Human Neonatal Neutrophils Are Resistant to Apoptosis with Lower Caspase-3 Activity

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The incidence of neonatal inflammatory diseases remains high despite improved strategies for dealing with infection. Neutrophils are believed to play a significant role in neonatal inflammatory diseases due to their secretion of harmful mediators. Neutrophils rapidly undergo apoptosis following activation; dysregulation of the neutrophil apoptotic pathway may be an underlying mechanism of neonatal inflammatory disorders. In this study, we determined whether neonatal neutrophils are intrinsically resistant to apoptosis relative to their adult counterparts. Twelve healthy full-term newborn infants and 12 healthy adult volunteers, aged 20 to 45 years, were enrolled in this study. Neutrophils were isolated from umbilical cord blood or fresh venous peripheral blood, and neutrophils of each subject were cultured for up to 48 hours. Flow cytometric analysis revealed that spontaneous apoptosis of adult neutrophils increased with culture time (23% ± 2% at 12 h, 49% ± 4% at 24 h and 76% ± 3% at 48 h), whereas the frequency of apoptosis was significantly lower in neutrophils from neonates (9% ± 2% at 12 h, 30% ± 4% at 24 h and 49% ± 3% at 48 h) (p < 0.01 for each time point). Importantly, the expression levels of caspase-3 mRNA and a precursor form of caspase-3 protein were lower in neonatal neutrophils than adult neutrophils, as judged by RT-PCR and Western blot analyses. Moreover, caspase-3 activity was lower in neonatal neutrophils, compared to adult neutrophils. These findings suggest that neonatal neutrophils are intrinsically apoptosis-resistant, which may be due to the low expression of caspase-3.

Keywords: adult; apoptosis; capase-3; neonate; neutrophil


Neutrophils are the first line of defense against microbial infections; they rapidly migrate from the bloodstream to injured sites, where they are activated and secrete a variety of inflammatory mediators (Nathan 2006; Luo and Loison 2008; Fox et al. 2010). However, some of the secreted factors, such as proteases and oxygen radicals, can be harmful and may induce tissue injury (Liu et al. 2005; Filep and El Kebir 2009). To avoid such damage, activated neutrophils are programmed to undergo apoptosis rapidly, and are cleared via macrophage-mediated phagocytosis (Savill et al. 1989; Hallett et al. 2008; Leitch et al. 2008). Apoptosis is a form of cell death characterized by morphological changes and endonuclease-mediated DNA fragmentation. Although the cellular mechanisms underlying apoptosis are not completely understood, it is generally believed that apoptosis can be mediated by the activation of the caspase cascade or via inhibition of survival signaling.

The incidence of neonatal inflammatory diseases remains high despite continuous advances in neonatal care and improved strategies for dealing with infection. The reason for this phenomenon seems to be multifactorial including leukocyte dysfunctions (Koenig et al. 2005; Nguyen et al. 2010). Inflammatory diseases in neonates are characterized by the persistence of neutrophils in injured tissues, which was thought to contribute to the pathogenesis of neonatal conditions such as bronchopulmonary dysplasia, necrotizing enterocolitis, and sepsis (Molloy et al. 2005). Despite defective chemotaxis, phagocytosis, and oxidative metabolism in neonatal neutrophils, infants are at high risk for neutrophil-mediated tissue injury (Qing et al. 1995; Nupponen et al. 2001; Weinberger et al. 2001). Given that apoptosis plays an important role in the clearance of neutrophils, it is reasonable to speculate that neonatal neutrophils are more resistant to apoptosis, thus contributing to the pathological processes of neonatal inflammatory diseases. In the current study, we investigated whether there are differences in apoptosis between neonatal and adult neutrophils and evaluated potential
mechanisms mediating the differences.

Materials and Methods

Neutrophils isolation and preparation

Twelve healthy full-term newborn infants from healthy mothers, appropriate for gestational age, after an uneventful pregnancy and delivery, and 12 healthy adult volunteers, aged 20 to 45 years, were enrolled in this study. Fresh venous peripheral blood and umbilical cord blood was collected under sterile conditions from healthy adult volunteers and normal full-term neonates, respectively, and treated with heparin (10 U/mL). After sedimentation using 3% dextran (Sigma-Aldrich, USA), the leukocyte enriched-plasma was layered on to Ficoll-Hypaque and centrifuged at 400 × g for 30 min, as previously described (Vancurova et al. 2001). The supernatant was discarded and the pellet was subjected to hypotonic lysis with 0.15 M NH₄Cl to free the neutrophils from contaminating red blood cells. After washing with phosphate-buffered saline (PBS), neutrophils were suspended in RPMI-1640 (Gibco, USA) supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 100 μM penicillin, and 100 μg/mL streptomycin. The collected neutrophils were 96% pure based on morphologic examination and displayed a viability of 98% using the trypan blue dye exclusion assay. This study was approved by the ethical approval committee of Bengbu Medical College.

Flow cytometric measurement of neutrophil apoptosis

The binding of annexin V-FITC to phosphatidylserine in a Ca²⁺-dependent manner was used as a sensitive measure of neutrophil apoptosis. Neutrophils were washed in sample wash buffer and stained with either annexin-V-FITC alone or in combination with Propidium iodide (PI) according to manufacturer’s recommendation (Annexin V-Fluorescein Apoptosis Detection Kit, eBioscience, USA). PI was added as an indicator of viability, and no distinction was made between intermediate and late apoptosis. Cells that were stained positively for annexin V-FITC were considered apoptotic. Data acquisition and analysis was performed by flow cytometry (FACS Calibur, BD Biosciences, USA) using CellQuest software. At least 10,000 cells were counted in each sample and a gate based on forward and side scatter was set to exclude cell debris.

Morphological assessment of apoptosis

At specific time points, aliquots of neutrophils (100 μL) were collected and stained with Giemsa staining solution. Cytospins were examined by oil-immersion light microscopy (Olympus BX3, Japan) at × 1,000 magnification. Apoptotic neutrophils were identified by characteristic morphological changes (pyknosis and diminished cell size), as previously described (Kang et al. 2006). Blinded samples were counted in duplicate, and at least 200 cells were scored in each sample to determine the percentage of apoptotic neutrophils.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from neutrophils using TRIzol solution (Invitrogen, USA) according to the manufacturer’s instructions. RT-PCR procedure was used to determine the expression of mRNA (One-step RT-PCR kit, Qiagen, Germany). The primer sequences were as follows: human caspase-3 (sense 5′-TGAAACAAATGG ACCTGTTGACC-3′ and antisense 5′-AGGACTCAAATTCTG TGGCCACC-3′) and human β-actin (sense 5′-TCTCTGTTGGCATC CACGAACT-3′ and antisense 5′-GAAGCATTTGCGGTG GACGAT-3′). The PCR products were analyzed on a 1% agarose gel containing ethidium bromide and quantified by densitometry.

Western blot analysis

Western blot analysis was performed as described previously (Huang et al. 2008). Briefly, neutrophil lysates and prestained molecular weight markers were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.5% of Triton X-100), and probed with anti-caspase-3 or β-actin antibodies (Cell Signaling, USA). After incubation with the secondary antibody conjugated with HRP, membranes were extensively washed, and the blots were visualized by enhanced chemiluminescence according to the manufacturer’s protocol (ECL kit, Santa Cruz, USA).

Caspase-3 activity assays

Caspase-3 activity in neutrophils lysates was measured using a colorimetric assay. Briefly, neutrophil aliquots were lysed and the supernatants assayed using conjugated substrate (2 mM Ac-DEVDD-pNA), following the manufacturer’s protocols (Caspase-3 Colorimetric Assay Kit, Beyotime, Haimen, China). The cell lysis buffer consists of 1 mM EDTA, 0.5% Triton X-100, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 100 μM PMSF, 3 μg/mL aprotinin in PBS (pH 7.2-7.4). For spectrophotometric analysis, samples were transferred to 96-well plates, and the colorimetric reaction assayed at 405 nm. Quantification of relative protease activity in OD units was determined from a calibration curve generated using standard concentrations of pNA, the product resulting from cleavage of Ac-DEVD-pNA. This cleavage product is directly proportional to enzymatic activity of caspase-3.

Statistical analysis

Data are presented as the mean ± s.d. unless otherwise indicated. Results from paired experiments were evaluated with the use of student’s t test or the one-way ANOVA; multiple pairwise comparisons were analyzed using the Student-Newman-Keuls test. A p value < 0.05 was considered statistically significant.

Results

Neonatal neutrophils are resistant to apoptosis compared to adult neutrophils

Neutrophils isolated from venous and umbilical cord blood were cultured in media only, and aliquots tested after 12, 24, and 48 h. An important feature of apoptosis is the cell surface exposure of phosphatidylserine (PS), which can be detected using FITC-conjugated annexin V. Flow cytometric analysis revealed that spontaneous apoptosis of neutrophils isolated from adults increased with time (23% ± 2% at 12 h, 49% ± 4% at 24 h and 76% ± 3% at 48 h). However, the frequency of apoptosis was significantly lower in neutrophils from neonates (9% ± 2% at 12h, 30% ± 4% at 24 h and 49% ± 3% at 48 h) (Fig. 1A, B). The flow cytometric assessment of apoptosis was further confirmed by morphological methods, which provided similar results. As shown in Fig. 1C, apoptotic neutrophils displayed typical morphological changes, such as chromatin condensation, accompanied by the loss of the characteristic multilobular nuclear structure.
The expression level of caspase-3 is lower in neonatal neutrophils

A common feature of cells undergoing apoptosis is the caspase activation, specifically, caspase-3 in neutrophils. To determine whether the lower frequency of apoptosis in neonatal neutrophils was the result of a reduced expression of pro-apoptotic genes, we examined caspase-3 expression in neonatal neutrophils. Using RT-PCR and freshly isolated neutrophils, we found that the level of caspase-3 mRNA was noticeably lower in freshly isolated neutrophils from neonates than the level in neutrophils from adults (Fig. 2A). Next, we examined the expression of caspase-3 protein. Western blot analysis of total cell lysates revealed higher expression levels of the inactive precursor, procaspase-3, in adult neutrophils cultured for 0 h and 24 h compared to cultured neonatal neutrophils. In addition, the level of procaspase-3 tended to be lower in adult neutrophils at 24 h compared with its expression level at 0 h. Similarly, the level of procaspase-3 protein in neonatal neutrophils was decreased at 24 h, compared to 0 h (Fig. 2B).

Neonatal neutrophils have lower enzymatic activity of caspase-3

Caspase-3 is a key cleavage enzyme in the spontaneous apoptosis of neutrophils. Next, we determined if lower expression of caspase-3 was associated with diminished enzymatic activity. The enzymatic activity of caspase-3 in neutrophil lysates was measured using a caspase-3 substrate (Ac-DEVD-pNA), which gives rise to a chromogenic product (pNA) after cleavage by the activated enzyme. This colorimetric assay was used to measure relative enzymatic activity of caspase-3 in neutrophils cultured for 0-48 h. The results demonstrated substantial caspase-3 activity that peaked at 24 h during the spontaneous apoptosis of both adult and neonatal neutrophils. Moreover, relative caspase-3 activities were higher in adult neutrophils than those in neonatal neutrophils at 12 and 24 h of culture. However, there was no apparent difference in caspase-3 activity at 48 h between the two groups of neutrophils (Fig. 3).
Discussion

Neutrophils play a very important role in the cellular pathology of inflammatory diseases in neonates. Accumulated neutrophils are activated and induce tissue injury through the secretion of harmful mediators such as oxygen radicals, proteases, and cytokines (Liu et al. 2005; Filep and EL Kebir 2009). This is thought to contribute to the tissue injury associated with inflammatory diseases (Schultz et al. 2002). In the present study, we compared the longevity of neutrophils from neonates to that of adult-derived cells and investigated the potential mechanisms underlying survival difference. Using flow cytometry and morphological methodology, we observed that spontaneous apoptosis of neutrophils obtained from neonates was substantially diminished compared with those from adults. This observation is consistent with previous studies suggesting a functional immaturity of neonatal neutrophils (Bortolussi et al. 1993; Koenig et al. 1996). For example, compared with neutrophils from adults, neonatal neutrophils have decreased functional expression of the surface adhesion molecules, Mac-1 (Anderson et al. 1990; Abughali et al. 1994) and L-selectin (Koenig et al. 1996). Differences have also been observed in the apoptosis of murine T cells from neonates and adults (Adkins et al. 1996). Although these observations suggest that cellular immaturity occurs in neonates, the underlying mechanisms responsible for such functional differences remain unclear.

The initiation of an apoptotic program is mediated by caspases; there is evidence supporting a major role for caspase-3 in neutrophil apoptosis (Ge et al. 2005; Van Raam et al. 2008). In the present study, we demonstrated that the expression of caspase-3, at both mRNA and protein levels, was significantly lower in neonatal neutrophils compared to adults. Moreover, we observed that the diminished expression of caspase-3 in neonatal neutrophils was associated with lower caspase-3 enzymatic activity. Apoptosis of neutrophils is delayed in caspase-3-deficient mice (Woo et al. 1998) and, also, decreased caspase-3 expression has been linked to prolonged survival of neutrophils in the inflamed lung (Watson et al. 1997). As caspase-3 is a critical component in the apoptosis of neutrophils, our present data suggest that low functional caspase-3 expression may confer the apoptosis resistance on neonatal neutrophils.

In summary, we have determined that neonatal neutrophils display apoptosis resistance compared with adult neutrophils. In addition, the observed low caspase-3 activity indicates that specific developmental deficiencies are responsible for the delayed apoptosis of these cells. This delay may lead to prolonged inflammation and tissue injury, which may account, in part, for the severity of inflammatory diseases observed in neonates despite reduced neutrophil function. Therefore, these findings may aid in the development of more specific and effective therapeutic and preventive strategies to regulate neutrophil apoptosis in neonatal inflammatory diseases.

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Conflict of Interest

The authors declare no conflict of interest.

References


