apoptosis in healthy patients. However, this has not, to our knowledge, been shown to markedly decrease neutrophil apoptosis in healthy patients. The LPS-induced inhibition of apoptosis in normal neutrophils results in relatively increased percentages of apoptotic cells, with resulting decreased functional longevity. This would promote decreased microbicidal function.

The possible significance of our findings is that the absence of LPS-induced inhibition of neutrophil apoptosis in diabetes may be another cause of the increased susceptibility and severity of infections in diabetic patients. The LPS-induced inhibition of apoptosis in normal neutrophils results in fewer apoptotic cells and therefore more nonapoptotic neutrophils, with resulting increased functional longevity. This would promote increased microbicidal function. Conversely, the absence of LPS-induced inhibition of apoptosis in diabetic neutrophils results in relatively increased percentages of apoptotic cells, with resulting decreased functional longevity and relatively fewer nonapoptotic cells. This would promote increased microbicidal function.

In addition, our data suggest that a high-glucose environment may mediate the apoptotic findings in diabetic patients. This theory might be supported by the clinical finding that optimal glycemic control in diabetic patients appears to decrease infectious susceptibility. We thank Stephen Lerman, PhD, Eric Van Buren, and Evano Piasentin, of the Flow Cytometry Facility, Department of Immunology and Microbiology, Wayne State University School of Medicine. Statistical assistance by Bradley Axelrod, PhD, Psychology Service, John D. Dingell (Detroit) Veterans Affairs Medical Center, is gratefully appreciated.

Patients with diabetes mellitus are generally considered to be at increased risk for infections and often experience more serious and prolonged infections. Defects in neutrophil microbicidal function have been identified in diabetic patients and are believed to contribute to this problem. Neutrophil apoptosis, or programmed cell death, regulates neutrophil functional longevity, and is an integral component of inflammation and its resolution. We found that diabetic neutrophils undergo normal spontaneous apoptosis but do not demonstrate lipopolysaccharide-induced inhibition of apoptosis. This latter finding may promote decreased functional longevity and increased clearance of neutrophils from infectious sites, and contribute to the increased susceptibility and severity of infections in diabetic patients. Optimal glycemic control (as suggested by our data) and pharmacologic methods to restore normal neutrophil apoptosis may prove beneficial to diabetic patients.

Reprints: Steven D. Tennenberg, MD, Department of Surgery (112), John D. Dingell (Detroit) Veterans Affairs Medical Center, 4646 John R, Detroit, MI 48201 (e-mail: tennenberg.steven@allen-park.va.gov).

REFERENCES


DISCUSSION

Jan Horn, MD, San Francisco, Calif: This was a fine presentation and the manuscript is concise and well written. I commend you for your efforts to elaborate on the clinical status of neutrophil apoptosis in diabetic patients. This study documents, with 2 alternative assay methods, that 24-hour spontaneous apoptosis is normal in diabetic and normal individuals; however, diabetic neutrophils fail to respond to LPS in vitro with suppression of apoptosis. Furthermore, normal neutrophils can be rendered “diabetic” by exposure to higher glucose levels.

It is clear that diabetic neutrophils are intrinsically different from normal neutrophils; however, before we accept the concept that this constitutes an immunologic defect, I believe that further studies will be required. Bear in mind that numerous pro- and anti-apoptotic mediators may coexist in vivo. For example, TNF-α and C5a promote neutrophil apoptosis, whereas GM-CSF and leukotriene B4 suppress neutrophil apoptosis.

I have 2 issues that I would like the authors to clarify. First, what were the glucose levels at the time of neutrophil sampling? Were these neutrophils in the diabetic individuals actually exposed to high glucose concentrations?

Second, how do you explain the higher baseline levels of apoptosis observed in diabetic patients assayed by the TUNEL method? You didn't show the baseline levels here to this audience, but it was in your manuscript. You described their levels of 12% to 14%, which seem relatively high to me. Does this finding imply that the clearance of apoptotic neutrophils is sluggish in diabetic patients? Please clarify this disparity with the 2 methods that you have employed.

In summary, I believe that studies like this will be important to help us clarify the nature of neutrophil dysfunction in immunocompromised patients, and I encourage you to continue with such work.

Dr Tennenberg: Regarding the glucose levels of diabetic patients, we did not perform simultaneous glucose measurements on them when we harvested their neutrophils, but they were all insulin-dependent diabetics with elevated hemoglobin A1cs, and we assumed that they had been exposed to a higher glucose environment than normal, but we don't have documentation of that.

Regarding the higher levels of apoptosis seen with the TUNEL assay, your references to higher levels seen at zero hours, which, with the TUNEL assay, appeared to be about 12% to 14% in diabetics compared with about 5% in normal neutrophils, might suggest an alteration in neutrophils even at zero hours; but it is unclear what the biological significance of that finding is at 0 hours. At 24 hours, we actually find slightly lower overall numbers of apoptosis with the TUNEL assay compared with, I think, the more sensitive Hoescht assay.

E. Patchen Dellinger, MD, Seattle, Wash: It is interesting to speculate about the relevance of this difference in apoptosis to the generation of multiple organ dysfunction or ARDS. Two papers ago, Dr Wright showed less permeability changes in the lung in diabetic animals, and if the apoptosis was not suppressed by LPS and by other stimuli, that might imply that white cells would be removed from the circulation without the inflammatory stimulus and therefore, reduce the incidence of multiple organ dysfunction syndrome.

Dr Tennenberg: That is an interesting comment. Neutrophil apoptosis, as with many aspects of neutrophil function, has already been shown to be the classic double-edged sword. Depression in neutrophil apoptosis has been postulated as a mechanism for lung injury in ARDS. I view neutro-
phil apoptosis as a more normal and beneficial response of the neutrophil, as seen in the response to infection, but I think in extremes of physiology, one effect may take predominance over the other.

Cathy L. White-Owen, MD, Cleveland, Ohio: Steve, I enjoyed your paper a great deal, and I have to profess a tremendous amount of ignorance about apoptosis in neutrophils, and I am a little embarrassed to say that. I wonder if you can enlighten me about the actual functional capacity of early and late apoptotic cells, and how you interpret your data, in light of the fact that your neutrophils really haven’t undergone any kind of activation process; they haven’t gone to a diapedesis and entered the tissues? How do you put this, in the context of what actually happens to neutrophils in vivo? Does the inhibition of apoptosis affect the function of these cells in vivo in tissues?

Dr Tennenberg: We have not specifically looked at functional activity, correlating that with a positive apoptotic cell, although we plan on doing that. That has been looked at briefly by some other investigators, and in general, apoptotic cells appear to have decreased overall function; therefore, these cells are thought to be senescent, and are in the process of being engulfed by macrophages and eliminated from the circulation.

Your point about the in vivo response I think is very important, and I think that is actually where we are more interested in looking at neutrophil apoptosis. It has been well shown that endothelial transmigration markedly depresses neutrophil apoptosis, and how these effects are then modulated in tissue where the baseline is now much different remains to be seen. But I think that is an important area to look at. We have obviously just looked at circulating neutrophils here.

Dr White-Owen: Does an activated neutrophil that has “done its thing” in respiratory burst or in phagocytosis and bacterial killing, does that automatically initiate an apoptotic response in that cell, and is it automatically going to go on, and does it show the similar phases of early and late apoptosis, or is that a whole different process of cell death?

Dr Tennenberg: I think the assumption is that what you said is correct, but we do not have the technology yet to look at a single cell with a real-time assay of apoptotic changes to see whether activation, by definition, leads to apoptosis or, more intriguing, if there will be ways to prevent apoptosis after cells are activated or to alter the course of apoptosis in resting cells.