Reduction in Neutrophil Cell Surface Expression of Tumor Necrosis Factor Receptors but Not Fas After Transmigration

Implications for the Regulation of Neutrophil Apoptosis

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Objectives: To test the hypothesis that loss of polymorphonuclear neutrophil tumor necrosis factor α (TNF-α) receptors during transmigration renders the exudate neutrophil refractory to TNF-α–mediated stimulation of apoptosis; and to investigate the surface expression of Fas on both circulating and exudate neutrophils.

Design: A prospective cohort study.

Setting: Surgical laboratory of a tertiary care hospital.

Participants: Twenty-one healthy human volunteers.

Interventions: All subjects had circulating neutrophils and exudate neutrophils collected by venipuncture and skin window methods, respectively.

Main Outcome Measures: Circulating and exudate neutrophils were incubated in culture medium (1.0 × 10^6 neutrophils per milliliter) alone or with TNF-α (100 ng/mL). Apoptosis was evaluated by flow cytometry (annexin V–fluorescein isothiocyanate and propidium iodide). Tumor necrosis factor α–phycoerythrin and anti–human Fas–fluorescein isothiocyanate were used to evaluate neutrophil TNF-α receptors and surface expression of Fas.

Results: Exudate neutrophils had a significant delay in apoptosis rates when compared with circulating neutrophils. The percentage of neutrophils expressing TNF-α receptors was significantly diminished after exudation (80% ± 15% vs 33% ± 9%; P < .001), as was the median channel number of TNF-α phycoerythrin fluorescence (8.1 ± 1.6 vs 5.2 ± 0.5; P = .001). However, the expression of Fas was unchanged after transmigration (percentage positive for Fas: 98.7% ± 0.7% vs 92.8% ± 3.4%, P = .89; Fas antibody–fluorescein isothiocyanate median channel fluorescence: 12.2 ± 1.1 vs 13.1 ± 1.2; P = .80). Exposure of exudate neutrophils to TNF-α failed to increase their rate of apoptosis.

Conclusions: Exudate polymorphonuclear neutrophils are confirmed to have delayed apoptosis. Loss of TNF-α receptors during transmigration is necessary for neutrophil survival in the extravascular inflammatory milieu.

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The study of neutrophil apoptosis has been stimulated by the belief that neutrophils are implicated in the pathogenesis of persistent inflammatory states, including multiple organ dysfunction syndrome and adult respiratory distress syndrome. Neutrophils possess a variety of means of inducing tissue injury, including the secretion of connective-tissue proteases capable of tissue destruction in septic shock. There is an association between neutrophil number and level of activation and the degree of inflammation, as well as pulmonary vascular permeability, in patients with adult respiratory distress syndrome. Prevention of neutrophil sequestration will lead to reduced tissue injury in multiple animal models. In addition, clearance of neutrophils is associated with resolution of inflammation. Neutrophil apoptosis, a genetically programmed form of cell death fundamentally distinct from necrosis, is the principal mode by which senescent neutrophils are cleared from circulation. It has been suggested that an imbalance of granulocyte apoptosis and necrosis may be important in the pathogenesis of inflammatory disease. Thus, the study of neutrophil apoptosis is vital to better understand the persistent inflammation common to patients with multiple organ dysfunction syndrome and adult respiratory distress syndrome.

As a check on our techniques for assessment of neutrophil apoptosis, we carried out a dose-response curve for TNF-α with...
SUBJECTS, MATERIALS, AND METHODS

SUBJECTS

Twenty-one healthy human volunteers were recruited for the study. Exclusion criteria included history of infection within the previous 48 hours, severe chronic illness, immunosuppressive medication, and known malignant neoplasm. The study was approved by the Committee of Human Experimentation, Royal Victoria Research Institute, Montreal, Quebec.

MATERIALS AND REAGENTS

All preparations of neutrophils were kept in polypropylene tubes to prevent adherence. Neutrophil incubation was in dMEM (Gibco, Mississauga, Ontario), supplemented with 10% fetal bovine serum, 1% streptomycin-penicillin, and 1% L-glutamine (Gibco). Recombinant human tumor necrosis factor alpha (TNF-alpha) (Sigma Chemical Co, St Louis, Mo) was stored at −70°C in aliquots of 60 mL at a concentration of 2 ng/mL.

ISOLATION OF CIRCULATING NEUTROPHILS

Whole blood, 7 mL, was obtained from the subjects with the use of heparinized vacuum-sealed tubes (Becton Dickinson, Franklin Lakes, NJ), and circulating neutrophils were isolated with Macrodex/dextran-70 (Pharmacia Laboratories, Piscataway, NJ) and gravity sedimentation (60 minutes) followed by Ficoll-Hypaque (Pharmacia Laboratories) centrifugation. Cells were counted with a hemocytometer after staining with Turk solution and suspended in medium at a concentration of 1 x 10^6 cells per milliliter.

ISOLATION OF EXUDATE NEUTROPHILS

Skin window chambers were manufactured at the McGill University Workshop and used as previously described.11 The technique follows the one described by Zimmerli and Gallin.12 Briefly, exudate neutrophils were collected from skin windows placed on the volar aspect of the forearm. The forearm was sterilized with 10% povidone-iodine topical antiseptic followed by 70% isopropyl alcohol. Vacuum suction, 360 mm Hg, was applied through a plastic template for 60 to 90 minutes until 4 even 1.0 x 1.0-cm open-bottomed chambers was tightly applied with wide adhesive tape. The chambers were filled with 10% autologous serum, and the superior apertures were sealed with a sterile covering. After 16 to 18 hours, the exudate fluid, consisting of an almost pure (>98%) suspension of neutrophils, was aspirated. The chambers were rinsed 3 times with isotonic sodium chloride solution, and the fluid was transported immediately on ice to the laboratory. The neutrophils were sedimented at 400 g at 4°C for 5 minutes. Neutrophil viability was confirmed to be greater than 95% by propidium iodide exclusion. Neutrophils collected from skin windows were counted with a hemocytometer after staining with Turk solution and suspended in medium at a concentration of 1 x 10^6 cells per milliliter.

NEUTROPHIL INCUBATION

Both circulating and exudate neutrophils were incubated in culture medium at 37°C with 5% carbon dioxide for varying periods of time. The cells were shaken gently with a mixer (Thermolyne Rotomix; Diamed, Mississauga, Ontario). Next, 1 x 10^6 cells were incubated in 1 mL of medium in polypropylene tubes. Varying concentrations of TNF-alpha (see the "Results" section) were added to the cell suspension immediately before cell incubation.

QUANTIFICATION OF NEUTROPHIL APOPTOSIS AND NECROSIS

After incubation, cells were removed from the incubator, sedimented (400 g at 4°C for 5 minutes), washed with phosphate-buffered saline, sedimented, and suspended in 1 mL of binding buffer (10-mmol/L Hepes/sodium hydroxide, pH 7.4; 140-mmol/L sodium chloride; 2.5-mmol/L calcium chloride). Then 100 mL of the cell suspension in binding buffer was added to 5 mL of annexin V–fluorescein isothiocyanate (FITC). The median intensity of fluorescence as well as the number of cells that were positively expressing Fas, however, were not significantly different between circulating and exudate neutrophils (Figures 3 and 4, respectively).

COMMENT

There are 2 potential mechanisms suggested by previous studies to explain the observed delay in apoptosis after transmigration. First, neutrophil adhesion to the endothelial cell during transmigration may cause a delay in apoptosis. Watson et al16 demonstrated a delay in apoptosis after cross-linking CD11a and CD11b; however, cross-linking 1-selectin accelerated neutrophil apoptosis. The authors concluded that adhesion molecules may serve as modulators of neutrophil apoptosis. The second mechanism capable of causing delayed neutrophil apoptosis in exudate cells involves the products of the
Neutrophil adherence, involving L-selectin and engagement of adhesion molecules. One report suggested that neutrophils shed their TNF-α receptor before even entering the exudate environment. This would suggest that TNF-α receptor shedding might lead to a delay in apoptosis during transmigration, whereas neutrophils are adherent, rather than in suspension. As TNF-PE was used to detect both TNF receptors on the surface of neutrophils, this method may help explain the uncontrolled neutrophil activation seen in severe sepsis when systemic levels of TNF-α are elevated.

Neutrophil activation in the inflammatory milieu may provide the second mechanism for loss of neutrophil expression of TNF-α receptors. The TNF-α receptors are shed when neutrophils are activated in vitro with f-met-leu-phe (FMLP). The authors also noted that neutrophils shed their TNF-α receptors rather than internalize them and/or decrease their synthesis of the TNF receptor. The TNF-α receptor has also been demonstrated to cause a loss of neutrophil TNF receptors (because of receptor endocytosis), in addition to other inflammatory mediators, including C5a, platelet-activating factor, leukotriene B4, endotoxin, and FMLP. The same authors found that activation mediated by TNF-α in vitro is more potent when neutrophils are adherent, rather than in suspension. As TNF-PE was used to detect both TNF receptors on the surface of neutrophils, this method may help explain the uncontrolled neutrophil activation seen in severe sepsis when systemic levels of TNF-α are elevated.

Flow cytometry was performed on whole blood collected in 7-mL sterile specimen tubes with EDTA as anticoagulant (Becton Dickinson). In addition, QC3 beads (Becton Dickinson) were used to calibrate the intensity of fluorescence for every use of the cytofluorometer. The QC3 beads allowed for standardization of the intensity of fluorescence, which is proportional to the density of surface receptors. The settings for both apoptosis and for cell surface analysis were altered when required to ensure that the intensity of fluorescence of the beads remained the same throughout the study. For propidium iodide to stain DNA, the cellular membrane must not be intact. These cells are thus determined to be necrotic. Propidium iodide fluorescence was plotted against annexin V–FITC fluorescence; the data were registered on a logarithmic scale. At least 5000 events were recorded for each sample.

**CELL SURFACE ANALYSIS**

Whole blood, collected in 7-mL sterile specimen tubes with EDTA as anticoagulant (Becton Dickinson), was kept on ice until analysis (begun immediately). Exudate neutrophils were analyzed as soon as they were suspended in medium at a concentration of 1 × 10⁶ per milliliter. The TNF receptor density was analyzed by means of TNF-phycocerythrin (PE) (R&D Systems, Minneapolis, Minn) in the following manner. In 5-mL tubes, 10 µL of TNF-PE was incubated along with 100 µL of whole blood for 30 minutes at room temperature in the dark. Subsequently, 2 mL of monoclonal lysing solution (1 L of distilled water, 8.26 g of ammonium chloride, 1 g of potassium bicarbonate, and 0.037 g of sodium EDTA) was added to the mixture, and again it was stored in the dark for 10 minutes. After the neutrophils were sedimented (400 × g for 5 minutes), the cells were washed twice with phosphate-buffered saline with 1% fetal bovine serum and 0.1% azide (Sigma). Cells were finally suspended in 2% paraformaldehyde (Fisher Scientific, Mississauga) and promptly analyzed by flow cytometry. Specificity of the TNF-PE reaction was ascertained by means of a TNF blocking antibody (R&D Systems) before incubation.

**DATA ANALYSIS**

All results are expressed as mean ± SD. Statistical significance (P<.05) was determined by the paired Student t test, with the Bonferroni correction when multiple tests were performed simultaneously. Statistical significance is indicated on the figures to follow. The number of subjects used for each test may vary, as the yield of the exudate cells would also vary.

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neutrophil in this experiment, the observed reduction in TNF binding may be secondary to down-regulation of either the 55-kd or the 75-kd receptor, or both. Nonetheless, the loss of TNF receptors on neutrophils appears to be fundamentally important to neutrophil survival in the inflammatory exudate environment.

Although the loss of TNF-α receptors helps explain neutrophil survival in the extravascular inflammatory environment, the mechanism leading to neutrophil apoptosis and clearance and the resolution of inflammation remains unsolved. The Fas–Fas ligand (FasL) apoptotic pathway has received considerable attention in the regulation of phagocyte apoptosis, as it plays a critical role in the regulation of T-cell development and apoptosis.20-22 Although neutrophils are highly susceptible to rapid apoptosis after incubation with anti–Fas IgM antibody, this may be suppressed with a variety of inflammatory mediators, including granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interferon gamma, and TNF-α.23 Thus, elevated levels of TNF-α in the exudate inflammatory environment may prolong neutrophil survival by inhibiting the Fas-FasL apoptotic pathway. The importance of soluble FasL vs membrane-bound FasL in the exudate environment remains under investigation. Activated T cells have been shown to secrete soluble FasL.24 Constitutive expression of both Fas and FasL on the neutrophil cell surface (a unique feature among the phagocytes) suggests that the neutrophil may be irrevocably committed toward an apoptotic cell death.23 Regardless of the exact mechanism, our demonstration that expression of Fas is maintained after transmigration lends further support to the Fas-FasL pathway in neutrophil clearance in the exudate environment. Neutrophils also undergo apoptosis after bacterial ingestion,25 providing a second possible mechanism for neutrophil clearance in the inflammatory milieu. The full understanding of neutrophil transmigration and subsequent clearance from the exudate inflammatory environment...
may provide important clues to prevent or treat persistent inflammatory states responsible for significant morbidity and mortality in the surgical intensive care unit.


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REFERENCES

date neutrophils on the skin; however, we have no way of knowing whether or not there is any alteration due to the fact that we are allowing the cells to stay in this chamber for 18 hours. The means of collecting the neutrophils does not allow us to measure precisely the mechanisms leading to TNF receptor loss. There is a lack of control because of the in vivo nature of the model.

You asked if the TNF receptors, which one is affected. Using our technique, the binding was on both TNF receptor 1 and TNF receptor 2. We have since evaluated which one is down-regulated and found that it is specifically the TNF receptor 1, or the p55 receptor, which is down-regulated through the process of exudation, which makes sense, since that is the receptor which mediates neutrophil apoptosis.

Lastly, you asked if we could evaluate monocytes or macrophages, and the problem with evaluating the transmigration of monocytes using this technique is that you need to leave the skin window on for prolonged periods of time, and it is a somewhat uncomfortable technique and it would be unacceptable to ask control subjects to leave the skin window on for 24, 48, or 72 hours, and that is why we have not evaluated macrophages or monocytes using this technique.

John C. Marshall, MD, Toronto, Ontario: Apoptosis in the neutrophil is kind of a default position, and inhibition of apoptosis is actually an active process. And one of the ways that you could get at, whether or not the cause of inhibited apoptosis was a reduction in the number of receptors or not, would be to inhibit protein synthesis. Have you looked at the effects of a protein synthesis inhibitor on the apoptotic delay that you see in your model?

Dr Seely: We did not evaluate that.

Carl Hauser, MD, Newark, NJ: Presumably, you obtained your peripheral blood neutrophils by Ficoll followed by dextran, or some such procedure. Did you handle the skin window exudate neutrophils identically?

Dr Seely: No, we did not. We assumed that there was going to be no alteration in apoptosis due to the separation techniques that we used.

George W. Machiedo, MD, Bronx, NY: Do you have any data on the functionality of the white cells in the exudate, based on the TNF receptor data that you have? In other words, once they shed or in other ways modulate that TNF receptor, are they as functionally equivalent as either the circulating neutrophils or the neutrophils that do not shed and go into the exudate?

Dr Seely: Our previous data from our laboratory has demonstrated that these cells are in fact activated or that exudate neutrophils have an increased microbicidal activity and demonstrate other increased markers of activity.

Timothy R. Billiar, MD, Pittsburgh, Pa: Although you showed that Fas receptor levels did not change, but did apoptosis induced by Fas change? Perhaps there is a mechanism involved in the suppression of apoptosis that has nothing to do with the TNF receptor levels. Have you tried to induce apoptosis by a non-receptor-dependent mechanism to determine whether the cells have other protective pathways?

Dr Seely: It’s an excellent question, and to date we have only evaluated whether Fas is present on the cell surface, and we are in the process of evaluating whether exudate cells respond to Fas-mediated apoptosis. But I am afraid I don’t have that data for you at present.

Jan K. Horn, MD, San Francisco, Calif: It occurred to me that neutrophils express phosphatidylserine on their surface when they become apoptotic, and this signals their recognition and removal by macrophages. Is it possible that, in the exudative process, the apoptotic cells are actually removed during dermal transmigration and you are only seeing on exudation a population of cells that has escaped this sort of terminal elimination?

Dr Seely: Absolutely correct in that phosphatidylserine is the marker of macrophages used to detect apoptotic neutrophils and subsequently phagocytose them. There is no doubt that there is probably some phagocytosis of apoptotic neutrophils in that dermal segment; however, we assume that enough neutrophils are escaping the phagocytosis of macrophages in order for us to detect them in the exudate in the skin window chamber. But using this technique, we have no way of directly evaluating that question.

Steve E. Calvano, PhD, New Brunswick, NJ: You make it sound like the exudate cells specifically are down-regulating their TNF receptors in response to transmigration. I would just like to point out that almost any neutrophil-activating stimuli will cause a rapid down-regulation of TNF receptors.

Dr Seely: Absolutely, and in fact there are 2 potential mechanisms whereby the TNF receptors are down-regulated; one is the activation, as you mentioned, of the neutrophil in the exudate environment, and, secondly, there is data to suggest that binding of L-selectin will down-regulate TNF receptors as well. Because we evaluate the receptors, as it were, at the end stage of exit transmigration, we cannot identify precisely the mechanism of shedding of TNF receptors. But I agree with you.