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Higher Spontaneous and TNFα-Induced Apoptosis of Neonatal Blood Granulocytes

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ABSTRACT

Granulocytes play an important role in inflammatory diseases. Neonates tend to develop granulocytopenia under sepsis and stress. It remains unclear whether apoptosis of neonatal granulocytes is different from that of adult granulocytes. In this study, we analyzed the discrepancy of granulocyte apoptosis between cord blood (CB) and adult blood (AB). We found that spontaneous and tumor necrosis factor-α (TNFα)-induced granulocyte apoptosis as determined by phosphatidylserine expression and DNA fragmentation were more prominent in CB granulocytes than AB granulocytes. CD95 ligand and TNFα levels were significantly higher in CB than in AB ($p = 0.001$ and $p < 0.001$, respectively). TNF receptor-2 and CD95 expression on CB granulocytes were not different from those on AB granulocytes. However, the TNF receptor-1 (TNFR1) expression was lower on CB granulocytes than that on AB granulocytes (69.98 $\pm$ 7.32 *versus* 89.04 $\pm$ 3.73%, $p = 0.029$). This decrease of TNFR1 expression on neonatal granulocytes might be related to a higher plasma TNFα level associated with an intrinsic defect on the dynamic change of membrane TNFR1 expression in neonatal granulocytes. The expression of anti-apoptotic molecule Bcl-2 in neonatal granulocytes was also lower than that in adult granulocytes (4.64 $\pm$ 0.51 *versus* 7.24 $\pm$ 1.17 unit/mg protein, $p = 0.032$). Moreover, another anti-apoptotic signal, nuclear factor-κB nuclear translocation, was also lower in CB than AB granulocytes. Results from this study suggest that higher plasma death ligands associated with lower anti-apoptotic molecules in granulocytes may act an important role in triggering neonatal granulocyte apoptosis. (***Pediatr Res* 58: 132–137, 2005**)

Abbreviations

AB, adult blood
CB, cord blood
CD95L, CD95 ligand
EMSA, electrophoretic mobility shift assay
PS, phosphatidylserine
RBC, red blood cells
TNFR, tumor necrosis factor receptor

Granulocytes play an important role in inflammatory diseases and provide a first-line defense against bacterial infections. They, however, have a short life span, about 6 h, and rapidly undergo apoptosis in blood circulation (1). Granulocytopenia is a poor prognostic factor for patients in neonatal intensive care units (2). It is known that the counter-regulation between apoptotic and anti-apoptotic signals determines the death program of granulocytes (3,4). It is not known whether abnormal plasma death ligands or intracellular anti-apoptotic molecules are involved in the susceptibility of granulocyte apoptosis in neonates.

Newborns tend to develop granulocytopenia in overwhelming infections or stress. Certain studies have indicated that neonatal leukocytes are functionally immature and have deficient immune responses compared with adult ones (5,6). However, some studies demonstrated that the immune responses of newborns are similar to those of adults but are diminished under stress (7). It remains unclear whether susceptibility to granulocytopenia in neonates is related to faster cell program death (apoptosis) or to stress-induced alteration and consumption. Allgaier *et al.* (8) reported that neonatal granulocytes underwent apoptosis slower than adult ones. In contrast, Uguz *et al.* (9) found that granulocytes from neonates underwent faster apoptosis than adult granulocytes. These studies used purified granulocytes. Evidence has shown that apoptosis of granulocytes can be affected not only by death receptor expression but also by soluble death ligands. It is possible that a disproportion of plasma death ligands and blood granulocyte death receptors might be involved in the discrepant apoptosis programs between adult and neonatal granulocytes.

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There are many molecules that can mediate cell apoptotic and anti-apoptotic signals. The death ligands, CD95L and TNFα, can trigger apoptosis, respectively, *via* their CD95 and TNF receptors (TNFR1 and TNFR2), especially TNFR1 (10,11). In contrast, the anti-apoptotic molecules such as Bcl-2 can prevent cells from undergoing apoptosis, including hematopoietic cells (12,13). TNFα is the major pro-inflammatory mediator in response to infections or stress that can promote cell apoptosis through TNFR (11,14). Recently, evidence showed that TNFα could also activate anti-apoptotic pathways through the nuclear factor (NF)-κB, which is a critical regulator in protection of human granulocyte apoptosis (15–17). Taken together, these studies suggest that an optimal balance among plasma death ligands, cell surface death receptors, and intracellular anti-apoptotic molecules may play an important role in the homeostasis of neonatal granulocyte apoptosis (programmed cell death). Employing a whole-blood model with human umbilical CB and healthy AB, we investigated whether an imbalance among plasma TNFα levels, cell surface TNFR expression, and TNFα-induced intracellular Bcl-2 and NF-κB expression was involved in altered neonatal granulocyte apoptosis.

METHODS

Subjects. Paired fresh AB from healthy adult volunteers and normal umbilical CB from the placenta site of umbilical cords immediately after normal full-term delivery were collected for study. Neonates with prematurity (gestational age <37 wk), premature rupture of membrane over 18 h, or clinical chorioamnionitis (maternal fever >38°C and/or foul-smelling cervicovaginal discharge) were excluded for the study. Blood was collected in heparinized tubes after informed consent was obtained from parents. The study protocol was approved by the Institutional Review Board of Chang Gung Memorial Hospital at Kaohsiung.

Reagents and buffers. Ammonium chloride lysing buffer containing NH4Cl (150 mM) was used to deplete RBC from whole blood (18). Normal saline-containing calcium chloride (2.5 mM) and HEPES (10 mM) was run as a binding buffer for the detection of PS expression with FITC-labeled annexin-V (BD PharMingen, San Diego, CA). PBS containing NaCl (150 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM), and KH_2PO_4 (1.1 mM) was used as isotonic buffer.

Flow cytometric analysis of blood granulocyte apoptosis as determined by cell surface PS expression. When cells undergo apoptosis, an early stage of change is the translocation of PS from the inner side of plasma membrane to the outer layer (19). Thus, detection of the PS exposure on the cell surface is recognized as an early indicator of granulocyte apoptosis (20). Annexin-V, a Ca^{2+}-dependent phospholipid-binding protein, has a high affinity for PS and is a sensitive probe for detecting apoptotic cells by flow cytometry when conjugated to FITC. For studies, heparinized whole blood (100 μL) was washed twice in Ca^{2+}-containing binding buffer and stained with FITC-labeled annexin-V in a 12 × 75 mm polypropylene tube for 10 min at room temperature in the dark according to the manufacturer's direction (BD PharMingen). After staining, the cells were fixed with 1% paraformaldehyde, followed by hemolytic depletion of the RBC using ammonium chloride lysing buffer (18). The blood cells were washed in PBS once and suspended to 0.5 mL for flow cytometric analysis of PS expression by FITC-labeled annexin-V staining, as previously described (19). Whole-blood samples treated with TNFα (50 ng/mL) (BD PharMingen) for 6 h in an incubator at 37°C were subjected to the study on the effect of TNFα on granulocyte apoptosis. Adjacent controls were run with blood cells stained with an irrelevant FITC-labeled control antibody. At least 10^4 granulocytes were collected for analysis. Results were reported as percentage of annexin-V–positive granulocytes. Mean fluorescence intensity (MFI) was also shown with a histogram to demonstrate the different PS expression densities between AB and CB granulocytes.

Determination of granulocyte apoptosis by DNA fragmentation. Heparinized whole blood from healthy adult volunteers and umbilical cord blood (within 4 h after collection) was mixed with 4.5% dextran in a ratio of 5:1 for RBC sedimentation at 37°C for 30 min. The buffy coat was layered onto the Ficoll-Hypaque density gradient and centrifuged at 400 *g* at room temperature for 30 min. Granulocytes were recovered from the pellet of the gradient centrifugation by hemolytic depletion of contaminated RBC in 0.83% NH_4Cl buffer. The purity and viability were >97%, as we previously described (21). Granulocytes isolated from whole blood with and without TNFα for 6 h were subjected to DNA extraction with 0.5% SDS lysing buffer followed by proteinase K digestion (1 mg/mL) of nuclear protein for 4 h at 60°C, as previously described (22). Total DNA was harvested by the standard phenol-chloroform extraction procedure followed by alcohol precipitation. The DNA (20 μg) was subjected to 1.5% agarose gel electrophoresis. The DNA fragmentation ladders were confirmed by a series of 100-bp DNA markers.

Immunoenzymometric assay for the quantitative measurement of soluble death ligands (CD95L and TNFα). Plasma from umbilical CB and normal AB was collected by centrifugation at 1500 *g* for 30 min. All samples were stored at –70°C before analysis. The plasma CD95L and TNFα levels were measured by sandwich ELISA kit (Medical & Biologic Laboratories Co., Nagoya, Japan) and enzyme amplified sensitivity immunoassay kit (BioSource Europe S.A., Nivelles, Belgium), respectively. The detection sensitivity of CD95L measurement was ≥0.1 ng/mL, and the detection sensitivity of TNFα measurement was ≥4.4 pg/mL. All procedures were performed according to the manufacturers' recommendations.

Measurement of death receptors (CD95 and TNFR) on the surface of granulocytes by flow cytometry. Whole blood (100 μL) was washed once with PBS before staining with PE-conjugated mouse MAb to human CD95 (clone DX2, BD PharMingen), TNFR1 (clone 2H10, Caltag Laboratories, Burlingame, CA), TNFR2 (clone 4D1B10, Caltag Laboratories), and PE-conjugated isotype control IgG (BD Biosciences, San Jose, CA), respectively, for 15 min at room temperature in the dark. After depletion of RBC by ammonium chloride lysis buffer, leukocytes were washed with PBS and fixed with 1% paraformaldehyde for flow cytometric analysis of CD95, TNFR1, and TNFR2 expression on granulocytes, as determined by subtraction of the nonspecific fluorescence as gated by irrelevant isotope IgG fluorescence (23). To investigate the TNFα-induced down-regulation of TNFR1 expression between AB and CB granulocytes, whole blood from AB and CB was also exposed to different doses of exogenous TNFα to assess the dynamic changes of TNFR1 expression.

ELISA for the quantitative measurement of Bcl-2 in granulocytes. Granulocytes were isolated from heparinized whole blood by a 4.5% dextran sedimentation of RBC, followed by a density gradient separation by Ficoll-Hypaque solution as previously described (21,24). Granulocytes were harvested from the bottom layer and washed in PBS before being subjected to disruption of the granulocytes by a lysis buffer containing 10 mM Tris, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 0.7% NP-40. The cell lysates were frozen at –70°C until quantitative measurement of Bcl-2 expression by using an ELISA kit (Endogen Corp., Woburn, MA) was carried out according to manufacturer's direction.

Western blot analysis of cytosolic IkB level and EMSA of NF-κB nuclear translocation in granulocytes. To investigate the activation of NF-κB in blood granulocytes, cytosolic and nuclear protein extracts of blood granulocytes from AB and CB with and without TNFα (50 ng/mL) for 30 min were collected using a modification of the method of Dignam *et al.* (25). Briefly, granulocytes (1×10^7 cells) isolated from dextran sedimentation of RBC, followed by Ficoll-Hypaque density gradient centrifugation, were harvested into a 200 μL ice-cold lysing buffer containing 10 mM Tris, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 0.7% NP-40 for 10 min and centrifuged at 500 *g* for 5 min. The cytosolic fraction was harvested by centrifugation for Western blot analysis of cytosolic IkB level, and the nuclear pellets were further lysed with another lysing buffer containing 40 mM Tris, pH 7.9, 350 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EDTA, 20% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM DTT, 2 μg/mL leupeptin, and 1 μg/mL aprotinin on ice for 20 min and centrifuged at 12,000 *g*, 4°C for 10 min. The resulting cytosolic protein fractions and nuclear protein extracts were aliquoted and stored at –80°C until studies could be performed. Protein concentrations in cytosolic or nuclear protein extracts were determined by Bio-Rad assay kit (Bio-Rad, Hercules, CA).

Cytosolic protein extracts were subjected to Western blot analysis of cytosolic IkB concentrations by a specific antibody directed against IkB. Nuclear extracts were subjected to EMSA assay with double-stranded NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG-3', Promega, Madison, WI). The NF-κB consensus oligonucleotides were 5' end-labeled with [γ^{-32}P]ATP (3000 Ci/mmole at 10 mCi/mL; Amersham Pharmacia Biotech, Uppsala, Sweden) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and purified with QIAquick nucleotide removal kit (QIAGEN, Valencia, CA). Nuclear protein extracts (10 μg) were incubated with buffer containing 10 mM HEPES, pH 7.9, 1 mM DTT, 1 mM EDTA, 80 mM KCl, 20% glycerol, 0.25 mg/mL poly(dI-dC) (Amersham Pharmacia Biotech) and 1.75 *p*mole [γ^{-32}P] labeled oligonucleotide probe (30000–40000 cpm, 2 μL) at 25°C for 30 min. Samples were electrophoresed through 6% polyacrylamide gel in 0.5% TBE (45 mM Tris, 45 mM boric acid,

10 mM EDTA, pH 8.3) at 250 V for 90 min. The gel was dried and the radioreactive bands on dried gels were visualized using Kodak Bio-Max film (Eastman Kodak, Rochester, NY) with intensify screen at –70°C for 16 h (26).

Sample size estimation and statistical analysis. We initially estimated the sample size for this study at 12 pairs of AB and CB samples, based on the power of 0.8, the α level of 0.05, and the effective size of 0.5 (50%). We finally collected 12 plasma pairs for measurement of soluble death ligands and included 11 pairs of AB and CB granulocytes for measurement of cell surface death receptor expression and intracellular anti-apoptotic molecules. Results were presented as mean ± SD and statistically analyzed by *t* test to determine the significance of different parameters between AB and CB samples. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Spontaneous and TNFα-induced apoptosis of granulocytes higher in CB than in AB. Employing a dot plot sketched by size (forward scatter) *versus* granularity (side scatter), a homogenous cluster of granulocytes could be gated for analysis as shown in Figure 1*A*. The dot plot pattern of granulocytes did not change after *in vitro* incubation for 6 h. As determined by flow cytometric analysis of outer cell surface exposure of PS, it was found that some of the neonatal granulocytes underwent spontaneous apoptosis (Fig. 1*B*). Addition of TNFα (50 ng/mL) significantly promoted neonatal granulocyte apoptosis (Fig. 1*B*). In comparison to spontaneous and TNFα-induced granulocyte apoptosis in AB, neonatal granulocytes appeared to have a higher spontaneous apoptosis and TNFα-induced apoptosis (Fig. 1, *B* and *C*). Data summarized from 11 pairs of experiments (Fig. 2*A*) showed that spontaneous apoptosis of neonatal granulocytes in CB was significantly higher than that in AB (11.85 ± 4.15 *versus* 6.59 ± 4.43%, $p = 0.001$), and TNFα-induced granulocyte apoptosis was also found to be higher in CB granulocytes than in AB granulocytes (35.88 ± 9.49% *versus* 22.98 ± 6.11%, $p = 0.002$) (Fig. 2*A*). The higher spontaneous and TNFα-induced granulocyte apoptosis in CB was also demonstrated by DNA fragmentation (Fig. 2*B*).

Higher plasma CD95L and TNFα levels in CB than in AB. To explore the mechanism for a higher rate of early apoptosis in neonatal blood granulocytes, we further assessed death ligands in the AB and CB blood. We found that CD95L was higher in the CB than in AB (0.197 ± 0.048 *versus* 0.135 ± 0.017 ng/mL, $p = 0.001$) (Fig. 3*A*). This was also true for TNFα, the levels of which were also significantly higher in CB than in AB (21.01 ± 1.80 *versus* 8.74 ± 0.87 pg/mL, $p < 0.001$) (Fig. 3*B*).

Lower TNFR1, but not CD95 or TNFR2, expression on neonatal granulocytes. Employing flow cytometric analysis of cell surface receptors CD95, TNFR1, and TNFR2 expression on granulocytes, we found that there was no difference of the CD95 expression on blood granulocytes between CB and AB (92.29 ± 6.08 *versus* 97.58 ± 3.13%, $p > 0.05$). The mean fluorescence intensity (MFI) of CD95 expression on granulocytes did not differ between CB and AB (20.87 ± 3.62 *versus* 24.23 ± 5.64, $p > 0.05$). Expression of TNFR1 was significantly lower on CB granulocytes than that on AB granulocytes (69.98 ± 7.32 *versus* 89.04 ± 3.73%, $p = 0.029$) (Fig. 4). The MFI of TNFR1 expression on CB granulocytes was also significantly lower than that on AB granulocytes (16.49 ± 7.19 *versus* 30.82 ± 12.60, $p = 0.008$). Expression of TNFR2 was significantly lower than the expression of TNFR1 on both CB and AB granulocytes, but was not significantly different between CB and AB granulocytes (6.35 ± 6.20 *versus* 14.77 ± 9.15%, $p > 0.05$). The lower TNFR1 expression on neonatal granulocytes may be due to an intrinsic lower expression or a secondary down-regulation of TNFR1 by higher blood TNFα levels.

To discriminate these two possibilities, we added exogenous TNFα into the AB to mimic the elevation of TNFα levels in CB. It was found that TNFα did cause down-regulation of TNFR1 expression on AB granulocytes in a dose-dependent fashion. As shown in Figure 5, *A* and *B*, the TNFα concentration for a 40–50% decrease of TNFR1 expression on adult granulocytes was estimated around 25 pg/mL, which is close to the level in neonatal blood. A TNFα dosage higher than 50 pg/mL, even up to 50 ng/mL, did not cause further down-regulation of TNFR1 expression. In contrast, neonatal granulocytes had a lower TNFR1 expression in a resting status and revealed a less TNFR1 down-regulation in response to exogenous TNFα (Fig. 5, *C* and *D*). Taken together, these results suggest that a higher TNFα level associated with an intrinsic defect on the dynamic change of membrane TNFR1 expression in neonatal granulocytes may be implicated in faster apoptosis of neonatal granulocytes.

Lower Bcl-2 expression in CB granulocytes than AB granulocytes. Granulocyte apoptosis can be influenced by not only death receptor expression but also anti-apoptotic molecules, especially Bcl-2 expression. We extracted total protein from

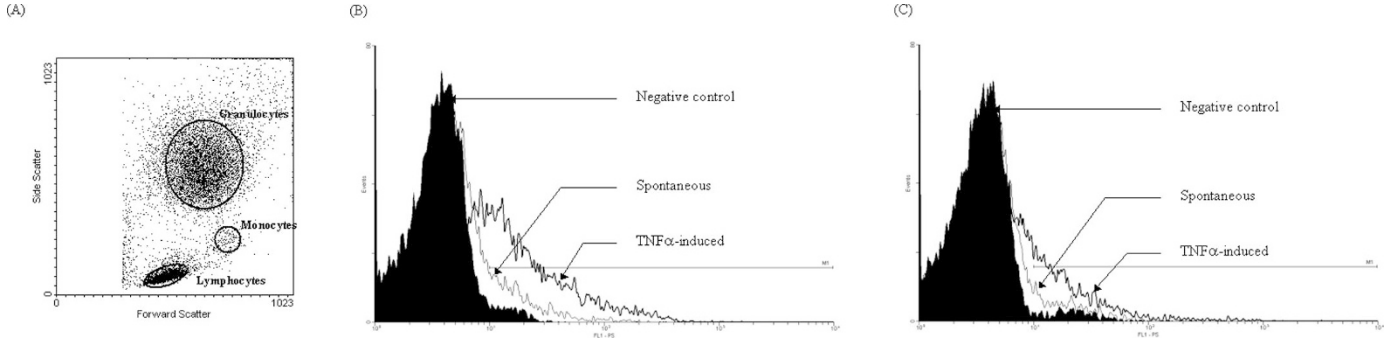

Figure 1. Spontaneous and TNFα-induced blood granulocyte apoptosis in CB and AB as demonstrated by flow cytometric analysis of PS expression. Identification of blood granulocytes was established according to a dot plot sketched by size (forward scatter) *vs* granularity (side scatter) by flow cytometric collection of 10,000 granulocytes, after hemolytic depletion of RBC (*A*). A significantly higher spontaneous and TNFα-induced blood granulocyte apoptosis was found in CB (*B*) than in AB (*C*), as shown by the histogram expression of PS expression.

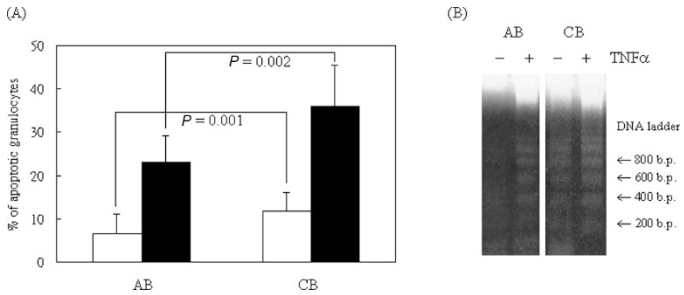

Figure 2. Spontaneous and TNFα-induced blood granulocyte apoptosis as demonstrated by PS expression and DNA fragmentation. Whole bloods from AB and CB were incubated with and without TNFα (50 ng/mL) for 6 h, and apoptotic granulocytes were determined by flow cytometric analysis of PS expression and gel electrophoresis of DNA fragmentation. In a summary of 11-paired experiments, TNFα induced significantly higher ($p = 0.002$) PS expression in cells of CB than AB granulocytes (*A*). As shown by DNA fragmentation, CB granulocytes also revealed higher spontaneous (□) and TNFα-induced (■) DNA fragmentation than AB granulocytes (*B*).

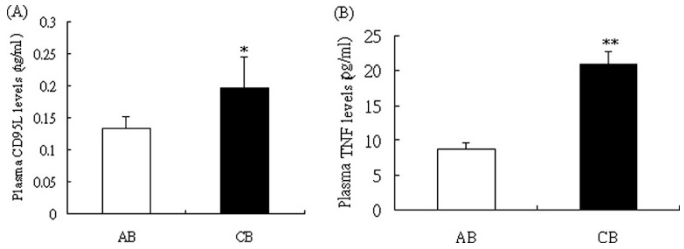

Figure 3. Plasma CD95L and TNFα levels in CB and AB. Plasma was collected from heparinized CB and AB by centrifugation (1500 *g* for 30 min). (*A*) Plasma CD95L levels in CB were significantly higher than those in AB. (*B*) Plasma TNFα levels in CB were also significantly higher than those in AB. Results were assessed by ELISA and presented as mean ± SE. *$p = 0.001$, **$p < 0.001$.

CB and AB granulocytes for measurement of Bcl-2 by ELISA. The Bcl-2 expression (unit per milligram protein) in the CB granulocytes was significantly lower than that in AB granulocytes (4.64 ± 0.51 *versus* 7.24 ± 1.17 Unit/mg protein, $p = 0.032$) (Fig. 6). Experiments were next performed to investigate the effect of TNFα on Bcl-2 expression in granulocytes. We found that TNFα treatment for 6 h could enhance the Bcl-2 expression both in AB and CB granulocytes. However, there was no significant difference of Bcl-2 expression between CB and AB granulocytes after TNFα stimulation (12.17 ± 1.06 *versus* 13.13 ± 2.9 Unit/mg protein, $p = 0.76$).

Higher cytosolic IkB concentration associated with lower NF-κB nuclear translocation in CB granulocytes than AB granulocytes after TNFα stimulation. We harvested cytosolic protein extracts and nuclear protein extracts from CB and AB granulocytes for assessment of cytosolic IkB concentration and NF-κB nuclear translocation. It was found that granulocytes from CB appeared to have higher cytosolic IkB level than those in AB. After TNFα stimulation, the IkB level in CB granulocytes remained higher than AB granulocytes (Fig. 7*A*). NF-κB translocation to the nuclei obtained from AB and CB granulocytes was not significantly different in resting. However, a significantly higher NF-κB translocation into nuclei after TNFα stimulation was detected in AB granulocytes than in CB granulocytes (Fig. 7*B*).

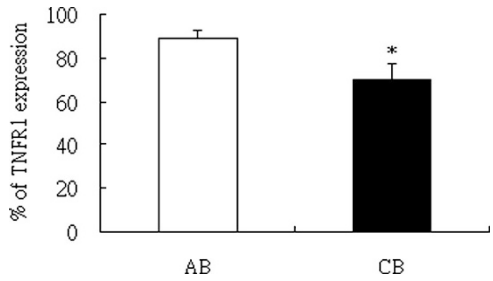

Figure 4. Flow cytometric analysis of TNFR1 expression in CB and AB granulocytes. TNFR1 expression in CB and AB granulocytes determined by a MAb directed against human TNFR1. Data are expressed as percentage of TNFR1-positive granulocytes (%). Results calculated from 11 paired experiments showed that CB granulocytes appeared to have lower TNFR1 expression than AB granulocytes ($p = 0.029$).

DISCUSSION

Using a whole-blood model, we found that both spontaneous and TNFα-induced granulocyte apoptosis was higher in CB than in AB. This result is compatible with the study by Uguz *et al.* (9), which showed that spontaneous apoptosis of CB granulocytes was higher than that from AB up to 48 h of culture, but contradictory to the study by Allgaier *et al.* (8), which showed that spontaneous and anti-Fas IgM Ab (300 ng/mL) induced apoptosis of granulocytes was higher in AB by 24 h of culture. The discrepancy among these studies may be due to different granulocyte isolation methods and apoptotic-inducing agents. Considering that granulocyte apoptosis can be regulated by both soluble death ligands and cell surface death receptors (4,5,9,14,15,27–30), we studied plasma TNFα and CD95L levels and cell surface TNFR and CD95 as well as intracellular anti-apoptotic molecules, Bcl-2, and NF-kB in AB and CB granulocytes. Results showed that higher death ligands, TNF-α and CD95L, associated with lower anti-apoptotic Bcl-2 and NF-κB levels were found in CB than in AB. This suggests that higher extracellular death ligands in conjunction with lower intracellular anti-apoptotic molecules in neonatal blood may contribute to the faster apoptosis in neonatal granulocytes. A better clarification on the homeostasis among soluble death ligands, death receptors, and anti-apoptotic molecules may be useful to control neonatal granulocyte apoptosis and possibly prevent granulocytopenia in neonates with stress or infection.

Evidence has previously shown that CD95 is constitutively expressed on normal human phagocytes (4,5). Our results demonstrated that CD95 expression on granulocytes did not differ between CB and AB. Similarly, TNFR2 expression was also not significantly different between CB and AB granulocytes (6.35 ± 6.20 *versus* 14.77 ± 9.15%, $p > 0.05$). However, the TNFR1 expression on neonatal granulocytes was lower than that on adult ones and revealed less down-regulation after TNFα stimulation. This might be related to an intrinsic defect on the dynamic change of membrane TNFR1 expression of neonatal granulocytes.

Effects of TNFα on promotion of, or prevention from, granulocyte apoptosis have been variably described in different studies (14,15,27,29,30). We found that TNFα could promote blood granulocyte apoptosis both in AB and CB. The effect

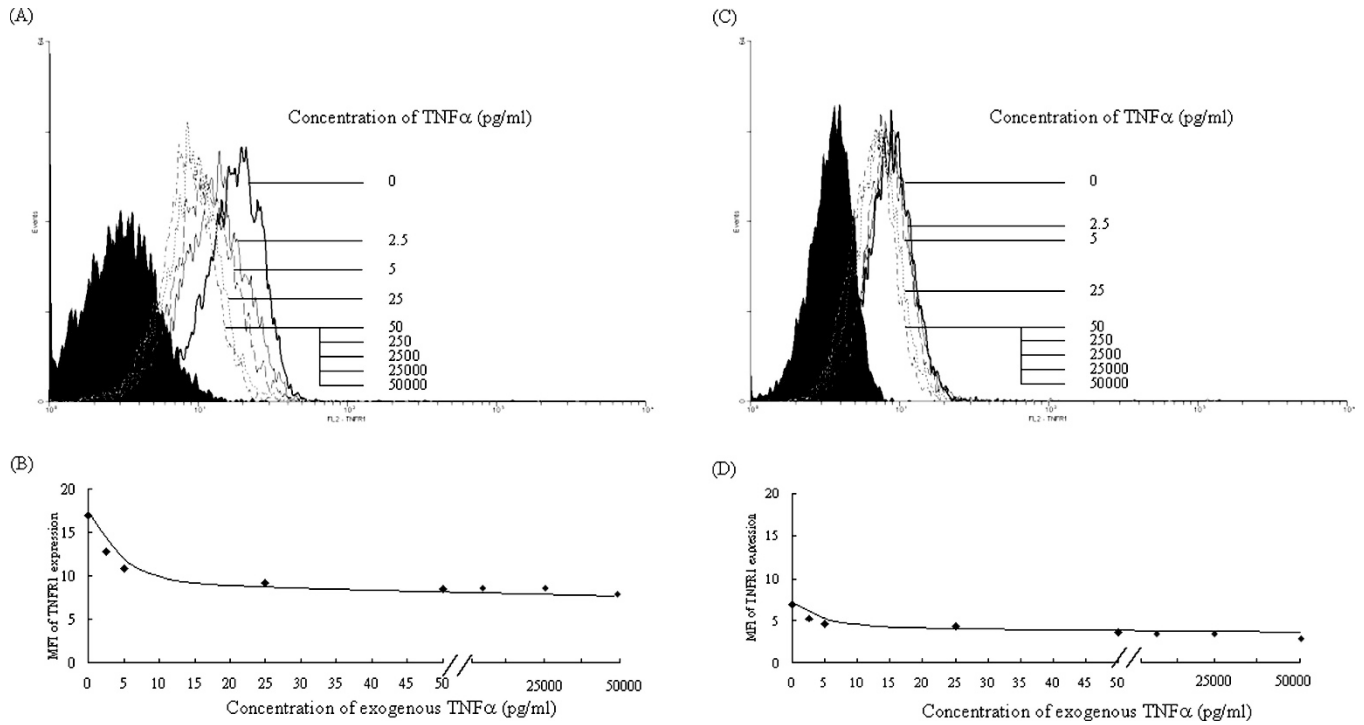

Figure 5. Down-regulation of TNFR1 expression in granulocytes by exogenous TNFα. Flow cytometric analysis of TNFR1 expression in granulocytes was performed after addition of exogenous TNFα (0, 2.5, 5, 25, 50, 250, 2,500, 25,000, and 50,000 pg/mL). (*A*) A representative study showed a dose-dependent down-regulation of TNFR1 expression in AB by exogenous TNFα. (*B*) A sketch from five replicate experiments showed that a 50% down-regulation of TNFR1 expression in AB granulocytes by exogenous TNFα was located at 25 pg/mL. (*C*) A representative study revealed less TNFR1 expression in CB by exogenous TNFα. (*D*) A sketch from five replicate experiments showed minimal down-expression after TNFα stimulation of CB granulocytes.

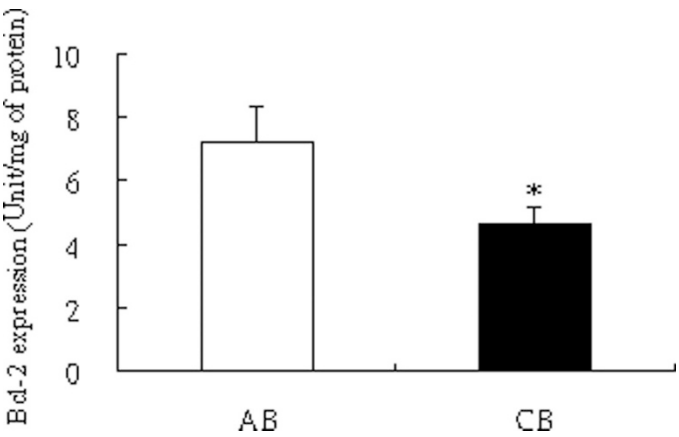

Figure 6. Expression of Bcl-2 in CB and AB granulocytes. Granulocytes from CB and AB were subjected to protein extraction for detection of Bcl-2 expression by ELISA. Data presented are calculated from 12 paired samples. The results were corrected by equalization with total protein levels (U/mg protein). $*p = 0.032$.

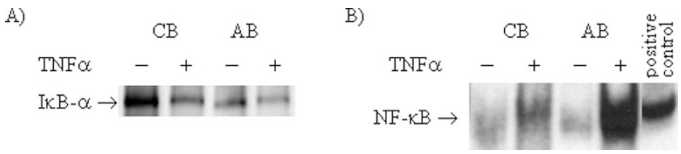

Figure 7. Cytosolic IkB concentration and nuclear translocation of NF-κB in AB and CB granulocytes. Cytosolic and nuclear protein extracts of granulocytes from CB and AB with and without TNFα treatment for 30 min were collected for Western blot analysis of IkB concentration, and EMSA analysis of NF-kB expression. A representative study from four reproducible paired experiments showed a higher cytosolic IkB concentration (*A*) associated with lower NF-κB activation (*B*) in CB granulocytes than in AB granulocytes.

was found to be more significant on CB granulocytes. To investigate whether the nuclear translocation of NF-κB after TNFα stimulation was related to the different susceptibility of granulocyte apoptosis between AB and CB, we found that AB granulocytes did have a significantly higher nuclear translocation of NF-κB after TNFα stimulation than CB granulocytes. In contrast, neonatal granulocytes have a higher cytosolic IkB level associated with lower NF-kB nuclear translocation. This might, at least in part, explain why CB granulocytes tend to have higher spontaneous and TNFα-induced apoptosis. A recent study by Bureau *et al.* (31) also showed that the basal NF-k B activity responsible for homeostasis of spontaneous apoptosis of granulocytes was regulated by the expression of distinct Bcl-2 family proteins. In our study, we found that higher spontaneous neonatal granulocyte apoptosis was associated with lower intracellular Bcl-2 level, but the higher TNFα-induced neonatal granulocyte apoptosis was not associated with significantly lower intracellular Bcl-2 levels. This suggests that the mechanism of spontaneous apoptosis is different from that of stimulus-induced apoptosis in neonatal granulocytes. Further studies to clarify the anti-apoptotic pathways of granulocytes under stimulation of different death ligands may provide a better strategy for preventing granulocytopenia in neonates under stress or with infections.

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