Comparative intracellular cytokine production by *in vitro* stimulated T lymphocytes from human umbilical cord blood (HUCB) and adult peripheral blood (APB)

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To date over 400 HUCB transplants have been reported from different centers. It has been suggested that there is a reduced graft-versus-host-disease (GVHD) with HUCB compared to bone marrow transplantation. Since cytokine production by a cell is an indication of the cells function it is important to determinate the differences between APB and HUCB with respect to production of these soluble factors. Our aim was to analyse the intracellular cytokine production by HUCB and APB T lymphocytes with and emphasize on their possible role in GVHD.

Heparinized HUCB samples from 8 normal full-term deliveries and 10 normal blood donors were stimulated 4 hours at 37°C and 5% CO_2 with phorbol 12-myristate 13-acetate (PMA) and Ionomycin in the presence of brefeldine. Afterwards cells were stained with CD3, CD4 or CD8 in different combinations. Finally, after cell permeabilization, cells were stained with Il-2, Il-4 or IFN-γ. Data acquisition was performed on a FACScan flow cytometer.

Compared to APB, HUCB T lymphocytes produced less Il-2, Il-4 and IFN-γ. In HUCB, Il-2, Il-4 and IFN-γ were produced predominantly by CD4+ T cells. In APB, Il-2 and Il-4 were also produced predominantly by CD4+ cells compared with CD8+ T lymphocytes, however, IFN-γ was produced by both CD4+ and CD8+ T cells.

These results indicate that there are clear differences in the cytokine profile between T cells in APB and HUCB.

1. Introduction

Human umbilical cord blood (HUCB) is a rich source of primitive hematopoietic stem cells and progenitor cells [4] with extensive proliferative and self-reneval capacity *ex vivo* [3] and possibly *in vivo* [10]. The first sucessfull HUCB transplantation was performed in 1988 in a patient with Fanconi anemia [10]. Subsequently, HUCB has been used in both related [21] and unrelated bone marrow transplantation (BMT) [16]. A potential advantage of this procedure is a lower possible incidence and severity of graft-vs-host disease (GVHD).

GVHD is a complication of allogeneic BMT (ABMT) and peripheral blood transplantation in which recipient mature T cells and maybe natural killer (NK) cells play an important role [9]. Both of these cells produce cytokines which are important mediators of this process [18]. Secretion of a variety of pro-imflamatory cytokines such as Il-2, TNF-α and IFN-γ, by the host-reactive T cells mediate tissue damage either directly or via activation of other effector cells such as cytotoxic T cells, antibody producing B cells and NK

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cells. Acute GVHD has been associated with a Th1 response, producing high amounts of IFN-γ, while chronic GVHD has been associated with a Th2 response, with high amounts of Il-4 and Il-5 [15]. Therefore, it is important to investigate if altered cytokine production of HUCB could justify the reduced incidence and severity of GVHD after HUCB transplantation. Il-2 is an important regulatory cytokine and there are some conflicting results regarding the expression of this cytokine in HUCB and APB [5,11]. Han et al. [11] found that the frequency of T HUCB producing Il-2 was higher than that of APB T lymphocytes. This is consistent with a previous report that HUCB T cells produced more Il-2 than APB T lymphocytes [19] but differs from a recent report which examined the intracellular cytokine profile of mononuclear HUCB [5].

In this study we have analysed the intracellular expression of interleukin-2 (Il-2), interleukin-4 (Il-4) and interferon-γ (IFN-γ) after phorbol 12-myristate 13-acetate (PMA)-and-ionomycin activation in HUCB and adult peripheral blood (APB) T lymphocytes by flow cytometry. In all experiments we have used heparin anticoagulated whole blood and T lymphocytes were selected according to their CD3 expression and low side scatter (SSC). Therefore, a comprehensive phenotypic analysis of cytokine producing cells has been performed using CD4/CD8 markers.

2. Material and methods

2.1. Samples

Heparinized HUCB samples were obtained from the placenta of eight normal full-term deliveries, following ethical committee approval. Heparinized APB were obtained from ten normal blood donors.

2.2. Intracellular cytokine analysis

Whole HUCB and APB samples were diluted with RPMI 1640 without serum. Cells were stimulated for 4 hours at 37°C and 5% CO_2 with phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldine (BFA) (10 μg/ml) which inhibits the intracellular transport of proteins, so antigens and cytokines produced during activation will be retained inside the cell. Cultured cells were washed twice in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) and then stained with monoclonal antibodies (MoAb) to the following cell-surface markers: CD3-FITC and PE, CD4-PerCP, CD8-PerCP and CD69-PE. All MoAb were purchased from Becton Dickinson (San José, CA, USA). Samples were incubated for 15 minutes at room temperature in the dark, in the presence of 20 μl of each of the above mentioned MoAb. Afterwards, 2 ml of FACS lysing solution (Becton Dickinson) was added and the samples incubated for 10 minutes in the same conditions. Then the samples were centrifuged for 5 minutes at 2000 rpm and the cells pellet was fixed and permeabilized with FACS Permeabilizing solution (Becton Dickinson) according to manufacturer's instructions. Finally, the cells were incubated with CD69 and with MoAb to the following cytokines: Il-2-FITC, Il-4-PE and IFN-γ-FITC purchased from Becton Dickinson. An isotype-specific anti-mouse IgG1 or IgG2 were used in all experiments to assess background fluorescence. In all experiments we used the next combinations of markers: Il-2-FITC/CD3-PE/CD4-PerCP; Il-2-FITC/CD3-PE/CD8-PerCP; CD3-FITC/Il-4-PE/CD4-PerCP; CD3-FITC/Il-4-PE/CD8-PerCP; IFN-γ-FITC/CD3-PE/CD4-PerCP; IFN-γ-FITC/CD3-PE/CD8-PerCP and CD3-FITC/CD69-PE/-. Data acquisition was performed on a FACScan flow cytometer (Becton Dickinson) equipped with an argon ion laser tuned at 488 nm and 15 mW. Acquisition was performed in two consecutive steps: in the first step a total of 10000 events/tube were acquired, and in the second step, cells were acquired through a "live gate" drawn on the side-scatter (SSC)/CD3-positive cells. In all experiments we used an activation control in the same conditions as previously described, but omitting BFA. Then cells were staining with CD69 (and early activation marker) and CD3. We considered a correct activation if more than 90% of T cells expressed CD69. For analysis the PAINT-A-GATE PRO software program (Becton Dickinson) with the polynomial SSC transformation capability was used. The calibration of the instrument as well as fluorochrome compensation was performed using CD4/CD8/CD3 lymphocytes prior to data acquisition using previously well established protocols.

2.3. Statistical analysis

Student's t-tests were used to compare HUCB lymphocyte versus APB lymphocytes cytokine production. P values < 0.05 were considered to be significant.

3. Results

There were no differences in the percentage of T lymphocytes between HUCB ($72.0 \pm 9.7\%$) and APB ($73.5 \pm 3.8\%$). There were more CD4+ lymphocytes ($53.8 \pm 7.8\%$) and less CD8+ T cells (16.0 ± 4.8) in HUCB than in adult PB (APB) (45.3 ± 5.2 and $29.6 \pm 4.7\%$, respectively) giving a higher CD4 : CD8 ratio in HUCB. After stimulation with PMA and Ionomycin more than 90% of T lymphocytes in HUCB and APB in all experiments expressed the early activation marker CD69.

3.1. Cytokine expression on HUCB and APB

Figure 1 shows the expression of Il-2, Il-4 and IFN-γ on individual samples of HUCB and APB T lymphocytes. The gates used for analysis of T lymphocytes are also shown in Fig. 1. These results show that there is reduced production of Il-2, Il-4 and IFN-γ in HUCB T lymphocytes compared with APB T lymphocytes. The percentage of positive cells for each cytokine is significantly lower in HUCB compared with APB (Fig. 2).

3.2. CD4/CD8 subset analysis

Further phenotypic analysis of the cytokine-producing T lymphocytes was also performed according to CD4 and CD8 expression. In HUCB, Il-2, Il-4 and IFN-γ are produced mainly by CD4+ T cells compared with CD8+ T lymphocytes. In APB, Il-2 and Il-4 are also produced predominantly by CD4+ cells compared with CD8+ T lymphocytes. However, IFN-γ is produced by both CD4+ and CD8+ cells (Table 1).

4. Discussion

In this study we have analysed the cytokine profile of T lymphocytes from HUCB, using a modification of the technique described by Jung et al. [14]. This technique allows a more accurate and detailed comparison of the cytokine profile of cord and adult T lymphocytes, at single-cell level in comparison with other methods as ELISA or bioassay techniques which measure cytokine production in cell-culture supernatants. The flow cytometric assay allows simultaneous determination of an individual T-cell's ability to produce multiple cytokines and its phenotype after only short *in vitro* incubation (4 hours) with an activating stimulus and the secretion inhibitor brefeldin A. In addi-

tion, T lymphocytes were selected according to their expression of CD3 and low SSC.

Mosmann et al. [17] described two groups of clones. "Th1 cells" secrete Il-2 and IFN-γ but not Il-4, and the other, labelled "Th2 cells", has a reciprocal cytokine secretion profile. Th1 cells mediate pro-inflammatory reactions and help B cells produce IgG2, whereas Th2 type cells help B cells in production of antibody IgG1 [20]. Although these functionally distinct T cell subsets were originally described in the mouse, human T cell clones have subsequently been shown to have a similar, restricted cytokine profiles [6]. However, Th1 and Th2 type cells in the human are usually defined according to the ratio's of IFN-γ to Il-4 produced.

Previous studies have shown that after HUCB stimulation, Il-2 and IFN-γ secretion into the supernatant is lower than or similar to activated APB [2,18]. The results of this study show that the proportion of T lymphocytes expressing Il-2 and IFN-γ was lower in HUCB than in APB. With regard to Il-4, this study shows that the proportion of Il-4-producing cells was lower in HUCB T lymphocytes compared with APB T lymphocytes in keeping with previous data [5]. In our study the intracellular expression of Il-4 is lower, in both HUCB and APB T lymphocytes, than in Chalmers' study [5]. This discrepancy could be explained by the kinetic of Il-4 synthesis with a peak in its production at 8 hours after stimulation. Then, our study performed at 4 hours after stimulation do not detect the maximum level of production of this cytokine. Chalmers et al. [5,7] found a lower expression of intracelullar Il-2 and IFN-γ in HUCB lymphocytes compared to APB lymphocytes similar to described by us. These results are in contrast with recent report [11] that HUCB T cells produced more Il-2 than APB T lymphocytes after 4 hours of stimulation. Using the same protocol of T cell stimulation and flow cytometry analysis we have found a lower frequency of HUCB T lymphocytes producing Il-2. This agrees with the observation that HUCB lymphocytes are naive (CD45RA) [8, 12], since they have not yet become primed to the Th1 or Th2 type cells (results from our laboratory, data not shown). However there are some CD45RA cells within APB and it is not clear wheter these cells are as naive as their equivalent in HUCB. A recent study by Hassan et al. [13] suggested that the CD45RA population within HUCB and APB differ in their cytokine production since purified CD4+ CD45RA+ cells derived from HUCB do not make Il-2 on stimulation, whereas purified adult CD4+CD45RA+ do. Thus HUCB CD45RA cells may be more naive than adult CD45RA cells.
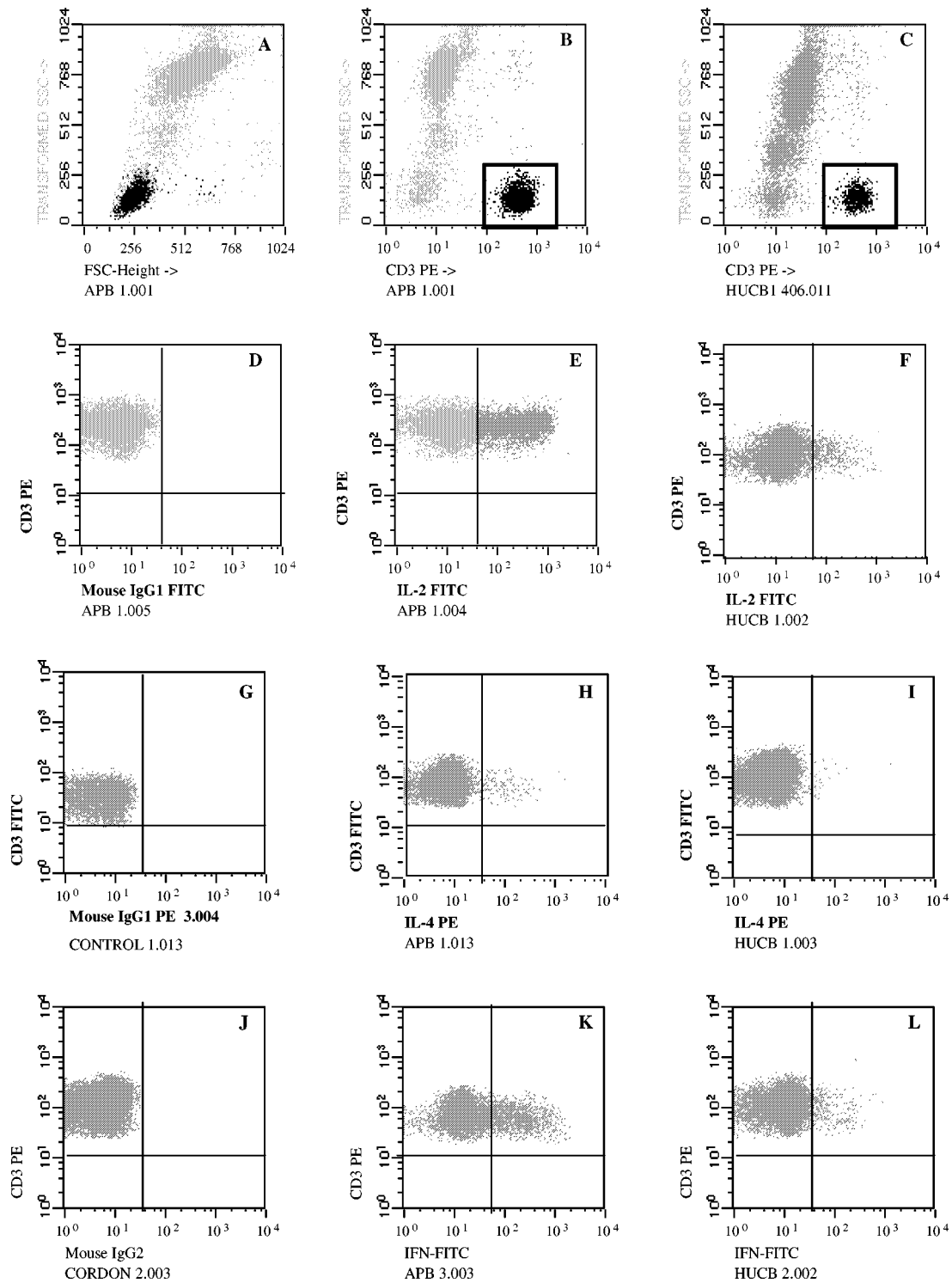
Fig. 1. Flow cytometric cytokine expression on HUCB and APBT lymphocytes. The top three dot plots (A, B and C) show the forward and transformed side scatter (SSC) distribution and the gate used to select T lymphocytes according to the expression of CD3 and low side scatter. The expression of Il-2 (E), Il-4 (H) and IFN-γ (K) on APB CD3+ lymphocytes is compared with the expression of Il-2 (F), Il-4 (I) and IFN-γ (L) on HUCB T lymphocytes. An isotype-specific negative control was used with each cytokine (D, G, and J, respectively).
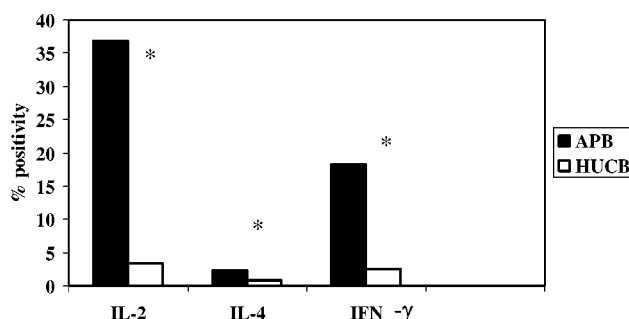
* P< 0.001

Fig. 2. Cytokine expression on HUCB and APB T lymphocytes. The percentage positivity of eight samples of HUCB and ten APB is shown following PMA/Ionomycin activation. Results are expressed as a percentage of CD3+ lymphocytes.

Table 1

T-cell subset analysis. The cytokine-producing T lymphocytes HUCB and APB were further characterized according to the expression of CD4 and CD8

	HUCB (n: 8)	APB (n: 10)
	$X \pm$ SD	$X \pm$ SD
CD3+CD4+Il-2+	$2.9 \pm 1.1\%$	$30.9 \pm 7.3\%$
CD3+CD8+Il-2+	$0.5 \pm 0.3\%$	$5.9 \pm 1.7\%$
CD3+CD4+Il-4+	$0.3 \pm 0.2\%$	$2.0 \pm 0.5\%$
CD3+CD8+Il-4+	$0.1 \pm 0.4\%$	$0.2 \pm 0.3\%$
CD3+CD4+IFN-γ	$2.0 \pm 1.5\%$	$8.2 \pm 2.3\%$
CD3+CD8+IFN-γ	$0.5 \pm 0.6\%$	$10.0 \pm 4.7\%$

We found a ten-fold decrease in the production of Il-2 by CD3+CD4+ HUCB lymphocytes compared with CD3+CD4+ APB lymphocytes. This reduced cytokine levels in HUCB could be implicated in the reduced GVHD after HUCB transplantation and make HUCB a particularly useful stem cell transplant.

Acknowledgement

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