Original Article

Crit Care & Shock (2006) 9: 9 - 15

Delayed neutrophil apoptosis in patients with multiple organ dysfunction syndrome

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Abstract

Aged neutrophils undergo spontaneous apoptosis and delayed apoptosis is associated with persistence of inflammatory disorders through release of toxic metabolites. We evaluated spontaneous apoptosis of neutrophils in patients with multiorgan dysfunction syndrome and neutrophil respiratory burst activity. Neutrophil apoptosis was assessed at study enrollment and after 24 hours incubation in culture medium by annexin-V assay, morphology and DNA fragmentation. Respiratory burst activity was measured using dihydrorhodamine. Twenty two patients with multiple organ dysfunction syndrome admitted to an intensive care unit and 22 healthy controls were studied. After 24 hours, a profound delay in spontaneous apoptosis in organ dysfunction patients was seen compared to controls for both annexin-V (26.9% versus 52.1%, p< 0.0001) and morphological assessment (25.0% versus 61.5%, p<0.0001). Respiratory burst activity was increased (86.3% versus 27.6%, p<0.001) suggesting that the delay in apoptosis was associated with prolongation of the functional activity of the cells. We conclude that in patient with multiple organ dysfunction syndrome there is a delay in spontaneous apoptosis together with a high functional activity of neutrophils.

Keywords: apoptosis, multiorgan dysfunction, sepsis, neutrophils.

Introduction

Despite advances in management and therapy, sepsis remains the main cause of morbidity and mortality in the intensive care unit, affecting around 500,000 to 750,000 patients each year in Europe and USA [1-3]. Sepsis is considered to be the initial event of a spectrum ranging from systemic inflammatory response syndrome (SIRS), to septic shock and multiple organ dysfunction syndrome (MODS). Mortality ranges from 40% for uncomplicated sepsis to 80% for septic shock and MODS and the main cause of death in patients with sepsis or SIRS is MODS.

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Neutrophils are one of the main mediators of tissue injury and they play an important role in the inflammatory response. Their release of proteolytic enzymes, toxic oxygen metabolites, and anti-microbial peptides is associated with extensive tissue damage. Under steady state conditions and in the absence of cytokines or other pro-inflammatory agents, aged neutrophils typically undergo spontaneous apoptosis [4,5]. Apoptotic neutrophils are then phagocytosed by macrophages that prevent them from releasing their cytotoxic contents into the extracellular milieu, which would occur if the cells died by necrosis. This safe removal of apoptotic neutrophils helps to limit tissue damage during the resolution of inflammation.

Although neutrophils are committed to apoptosis, their death can be delayed in sites of inflammation by external factors including pro-inflammatory cytokines, bacterial membrane components such as lipopolysaccharide (LPS; endotoxin), and pro-granulocyte differentiation factors such as granulocyte and monocyte colony stimulating factor (GM-CSF). Impaired function in aging neutrophils is associated with apoptosis [6] and so

delayed apoptosis is associated with the persistence of inflammation and localised tissue injury [7].

Several studies have investigated neutrophil activity in SIRS, but there is little work assessing neutrophil apoptosis in patients who have already developed MODS. We hypothesized that neutrophils in MODS patients will be in a highly active state with delayed spontaneous apoptosis, which might play an important role in the development and progress of the disease. We evaluated spontaneous neutrophil apoptosis and neutrophil functional activity in patients with established MODS.

Materials and methods

Patients

The study was approved by the local research ethics committee, and either assent from a near relative, or patient consent, was obtained in each case. After a power calculation, based on an estimated 30% difference in the percentage of apoptotic neutrophils between healthy controls and MODS patients ($p<0.05$, 80% power), 22 consecutive patients admitted to the intensive care unit (ICU) with MODS were recruited. Patient selection was based on dysfunction in two or more organ systems, scoring 1 or higher in the MODS score for each individual organ [8], including respiratory, renal, hepatic, cardiovascular, haematological and neurological systems. Healthy volunteers were recruited as controls.

Neutrophil isolation

Blood samples were obtained from patients within 24 hours of fulfilling the criteria for MODS as detailed above. Polymorphonuclear leucocytes (PMN) were separated from venous blood using density gradient centrifugation (Polymorphprep; Nycomed, Oslo, Norway). The resulting preparation contained 94-98% PMN with 93-98% viability. PMN were resuspended in RPMI 1640 medium (GIBCO-BRL; UK) supplemented with 10% heat inactivated fetal calf serum, 20mmol/L L-glutamine and 1% penicillin/streptomycin (GIBCO-BRL; UK). Each PMN sample was divided into two parts, the first was assayed for apoptosis immediately (time zero, T_0), and the other part was incubated at a concentration of 5-10 x 10^6 neutrophils/ml in 75cm^2 canted neck flasks (Nunclon surface, Fisher scientific; UK), for 24h (T_{24}) at 37ºC in a humid atmosphere of 5% CO_2/95% air. The cells were then harvested and washed twice with phosphate buffered saline (PBS), then assayed for apoptosis as follows:

Annexin-V assay

To assess membrane changes, one of the earliest features of apoptosis, annexin-V and propidium iodide (PI) staining was used, as we have described previously [9] and the samples were analysed by flow cytometery. Annexin-V has a high affinity to phosphatidylserine (PS), which undergoes translocation from the inner to the outer part of the plasma membrane early in apoptosis. Since necrotic cells also expose PS due to loss of membrane integrity, PI stain was used as an exclusion dye to discriminate apoptotic from necrotic cells. Flow cytometric analysis was performed using FACSCalibur (Becton Dickinson; San Jose, CA) with an argon laser exciting at 488nm; green fluorescence of fluorescein was detected at 530nm (FL-1), and red fluorescence of PI was detected at 610nm (FL-3). Typically 15,000 events per sample were collected in list mode, stored and analysed by CellQuest Pro (Becton Dickinson). Apoptotic PMN were calculated as the percentage of gated cells showing positive uptake of annexin-V (FL-1) and negative uptake of PI (FL-3).

Morphological assessment

Loss of plasma membrane integrity in late apoptosis was used as the basis of morphogical assessement [10]. Cells were inclubated for 1-2 min with a dye cocktail of equal volumes of ethidium bromide and acridine orange (Sigma) and examined with a fluorescence microscope (Axiolab, Carl Ziess, UK). Sample identity was blinded before counting, and at least 400 cells were counted per slide. Late apoptotic cells take up both the cell-permeable acridine orange and the plasma membrane-impermeable ethidium bromide.

DNA fragmentation assay

To identify the characteristic DNA intranucleosomal fragmentation typical of apoptosis [11], PMN were suspended in digestion buffer containing 10mM Tris, 100mM NaCl, 1mM EDTA, 1% sodium dodecyl sulfate and 0.2 mg/m proteinase K (Sigma, UK). After overnight incubation at 37°C the lysate was extracted with phenol:chloroform:isoamyl alcohol (25:24:1v/v/v) (Sigma). Low molecular weight DNA was then separated by centrifugation (12,000g, 15min, 0°C) and precipitated from the supernatant with ethanol. The pellet was redissolved in buffer containing 10mM Tris, 1mM EDTA, and 80µg/ml Rnase. Agarose gel electrophoresis was then performed using 1.8% agarose (Sigma), containing ethidium bromide (Sigma), run in TBE buffer

(890mM Tris, 890mM boric acid, 25mM EDTA, pH 8.0) at 120V for 1h. The gel was then viewed with an UV trans-illuminator.

Respiratory burst activity

The generation of reactive oxygen intermediates was assayed by modification of the technique of Smith and Weidemann [12]. PMN were stimulated with phorbol myristate acetate (PMA) (Sigma), which indirectly stimulates NADPH oxidase leading to the production of superoxide anions. Dihydrorhodamine 123 (DHR, Sigma) is a non-fluorescent probe that is oxidised by hydrogen peroxide to the highly fluorescent rhodamine 123. PMN were incubated with 200nM PMA at 37°C for 10 min prior to the addition of 1µM DHR. Samples were assessed within 30 min using FACSCalibur flow cytometer and CellQuest Pro software. Control samples containing no PMA were run in parallel and were used to set the negative and positive regions for the analysis. A minimum of 15,000 events was recorded. The green fluorescence of rhodamine 123 was detected using the 530nm filter (FL1).

Statistical analysis

Statistical analysis was performed using Analyse-it software for Excel (version 1.63). Data were not normally distributed so values were expressed as median [range]. For analysis of independent variables between groups, the Mann-Whitney U test was used. Differences in the same group was analysed using Wilcoxon Signed-Ranks test. A p value of < 0.05 was taken to be significantly different.

Results

Thirteen of the patients with MODS were females and 9 were males, with a median [range] age of 59 [22-75] years. Seventeen patients had sepsis, 2 had acute pancreatitis, 2 were post surgical patients and one had multiple trauma. Of the 22 healthy volunteers, 11 were females and the median age was 52 [35-62] years. Blood samples were obtained within 24h of fulfilling the criteria for MODS and after a median [range] of 4 [0-7] days following admission to the intensive care unit. Fourteen (63.6%) patients subsequently died.

Annexin-V assay

There was a significant increase in the percentage of apoptotic PMN using annexin-V assay at T_{24} samples compared to T_0 in both patients and controls ($p< 0.0001$). However, there were significantly less apoptotic PMN in patients than in controls at T_{24} ($p< 0.0001$). No such difference between the groups was observed at T_0 ($p= 0.76$, **Figure 1**).

Morphological assessment

Morphological analysis showed a significant increase in PMN apoptosis in both patients and controls at T_{24} compared to T_0 ($p< 0.0001$). However, the percentage of apoptotic PMNs at T_{24} was significantly lower in patients than in controls ($p< 0.0001$, **Figure 2**).

DNA fragmentation assay

Analysis of DNA samples from both control subjects and patients at T_0 did not show any apoptotic ladder pattern, indicating intact DNA of normal cells at this time point. However, analysis of T_{24} DNA samples revealed the characteristic ladder pattern of apoptosis in all control samples, but in none of the patients (**Figure 3**).

Respiratory burst activity

There was a significant decrease in respiratory burst activity of PMN in both patients and controls at T_{24} samples in relation to those at T_0 ($p<0.0001$). However, the respiratory burst activity in PMN from patient samples at T_{24} was significantly higher than that of controls at the same time point ($p<0.0001$). The delay in apoptosis seen in MODS patients was clearly associated with prolongation of the functional life of the neutrophil population (**Figure 4**).

Discussion

Using three different methods we have shown that neutrophil apoptosis is delayed in patients with MODS, associated with maintenance of neutrophil function. This ongoing functional activity may be related to the pathophysiological processes underlying organ dysfunction.

Apoptosis is the major mechanism controlling clearance of neutrophils from peripheral blood, and is considered to be the mechanism controlling the functional life of neutrophils at sites of inflammation [5]. Apoptosis of neutrophils may be delayed when inflammatory signals indicate a need for longer activity allowing for the prolonged elaboration of proinflammatory mediators and chemotactic factors. Senescent neutrophils undergo spontaneous apoptosis without eliciting any inflammatory re-

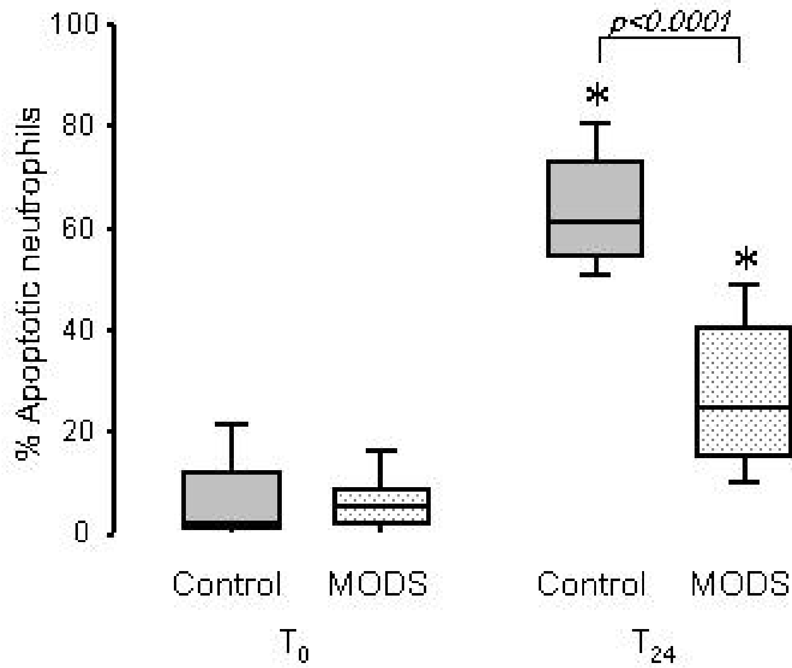

FIGURE 2. PERCENTAGE OF APOPTOTIC NEUTROPHILS IN PATIENTS AND CONTROL SAMPLES AS DETECTED BY MORPHOLOGICAL ASSAY AT T_0 AND T_{24}. N=22 IN EACH GROUP. BOX AND WHISKER PLOTS SHOW MEDIAN, 25TH AND 75TH PERCENTILES AND FULL RANGE.
* = SIGNIFICANTLY HIGHER THAN AT T_0 ($p<0.0001$).

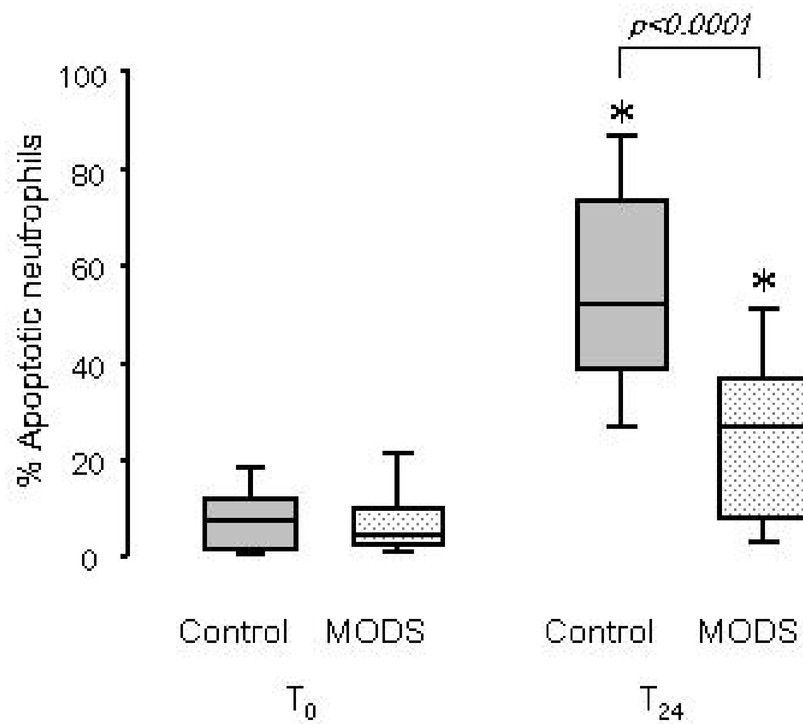

FIGURE 1. PERCENTAGE OF APOPTOTIC NEUTROPHILS IN PATIENTS AND CONTROL SAMPLES DETECTED BY ANNEXIN-V BINDING ASSAY AT T_0 AND T_{24}. N=22 IN EACH GROUP. BOX AND WHISKER PLOTS SHOW MEDIAN, 25TH AND 75TH PERCENTILES AND FULL RANGE.
* = SIGNIFICANTLY HIGHER THAN AT T_0 ($p<0.0001$).

FIGURE 3. AGAROSE GEL ELECTROPHORESIS OF LOW MOLECULAR WEIGHT DNA SEPARATED FROM $5x10^6$ NEUTROPHILS AFTER 24H CULTURE. DNA WAS ELECTOPHORESED IN A 1.8% AGAROSE GEL, AND DETECTED BY UV FLUORESCENCE AND ETHEDIUM BROMIDE STAINING. LANES 1-3 SHOW DNA FROM 3 MODS PATIENTS WITH NO DNA LADDERING, AND LANES 4-6 SHOW DNA FROM 3 HEALTHY CONTROLS SHOWING THE TYPICAL APOPTOTIC LADDER, CAUSED BY INTER-NUCLEOSOMAL FRAGMENTATION. RESULTS ARE REPRESENTATIVE OF ALL SAMPLES IN EACH GROUP. M: DNA MARKER.

action [5-7] such that delays in neutrophil apoptosis may result in senescent neutrophils dying by necrosis with the detrimental leakage of inflammatory mediators and destructive enzymes with the bystander effect of inflammation.

We analysed the degree of spontaneous neutrophil apoptosis in patients with diagnosed MODS at time of enrollment (T_0) and after 24 hours incubation in culture medium (T_{24}). The cellular mechanisms that result in apoptosis are complex, so three independent methods for apoptosis detection were used to provide a valid and precise measure of the different stages of apoptosis. These included flow cytometry using annexin-V assay, morphological assay and DNA fragmentation. In addition, we measured the functional activity of these neutrophils by analysis of the respiratory burst.

Annexin-V binding detects apoptosis-associated membrane changes on live cells before the appearance of the morphological or the nuclear changes that occur in apoptosis [13]. The high specificity of annexin-V, and the ability to perform analysis at the single cell level using FACS analysis, makes annexin-V a useful and convenient marker for early apoptosis [14]. Morphological assessment gives an accurate and detailed identification of the cells' morphology, differentiating viable and early apoptotic from late apoptotic cells [10]. DNA agarose gel electrophoresis is a highly specific late apoptotic marker, but although specific, this method lacks sensitivity, as cells may be phagocytosed before DNA fragmentation. Functional activity to generate reactive oxygen species was assayed using a highly sensitive fluorescent probe. Flow-cytometric analysis allowed detection of oxidative activity and disruption patterns of neutrophils at the single cell level.

We showed a profound delay in spontaneous apoptosis of neutrophils from MODS patients when compared to neutrophils from control subjects. Several other studies of patients suffering from SIRS or sepsis have also reported delayed neutrophil apoptosis [15-17]. There is evidence that the signal which delays neutrophil apoptosis is a soluble mediator since serum from patients suffering from SIRS and sepsis also delayed apoptosis in neutrophils from healthy controls [15-18]. Studies have established that apoptosis in the neutrophil is a constitutive process and its inhibition requires a signal resulting in expression of genes which inhibit the process. A recent study suggested that the delayed apoptosis seen in sepsis is associated with altered mitochondrial membrane potential and reduced activity of caspase-9 [16]. These findings might suggest future therapeutic use of agents to either protect mitochondria, such as mitochondria targeted antioxidants, or caspase-1 inhibitors.

Study of the respiratory burst activity in our study showed that the delay in apoptosis was clearly associated with prolongation of the functional activity of the cells. In line with our results, Jimenez *et al* [17] found that neutrophils of patients with SIRS had significant increased respiratory burst activity relative to controls, associated with delayed spontaneous neutrophil apoptosis.This clearly raises the possibility of prolonged free radical generation *in vivo* and suggests uncontrolled upregulation of pro-inflammatory mediators and dysregulation of down-regulating mechanisms of inflammation which may be responsible for the persistence of hyperinflammation resulting in multiple organ failure.

Although neutrophil apoptosis is delayed in sepsis, increased apoptosis in lymphocytes, endothelial cells and hepatoctyes has been shown to be increased [19, 20].

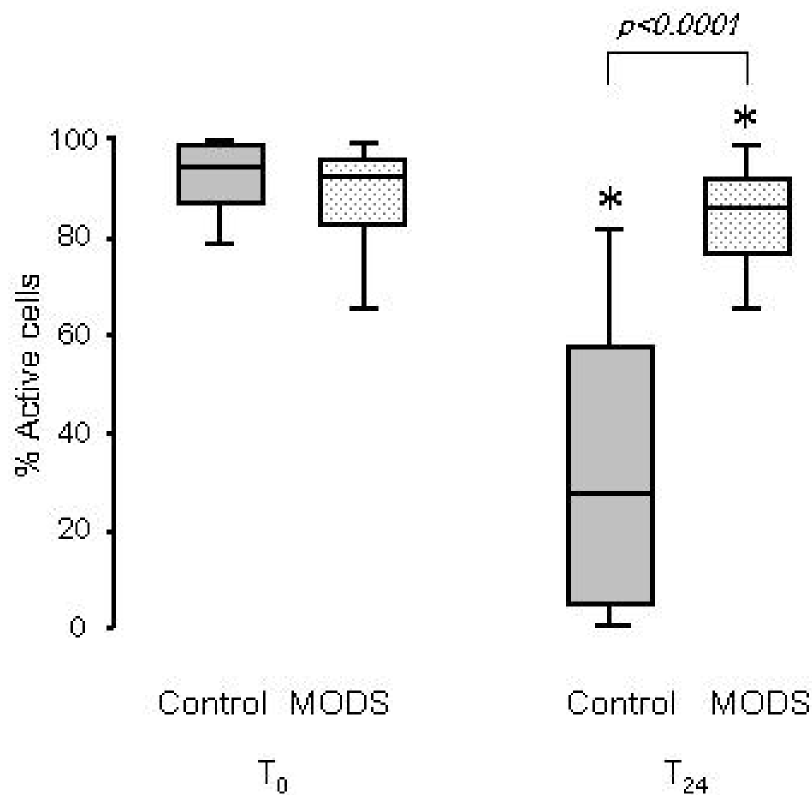

Figure 4. Respiratory burst activity of neutrophils of MODS patients and controls. N=22 in each group. Box and whisker plots show median, 25th and 75th percentiles and full range.
* = significantly higher than at T_0 ($p<0.0001$).

However in an animal model of neutrophil mediated lung injury, induction of apoptosis decreased lung injury and increased survival [21].

The pathophysiology of injury-induced organ dysfunction is poorly characterized but has been linked to systemic inflammation as a result of infection or massive tissue injury. Delayed apoptosis of neutrophils associated with maintenance of functional activity may contribute to organ failure. Simplistic *in vitro* models do not fully relect the clinical situation, reinforcing the need for studies in patients to identify the complex mechanisms involved.

Acknowledgment

This work was funded by the Egyptian Ministry of Higher Education Mission Department (FM).

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