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Insulin-Like Growth Factor 1 Promotes Cord Blood T Cell Maturation and Inhibits Its Spontaneous and Phytohemagglutinin-Induced Apoptosis Through Different Mechanisms

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Functional immaturity of neonatal T cells is related to their immature phenotype, with the majority of neonatal T cells of naive (CD45RA+ T cells). The progression of T cells from naive cells to effector cells is dependent on the survival of Ag-specific T cells and their resistance to apoptosis. In this study, we showed for the first time that insulin-like growth factor 1 (IGF-1) converted cord blood CD45RA+ T cells to CD45RO+ T cells and inhibited cord blood T cell apoptosis. We found cord blood T cells stimulated with PHA would result in gradual loss of CD45RA and gain of CD45RO expression. IGF-1 further increased the loss of CD45RA and enhanced CD45RO expression in PHA-stimulated cord blood T cells. In addition, IGF-1 prevented cord blood T cells from spontaneous apoptosis through a mechanism other than Fas/Fasl. In PHA-activated cord blood T cells, IGF-1 prevented both naive (CD45RA+) and memory/mature (CD45RO+) T cells from apoptosis. Moreover, cord blood T cells cultured with IGF-1 and PHA had a higher resistance to anti-Fas-induced apoptosis as compared with PHA-activated cord blood T cells. IGF-1 also significantly inhibited PHA-induced Fas expression on cord blood T cells. These results demonstrate that IGF-1 promotes the maturation and maintains the survival of cord blood T cells. Its antiapoptotic effect in PHA-activated cord blood T cells may be mediated through the down-regulation of Fas expression.


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4 Abbreviations used in this paper: IGF-1, insulin-like growth factor 1; MNC, mononuclear cell. PC5, PE-cyanin 5.1; PI, propidium iodide; PI 3-kinase, phosphorylcholine 3-kinase.
infants, with prior written informed consent of their parents. The protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Hong Kong. All samples were collected in heparinized flask. Cord blood MNC were isolated from whole blood by centrifugation, using Ficoll-Hypaque gradients purchased from Pharmacia Biotech (Uppsala, Sweden). The mononuclear cells at the interface were collected, washed three times with PBS, and resuspended at a density of 1 x 10^6 cells/ml in a serum- and hormone-free medium, DMEM Nutrient Mixture F-12 Ham (DME/F-12) obtained from Sigma (St. Louis, MO), which did not contain insulin, IGF-1, or other hormones, and supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin. Cell viability, as measured by trypan blue exclusion, was >99%.

Analysis of T cell phenotype changes

Cord blood MNC (1 x 10^6) were cultured in the presence or absence of PHA (1 μg/ml) with and without IGF-1 (100 ng/ml) purchased from R&D Systems (Minneapolis, MN), and incubated in DME/F-12 medium for 9 days with the culture medium replenished every 3 days. Cells were collected at defined times after culture, and then stained with different combinations of FITC-, PE-, or PE-cyanin 5.1-conjugated mAbs (CD3, CD4, CD8, CD45RA, and CD45RO) and isotype controls for 20 min in room temperature. mAbs and isotype controls were from Immunotech (Marseille Cedex, France). Three-color flow cytometric analysis was used to analyze T cell phenotype changes.

Induction and detection of T cell apoptosis

Cord blood MNC (1 x 10^6) were cultured in the presence or absence of PHA (1 μg/ml) with and without IGF-1 (100 ng/ml), and incubated in serum-free medium (DME/F-12) for 3 or 4 days. In some experiments, cord blood MNC were cultured with PHA and/or IGF-1 for 3 days, and then the anti-human CD95/Fas mAb (100 ng/ml, clone CH-11; Upstate Biotechnology, Lake Placid, NY) was added for 24 h to induce apoptosis. To define whether cord blood MNC underwent apoptosis or necrosis, the annexin-V and PI detection method was used (21–25).

In the early phase of apoptosis, the integrity of the cell membrane is maintained, but cells will lose the asymmetry of their membrane phospholipids. Phosphatidylycerine, a negatively charged phospholipid located in the inner leaflet of the plasma membrane, becomes exposed at the cell surface. Annexin-V, a calcium- and phospholipid-binding protein, binds preferentially to phosphatidylycerine, with high affinity. Apoptotic cells are stained by annexin-V before the dying cell changes its morphology and hydrolyzes its DNA (22–26). FITC annexin-V is a powerful tool for measuring apoptosis on a single cell basis by flow cytometry (24, 25). Staining cells simultaneously with FITC annexin-V and the PI, a nucleic acid dye and its DNA (22–26). FITC annexin-V is a powerful tool for measuring apoptosis, but not necrosis in this culture system, we analyzed the apoptosis of different T cell subsets using annexin-V and mAbs of various CD markers.

Cells were stained with PE-cyanin 5.1-conjugated-CD4/CD8, PE-conjugated CD45RA/CD45RO mAbs, and their isotype controls for 20 min in room temperature. After one washing step with cold PBS, cells were resuspended in 495 μl of ice-cold binding buffer (10 mM HEPES buffer, pH 7.5, 150 mM NaCl, 5 mM KCl, 1 mM MgCl 2, 1.8 mM CaCl 2). A total of 5 μl of FITC annexin-V (1 mg/ml; Immunotech) was added to the cell suspension. The cell suspension was kept on ice and incubated for 10 min in the dark, and then analyzed by flow cytometry.

Flow cytometric analysis

Flow cytometric analysis was performed with a Coulter Epics Elite Flow Cytometer (Couler, Miami, FL). The machine was optimized daily using Flow-Check beads and Standard-Brite beads (Couler). Cyto-Comp Reagent Kit (Couler) was used for adjusting color compensation settings on flow cytometer for two- or three-color analysis, according to the manufacturer’s instructions. Ten thousand events per sample were collected into listmode files and analyzed by EXPO II Cytometer Analysis Software.

Statistical methods

To determine difference between paired groups, the Wilcoxon signed rank sum test was used. The Mann-Whitney U statistic test was used to determine difference between unpaired groups.

Results

IGF-1 converts CD45 isoform from CD45RA to CD45RO expression on cord blood T cells

Our previous study demonstrated that IGF-1 promoted the functional maturation of cord blood T cells with respect to IFN-γ production and proliferation (12). In this study, we further observed the effects of IGF-1 on phenotype changes of CD45 isoform on cord blood T cells. The large majority of cord blood T cells expressed CD45RA (CD3^+CD45RA^+, 88.8 ± 1.7%), while only a minority of cord blood T cells expressed memory/mature phenotype (CD3^+CD45RO^+, 5.5 ± 1.6%). IGF-1 alone did not affect the CD45 isoform expression on cord blood T cells (data not shown). After cord blood MNC were stimulated with PHA over 9 days of culture, CD45RA^+ T cells gradually lost CD45RA expression and gained CD45RO expression (Fig. 1). IGF-1 signifi-

FIGURE 1. The phenotype changes of CD45 isoforms on cord blood T cells at different times of culture. The upper panels (A–D) indicate PHA-stimulated CD3^+ cells on days 0, 3, 6, and 9. The lower panels (E–H) indicate IGF-1 + PHA-stimulated CD3^+ cells on days 0, 3, 6, and 9. Phenotype changes were measured by three-color flow cytometric analysis. Results shown are of one case representative of four different cases.
cantly increased the loss of CD45RA and enhanced CD45RO expression on PHA-stimulated cord blood T cells during the same period (Fig. 1).

Cord blood MNC undergo apoptosis during culture in serum-free medium

To define whether cord blood MNC underwent apoptosis or necrosis, we stained cord blood MNC simultaneously with annexin-V and PI. After 4 days of culture in serum-free medium, most unstimulated cord blood MNC were annexin-V positive and PI negative, and a minority of unstimulated cord blood MNC was annexin-V positive and PI positive (Fig. 2). Similar results were shown in PHA-stimulated cord blood MNC after 4 days of culture (Fig. 2). These results indicated that both unstimulated and PHA-stimulated cord blood MNC underwent apoptosis, not necrosis in our culture system.

IGF-1 is a survival factor preventing cord blood T cell from spontaneous apoptosis

IGF-1 is a survival factor for many cell types (14, 18, 19, 26), but for T cells it is not clear. To determine whether IGF-1 is a survival factor for T cells, T cell apoptosis was studied using cord blood MNC cultured in serum-free medium with or without IGF-1. After 4 days of culture without any stimulator in serum-free medium, cord blood T cells underwent spontaneous apoptosis, with 36% of CD4+ and 30% of CD8+ cells annexin-V positive (Fig. 3). In further analysis of CD45RA/CD45RO subpopulations, CD45RO+ cells were more susceptible to spontaneous apoptosis as compared with CD45RA+ cells (Fig. 3). Thirty-four percent of CD4+CD45RA+ cells and 28% of CD8+CD45RA+ cells were annexin-V positive, whereas 53% of CD4+CD45RO+ cells and 85% of CD8+CD45RO+ cells were annexin-V positive (Fig. 3). In contrast, when cultured with IGF-1 for 4 days, apoptosis of cord blood T cells was significantly reduced. IGF-1 significantly prevented CD4+ and CD8+ cells from apoptosis (Fig. 3). It significantly inhibited apoptosis of all T cell subsets, including CD4+CD45RA+, CD4+CD45RO+, CD8+CD45RA+, and CD8+CD45RO+ cells (Fig. 3). These data clearly establish that IGF-1 is a survival factor for resting cord blood T cells.

IGF-1 inhibits PHA-induced apoptosis of cord blood T cells

Since PHA-induced cord blood MNC underwent apoptosis in our culture system (Fig. 2), we further analyzed the PHA-induced apoptosis in the subpopulations of cord blood T cells and observed the effect of IGF-1 on PHA-induced apoptosis of cord blood T cells. When cord blood MNC were cultured with PHA for 4 days, 58% of CD4+ and 44% of CD8+ cells underwent apoptosis (Fig. 4). CD45RO+ cells had a higher susceptibility to apoptosis as compared with CD45RA+ cells (Fig. 4). Fifty-five percent of CD4+CD45RA+ cells and 40% of CD8+CD45RA+ cells were annexin-V positive, whereas 61% of CD4+CD45RO+ cells and 66% of CD8+CD45RO+ cells were annexin-V positive (Fig. 4). In the presence of IGF-1, the PHA-induced apoptosis of cord blood T cells was significantly reduced in both CD4+ and CD8+ cells (Fig. 4). For the subpopulations of CD4+ and CD8+ cells, IGF-1 also significantly reduced the apoptosis of CD4+CD45RA+, CD4+CD45RO+, and CD8+CD45RO+ cells. The reduction of PHA-induced apoptosis of CD8+CD45RA+ cells by IGF-1 did not reach significance (Fig. 4). These results demonstrate that IGF-1 can prevent almost all subsets of cord blood T cells from PHA-induced apoptosis.

FIGURE 2. Cord blood MNC undergo apoptosis during the culture in serum-free medium. Cord blood MNC (1×10⁶) were cultured in serum-free medium with or without PHA (1μg/ml) for 4 days. Annexin-V and PI detection method was used to distinguish apoptosis and necrosis. Results shown are representative of five different cases. A. Shows results from unstimulated cord blood MNC. B. Shows results from PHA-stimulated cord blood MNC.

FIGURE 3. Effect of IGF-1 on spontaneous apoptosis of different types of cord blood MNC. Cord blood MNC (1×10⁶) were cultured in serum-free medium with or without IGF-1 (100 ng/ml) for 4 days. Cord blood MNC were stained with annexin-V and different CD mAb and analyzed by flow cytometry, as described in Materials and Methods. Results shown are of 10 different cases (mean ± SEM, n = 10). *, p < 0.05.

FIGURE 4. Effect of IGF-1 on PHA-induced apoptosis of different types of cord blood MNC. Cord blood MNC (1×10⁶) were cultured in serum-free medium with PHA (1μg/ml) and/or IGF-1 (100 ng/ml) for 4 days. Results shown are of 10 different cases (mean ± SEM, n = 10). *, p < 0.05.
IGF-1-treated cord blood T cells are resistant to anti-Fas-induced apoptosis

Anti-Fas mAb (clone CH-11), with similar function as FasL, can induce T cell apoptosis through the interaction with Fas Ag expressed on T cells (27, 28). We studied the resistance of IGF-1-treated cord blood T cells to anti-Fas-induced apoptosis by using anti-human Fas Ab. Cord blood MNC were cultured with IGF-1 and/or PHA for 3 days, then anti-Fas Ab was added for 24 h to induce apoptosis. Compared with unstimulated cord blood T cells, PHA-treated cord blood T cells were more susceptible to anti-Fas-induced apoptosis (Fig. 5). A total of 83% of CD4<sup>+</sup> cells and 61% of CD8<sup>+</sup> cells were annexin-V positive when cord blood MNC were cultured with PHA for 3 days and then challenged with anti-Fas Ab (Fig. 5). In further analysis of subpopulations of CD4<sup>+</sup> and CD8<sup>+</sup> cells, 84% of CD4<sup>+</sup>CD45RA<sup>+</sup> cells and 83% of CD4<sup>+</sup>CD45RO<sup>+</sup> cells were more susceptible to anti-Fas-induced apoptosis (Fig. 5). A total of 83% of CD4<sup>+</sup> cells and 61% of CD8<sup>+</sup> cells were annexin-V positive when cord blood MNC were cultured with PHA for 3 days and then challenged with anti-Fas Ab (Fig. 5). In further analysis of subpopulations of CD4<sup>+</sup> and CD8<sup>+</sup> cells, 84% of CD4<sup>+</sup>CD45RA<sup>+</sup> cells and 83% of CD4<sup>+</sup>CD45RO<sup>+</sup> cells showed annexin-V positive, whereas 60% of CD8<sup>+</sup>CD45RA<sup>+</sup> cells and 82% of CD8<sup>+</sup>CD45RO<sup>+</sup> cells were annexin-V positive (Fig. 5).

When cord blood MNC were cultured with PHA and IGF-1 for 3 days before challenged with anti-Fas Ab, cord blood T cells had a high resistance to anti-Fas-induced apoptosis. CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, CD4<sup>+</sup>CD45RA<sup>+</sup> cells, CD4<sup>+</sup>CD45RO<sup>+</sup> cells, and CD8<sup>+</sup>CD45RO<sup>+</sup> cells all showed significantly less anti-Fas-induced apoptosis in the presence of IGF-1 (Fig. 5). Although CD8<sup>+</sup>CD45RA<sup>+</sup> cells did not show a significant reduction of anti-Fas-induced apoptosis in the presence of IGF-1, there was still a trend toward reduction in apoptosis (Fig. 5). These results indicate that IGF-1 can inhibit anti-Fas-induced T cell apoptosis.

Anti-Fas cannot induce the apoptosis of cord blood T cells in the absence of PHA

Contrasting to that of PHA-treated cord blood T cells, anti-Fas cannot induce apoptosis of cord blood T cells in the absence of PHA (Fig. 6). There were no significant differences between the anti-Fas-treated and the non-anti-Fas-treated groups in the presence or absence of IGF-1 (Fig. 6).

Effect of IGF-1 on Fas expression on cord blood T cells

To understand how IGF-1 can inhibit cord blood T cell apoptosis as shown above, we studied the effect of IGF-1 on Fas expression on cord blood T cells. After cord blood MNC were cultured with IGF-1 and/or PHA for 3 days, Fas expression was analyzed by flow cytometry. In unstimulated cord blood MNC, only about 2% of CD4<sup>+</sup> cells and 2% of CD8<sup>+</sup> cells expressed Fas Ags (Fig. 7). For CD45RA/CD45RO subsets, a significantly higher proportion of CD45RO<sup>+</sup> T cells expressed Fas Ags compared with CD45RA<sup>+</sup> T cells (CD4<sup>+</sup>CD45RO<sup>+</sup> Fas<sup>+</sup> vs CD4<sup>+</sup>CD45RA<sup>+</sup> Fas<sup>+</sup>) (Fig. 7).
The phenotype change of CD45 isoform from CD45RA to CD45RO in neonatal T cells (Fig. 8) can be a result of preferential survival of CD45RO+ T cells over CD45RA+ T cells, without the conversion of CD45 isoforms. However, compared with CD45RA+ T cells, CD45RO+ T cells were more prone to both spontaneous and PHA-induced apoptosis either in the presence or absence of IGF-1 (Figs. 3 and 4). These results are consistent with published findings in adult T cells (6, 7, 29–31). Therefore, the phenotype change of CD45 isoforms induced by IGF-1 was not due to preferential survival of CD45RO+ T cells. It was indeed conversion of CD45 isoform from CD45RA to CD45RO induced by IGF-1. Our previous study also demonstrated that IGF-1 could increase neonatal IFN-γ production and T cell proliferation to normal adult level (12). These results together establish that IGF-1 can promote cord blood T cell maturation.

We demonstrated that IGF-1 prevented cord blood T cells from spontaneous apoptosis when cultured in serum-free medium (Fig. 3). IGF-1 significantly inhibited the spontaneous apoptosis of all the subsets of cord blood T cells (Fig. 3). These results establish that IGF-1 is a survival factor for resting cord blood T cells. The mechanism involved in the protective effect of IGF-1 against spontaneous apoptosis of cord blood T cells is not clear. It is known that Fas/FasL system is mainly involved in T lymphocyte activation-induced cell death (32–34). IGF-1 did not inhibit Fas expression on unstimulated cord blood T cells, but in fact slightly increased Fas expression on some subsets of cord blood T cells (Fig. 7). Anti-Fas mAb (clone CH-11), with similar function as FasL, can induce T cell apoptosis through the interaction with Fas Ag expressed on T cells (27, 28). We further found that anti-Fas could not induce apoptosis of cord blood T cells in the absence of PHA (Fig. 6). These results suggest that the Fas/FasL system is not involved in the protective effect of IGF-1 against spontaneous apoptosis of cord blood T cells and other mechanisms are involved in this process.

Teague et al. and Marrack et al. (35, 36) reported recently that IL-6 could rescue resting mouse T cells from apoptosis. Our previous study established that IGF-1 alone could significantly induce IL-6 production from cord blood MNC (12). Therefore, IL-6 may be involved in the protective effect of IGF-1 against spontaneous apoptosis of cord blood T cells. Additional experiments can be designed to test this hypothesis.

Recent studies demonstrated that phosphatidylinositol 3-kinase (PI 3-kinase) is a critical cellular protein that prevents apoptotic cell death in many cell types, including hemopoietic progenitor cells and T lymphocytes (15, 16, 37). IGF-1 can protect myeloid progenitor cells from apoptosis by activating PI 3-kinase, and the inhibition of IGF-1-induced PI 3-kinase results in apoptosis (16). These results suggest that the activation of PI 3-kinase by IGF-1 is a critical step in protecting cells from apoptosis. In cord blood T cells, whether the antiapoptotic effect of IGF-1 is also mediated through activation of PI 3 kinase needs further study.

Fas and FasL play an important role in T cell activation-induced cell death (32–34). The expression of Fas on T cells can be up-regulated by antigenic or mitogenic stimulation (6, 31, 38). Our results shown in this work indicated that PHA not only increased Fas expression on all subsets of cord blood T cells (Fig. 8), but also induced apoptosis of these cells (Fig. 4). These results suggest that cord blood T cells can employ the Fas/FasL system to proceed to apoptosis. We then found IGF-1 could inhibit apoptosis of PHA-activated cord blood T cells. It significantly prevented almost all subsets of these cord blood T cells from apoptosis (Fig. 4), and inhibited PHA-induced Fas expression on the surface of these cells (Fig. 8). These results suggest that the antiapoptotic effect of IGF-1 in PHA-induced cord blood T cell apoptosis may be mediated through down-regulation of Fas. We found that IGF-1 significantly inhibited anti-Fas-induced T cell apoptosis in PHA-activated cord

### FIGURE 8
Effect of IGF-1 on Fas expression on different types of cord blood MNC. Cord blood MNC (1 × 10⁶) were cultured in serum-free medium with PHA (1 μg/ml) and/or IGF-1 (100 ng/ml) for 3 days. Results shown are of 10 different cases (mean ± SEM, n = 10). *, p < 0.05.
blood MNC. This further supports that IGF-1 can prevent apoptosis of PHA-activated cord blood T cells through down-regulation of Fas expression. In contrast, the mechanism through which IGF-1 protects spontaneous apoptosis of cord blood T cells is not through Fas/FasL system; hence, the degrees of protection in these two conditions are quite different.

The mechanism of the down-regulation of Fas expression by IGF-1 is still not clear. However, more recently, Ayroldi et al. (39) reported that IL-6 could inhibit Fas expression of anti-CD3-induced OVA-specific T cell line 3D0 as well as its apoptosis. Our previous study indicated that IGF-1 could significantly increase IL-6 mRNA expression and protein production in PHA-activated cord blood MNC (12). These results suggest that the down-regulation of Fas expression on cord blood T cells by IGF-1 may be mediated through IL-6. The mechanism involved in the down-regulation of Fas expression deserves further study.

In conclusion, IGF-1 not only prevents cord blood T cell maturation, but also maintains cord blood T cell survival. IGF-1, as a survival factor, can protect cord blood T cells from spontaneous apoptosis, and its mechanism is unclear, but not mediated through the Fas/FasL system. IGF-1 can also inhibit PHA-activated cord blood T cell apoptosis, and this effect is mediated through the down-regulation of Fas expression. Its antiapoptotic effect in activated T cells suggests that IGF-1 may have an important role in antigenic T cell clonal expansion. IGF-1 may be useful for expansion of T cells for adoptive immunotherapy, and its role in combating certain viral infections that result in T cell apoptosis such as HIV also deserves further study.

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References