Comparison of Functional Competence of Umbilical Cord and Adult Peripheral Blood Dendritic Cells in Allogenic Mixed Leukocyte Reaction

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ABSTRACT

Background: Dendritic cells (DCs) are the most potent stimulators of primary T cell responses and play a key role in immune reactions after stem cell transplantation. Very little is known about the cord blood (CB) dendritic cells and their potential involvement in the low incidence and lower severity of acute graft-versus-host disease after CB transplantation. **Objectives:** The aim of this study was the isolation of cord blood and peripheral blood dendritic cells and comparison of their functional competence and determination of their probable role in graft versus host disease after stem cell transplantation. **Methods:** In this study, fresh peripheral blood DCs (PBDCs) were enriched as HLA-DR$^+$ cells, lacking the CD3, CD11b, CD14, CD16, CD19 and CD56, using immunomagnetic bead depletion. For cord blood dendritic cells (CBDCs) enrichment CD34$^+$ and CD66b$^+$ cells were needed to be depleted too. Immunomagnetically enriched PB/CB dendritic cells were co-cultured with adult T lymphocytes and cell proliferation was measured by ^{3}H-thymidine incorporation. **Results:** Results showed that CBDCs were significantly poor stimulators of the mixed leukocyte reaction as compared with PBDCs ($P < 0.05$). **Conclusion:** The demonstrated impairment of CBDCs function could be of importance in interpretation of the low incidence and milder severity of graft-versus-host disease (GVHD) in umbilical CB transplantation compared with peripheral blood or bone marrow stem cell transplantation.

Keywords: Cord Blood, Dendritic Cells, Mixed Leukocyte Reaction, Peripheral Blood

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INTRODUCTION

CB transplantation represents an encouraging alternative to bone marrow transplantation. There are a variety of reasons for this, the most remarkable being that for even HLA-antigen disparate grafts, the incidence and severity of acute graft versus host disease (GVHD) has been low, with unknown underlying mechanisms (1, 2).
In the afferent phase of GVHD, donor T cells recognizing host antigens are activated, which then either directly mediate or organize the efferent phase of the disease with a deregulation of cytokines (3). CB T cells have normal proliferative responses to primary allostimulation (4) and the frequencies of alloreactive T cells in CB are similar to or more than adult peripheral blood (5). CB T cells are mainly naive (4,6). Thus, they have to be activated by DCs, which are the only antigen presenting cells (APCs) capable of stimulating a primary T-cell response (7,8).
Unlike mouse, there is no specific marker for human DCs (9); therefore, they are identified as HLA-DR^+ cell population, lacking expression of antigens typical for other cell lineages (lin^-) (10,11). Some subpopulations for human DCs have been described based on the expression of CD11c and CD123 (8).
Although donor DCs are involved in graft rejection, very little is known about the potential role of donor DCs in stem cell transplantation- associated GVHD (12). DCs with potent allostimulatory capacity have been described in CB (13) but there are conflicting reports about comparative competence of PBDCs and CBDCs. Besides, in these few previous studies DCs have been enriched by overnight culture and density gradient method (14) or they are derived from PBMC and CBMC using cytokines (15). Because both of these methods alter DC characteristics, working on intact DCs is required for a more accurate understanding of the underlying mechanisms. In this article, we report the immunostimulatory competence of intact PB/CB DCs enriched by immunomagnetic depletion.

SUBJECTS AND METHODS

Blood Samples. CB samples were obtained from Hematology, Oncology and BMT Research Center, Shariati Hospital. The samples were taken at delivery from healthy full-term male and female newborns. Adult PB mononuclear cells were isolated from buffy coats obtained from local routine blood donations in Iranian Blood Transfusion Organization.
Mononuclear cells were separated from the cord blood and adult buffy coat by Ficoll-Hypaque density gradient centrifugation with three additional washing steps (10 min at 200 x g) to remove all platelet*s* from mononuclear cell suspension. The cells were counted and their viability were determined by trypan blue (Merck, Germany) staining.
Depletion of T Cells Using Rosetting Procedure. T cells were depleted through E-rosseting technique. In this technique, the mononuclear cells are separated into T cell (E-rosette-positive) and non-T cell (E-rosette-negative) populations.
Briefly fresh sheep RBC (SRBC) (Rami Saak Company, Iran), preserved in alsevers solution for a maximum of three weeks, were washed three times with PBS. One

volume of washed packed SRBC was incubated with 4 volumes of 4% AET (SERVA, USA) solution for 20 min at 37°C water bath. Then, the cells were washed with cold PBS three times and their optimal concentration was prepared. PB/CB mononuclear cells were resuspended in RPMI 1640 (Sigma, USA) containing 10% FCS (Gibco, UK) to a final concentration of 1 x 10^7 cells/ml. Equal volumes of mononuclear cells suspension and 4% SRBCs suspension and half volume of heat-inactivated FCS were mixed and centrifuged for 5 min at 200 x *g*, 4°C. The cell mixture was incubated for 2 hr on ice. Ficoll-Hypaque density gradient centrifugation in the next stage helped to separate the interface layer, which contains predominately non-T cells (E-rosette-negative population; consisting of B cells, monocytes, NK cells and DCs).

Titration of Monoclonal Antibodies for Isolating Dendritic Cells by Negative Selection. Monoclonal antibodies (mAbs) were used for depleting unwanted cells including T cells (mouse anti-human CD3), NK cells (anti-human CD16 and CD56), B cells (anti-human CD19), monocytes (anti-human CD14 and CD11b), stem cells (anti-human CD34) and myeloid precursors (anti-human CD66b) (Table 1).

The optimal working concentration of each mAb was determined by indirect immuno-fluorescence staining of normal PBMC using serial dilutions of the MAb and a fluo-

Table 1. mAbs used to label cells to be removed

Cell type	mAb	Isotype	Source
T cell	anti CD3	IgG1	IQ product
B cell	antiCD19	IgG1	IQ product
monocyte	antiCD11b	IgG1	IQ product
monocyte	antiCD14	IgG2a	IQ product
NK cell	antiCD16	IgG2a	IQ product
NK cell	antiCD56	IgG2a	IQ product
stem cell	antiCD34	IgG1	Becton Dickinson
myeloid precursors	antiCD66b	IgG1	Becton Dickinson

rochrome-conjugated anti-mouse Ig as the second antibody. Flowcytometric analysis was used to determine the saturating concentration (no increase in mean fluorescent intensity (MFI) with raising Ab concentrations). This initial screening will help to select appropriate antibody dilutions for subsequent experiments using immunomagnetic beads and would give the minimum concentrations that should actually be used.

Negative Selection of DCs from Peripheral Blood and Cord Blood Mononuclear Cells. PBDCs and CBDCs were isolated according to a slightly modified protocol described previously by Sorg et al. (13). E-rosette-negative population of mononuclear cells were incubated for 30 min. with a cocktail of saturating concentration of unconjugated monoclonal antibodies (anti-human CD3, anti-human CD16 and CD56, anti-human CD19, anti-human CD14 and CD11b) to coat unwanted cells. All the mAbs in this cocktail were from IgG class and were bounded to T cells, NK cells, B cells and monocytes respectively. For CBDC isolation, anti-CD34 and anti-CD66b reagents were included as well due to higher frequency of $CD34^+$ stem cells and myeloid precursors in CB. The cells were washed to remove any unbound mAb and resuspended in cold, freshly prepared coating medium (EDTA 2mM, BSA 0.1%) at

concentration of 25 x 10^6 cells/ml. An optimal amount of washed magnetic beads coated with goat anti-mouse IgG was added to cell suspension and incubated for one hour. Subsequent exposure to a strong magnetic field (Biotech, Dynal Norway) removes the unwanted bead-coated cells, leaving behind the desired cell population. The unbound cells were transferred to a fresh tube and a second round of magnetic separation was performed. The remained cells were analyzed by flowcytometry to assess DCs purity. All of the above steps were performed at 4°C.

T Cells Isolation. T cells were isolated from PBMC, after two hours of culture for monocyte adherence. Non–adherent lymphocytes were depleted from B cells by nylon wool technique. The purity of obtained T cells was determined by flowcytometry using anti-human CD3 monoclonal antibody.

Immunostaining and Flowcytometry. The purity of enriched PBDCs/CBDCs and T cells was determined through surface expression of leukocyte markers using fluorescence-activated cell sorting analysis. Because the obtained cells had already been treated with mAbs against lineage markers (as the first layer), they were incubated with saturating concentration of PE-conjugated anti-mouse IgG (as second layer) (Serotec, England) for 30 min at 4°C in FBS containing 1% FCS. The cells were then incubated for 10 min at room temperature in 10% mouse serum to occupay the remaining free binding sites of second layer Ab. Then, washed and finally labeled with FITC-conjugated mAb against HLA-DR (IQ product, Netherlands).

Enriched T cell population was incubated with saturating concentration of PE-conjugated anti-CD3. The cells were washed, fixed with 1% paraformaldehyde and analyzed by flowcytometer (Partec, Germany).

Allogenic Mixed Leukocyte Reaction (MLR). Graded numbers of lineage negative (Lin^-) enriched and irradiated (3000 rad), CBDCs/PBDCs were cultured with 10^5 allogenic PB T cells in a final volume of 200µl in round-bottom 96-well plates. After 5 days of culture in RPMI-1640 (Sigma, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, UK), 100 U/µl penicillin (Gibco, UK) and 100mg/µl streptomycin (Gibco, UK) at 37°C and 5% CO2, 1µCi [^{3}H] thymidine (Amersham, UK) were added per well for the final 18 hours before harvesting and liquid scintillation counting. All the assays were performed in triplicates.

Statistical Analysis. Non-parametric analysis was used to determine the statistical significance of the differences.

RESULTS

Flowcytometric Analysis. For determination of DCs purity, PBDCs and CBDCs were stained directly with FITC-conjugated anti HLA-DR and PE conjugated anti-mouse IgG which could bind mouse anti-human monoclonal antibodies (CD19, CD3, CD14, CD56, CD16, CD11b, CD34 and CD66b) in an indirect immunofluorescense assay (n=14).

The obtained results showed that 58.5% ± 4.5% and 45% ± 12% of enriched cells from PB and CB respectively did not express any of the above-mentioned lineage markers while expressing HLA-DR (Lin^- HLA-DR^+) (Fig. 1).

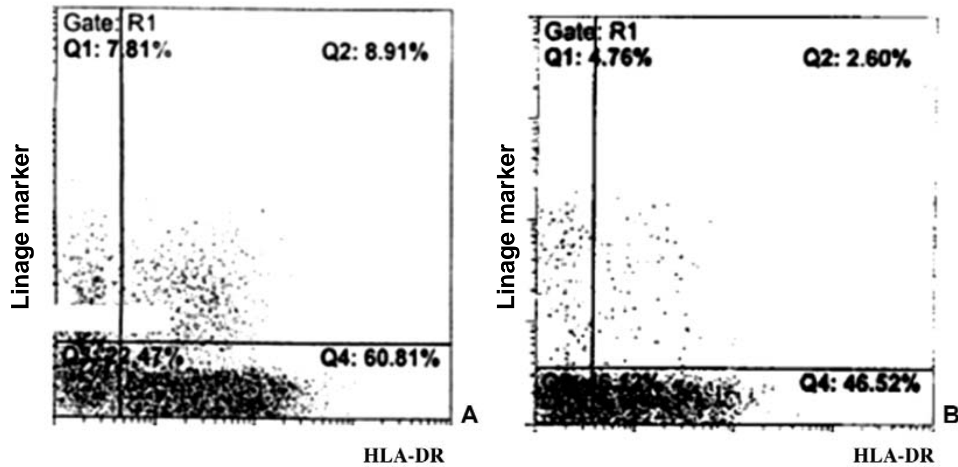

Figure 1. Frequency of Lin-/HLA-DR+ cells: (A) Lin-/HLA-DR+ in peripheral blood, (B) Lin-/HLA-DR+ in cord blood .The cells were double labeled with lineage marker (CD3, CD11b, CD14, CD16, CD19, CD56 for peripheral blood, in addition to CD34 and CD66b for cord blood) specific unconjugated mAb and, conjugated anti-IgG and anti-HLA-DR. Percentages of Lin-/HLA-DR+ , PBDCs and CBDCs are shown. The quadrants were set according to isotype controls. Lineage marker negative and HLA-DR positive cells appeared in quadrant Q4

T cell purity was determined through direct immunofluorsences staining with PE conjugated anti-human CD3. Flowcytometric analysis showed that 80.0% ± 0.5% of enriched cells were $CD3^+$.

Functional Competence of $Lin^-/HLA\text{-}DR^+$ PBDCs and CBDCs. To determine the minimal numbers of stimulatory cells required for a significant response, MLR experiment was carried out with graded doses of irradiated PBDCs as stimulators and purified adult T cells as responders, as mentioned earlier (n=3).

We observed a potent allostimulatory activity at DC numbers of 10^4 per well (Fig. 2).

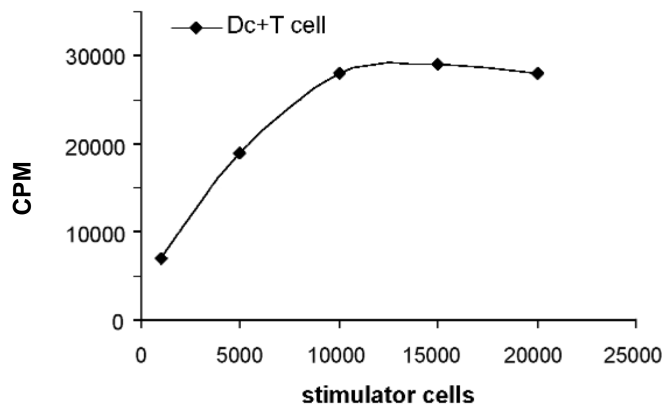

Figure 2. T cell stimulatory capacity of PBDCs
Graded doses of Lin-/HLA-DR+ enriched PBDCs were co-cultured with 10^5 allogenic PB T cells and after 5 days 3H-TdR uptake was determined

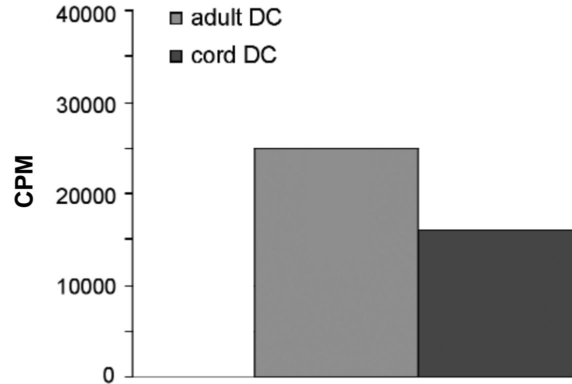

Figure 3. Proliferative response of allognic T cells
10^5 adult T cells co-cultured with either $1x10^4$ allogenic CBDCs or PBDCs. 3H-TdR incorporation is shown as count per minute (CPM). CPM in the absence of dendritic cells was 800 ±300 for T cells alone (n=7)

So the experiments were established with 10^4 PBDC or CBDC cultured for 5 days with 10^5 allogenic peripheral T cells, as mentioned earlier (n=7). Both HLA-DR^+ Lin^- PB and CB DCs revealed a potent allostimulatory activity but, as it can be seen in Fig. 3, the proliferative response of purified adult T cells to allogenic adult blood DCs was significantly greater than allogenic cord blood DCs ($P < 0.05$).

DISCUSSION

Fresh PBDCs and CBDCs were enriched as a lin^-/HLA-DR^+ population. Myeloid precursors were the main contaminating cells in cord blood MNC and could be eliminated using anti-CD33. Due to the expression of CD33 on myeloid subpopulation of DCs (8), we used anti-CD66b mAb and improved CBDCs purity to 45% ± 12%, which is better than that previously reported (13).

Although there are a few reports about T cell stimulatory potency of PBDCs and CBDCs, in this study, the functional properties of intact PBDCs and CBDCs have been compared and for the first time we report that intact or non-manipulated DCs isolated from adult blood are more efficient MLR stimulators than their equivalents in CB. The underlying mechanisms for the diminished potency of CBDCs are unclear but since the potency of DCs as APCs correlates with surface expression of MHC antigens and cell adhesion molecules including ICAM-1 and CD80 (13,14), we conclude that our observation is consistent with relatively low expression of HLA-A,B,C and HLA-DR molecules and ICAM-1 on CBDCs as reported by Hunt et al. (14). Furthermore, having a phenotype similar to immature DCs because of low expression of CD80, CD86 and CD40 (13) make it likely that their ability in antigen presentation is limited. In line with our results, Hunt et al. and Petty et al. have enriched PB and CBDCs through overnight culture and low-density gradient centrifugation method. Their results support our conclusion with reporting CBDCs as less efficient accessory cells than PBDCs (14,16) but Goriely et al. who generated DCs by adding cytokines to

adherent PB and CB mononuclear cells observed that the neonatal DCs were as efficient as adult DCs in inducing T cell proliferation (15). Since the applied method by Goriely could produce only myeloid dendritic cells, his reported results can not be extended to all DC's subsets. Sorg RV et al. has reported that there is more CD11c^{-} CD123^{+} DC (lymphoid DC) on CB than on PB (13). So our study on fresh CBDCs has more reliable judegement about what really happens in GVHD because of saving both DC's subsets in the used procedure.
In conclusion, our results show that CBDCs seem to differ functionally from PBDCs, consistent with the observation of Sorg RV et al. where HLA-DR^{+}/Lin^{-} CBDCs have diminished antigen presentation capability. This functional limitation of CBDCs could be the reason for less incidence and lower severity of GVHD in CB stem cell transplantation.

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