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The Phenotypic and Functional Characteristics of Umbilical Cord Blood and Peripheral Blood Natural Killer Cells

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Abstract

Allogeneic hematopoietic cell transplantation can be curative for patients with high-risk acute leukaemia. Umbilical cord blood (UCB) is an increasingly used source of allogeneic stem cells for patients who are in need of a transplant, but do not have a sibling donor. This review highlights the similarities and differences between the natural killer (NK) cells obtained from adult peripheral blood (PB) and UCB. These two cell sources show similar percentages of NK cells, including the major $CD56^{dim}$ and $CD56^{bright}$ subpopulations. UCB also contains an additional $CD56^{-}CD16^{+}$ subset, not typically found in PB. In addition, there are a number of progenitor cell populations in UCB that can give rise to NK cells. Some studies showed that UCB NK cells express a relatively higher percentage of inhibitory receptors (CD94/NKG2A and killer-cell immunoglobulin-like receptors) and less adhesion molecules. Resting UCB NK cells also show significantly less cytotoxicity compared to PB NK cells. However, following cytokine stimulation, the cytotoxicity of UCB NK cells can be rapidly increased to levels that are comparable to PB NK cells. Activation and expansion protocols for UCB NK cells are briefly reviewed. Lastly, we outline the early use of UCB NK cells in clinical trials.

Keywords

Umbilical cord blood; Natural killer (NK) cells; cytotoxicity; immunotherapy; expansion

Introduction

Allogeneic haematopoietic cell transplantation (allo-HCT) is curative for patients with leukaemia that is refractory to all other forms of therapy. Allo-HCT is efficacious for two reasons: the high doses of chemotherapy used and the allogeneic graft-versus-leukaemia (GVL) reaction that is mediated by the donor lymphocytes. Only about one third of all patients who require allo-HCT will have a matched sibling donor. Umbilical cord blood (UCB) is an increasingly used source of hematopoietic stem cells. The advantages of UCB include rapid availability and less stringent human leucocyte antigen (HLA) matching requirements, resulting in the identification of UCB units for the majority of patients. Also, despite considerable HLA disparity, UCB is associated with relatively less acute and chronic graft-versus-host disease (GVHD) and there is very low risk of viral transmission from donor to recipient (reviewed in Barker and Wagner, 2003).

Relapse remains a considerable obstacle to the success of allo-HCT. UCB contains at least ~10- to 100-fold fewer lymphocytes than does a bone marrow (BM) or peripheral blood

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(PB) stem cell graft. Given this; there have been concerns regarding the GVL capacity of UCB. However, the rates of leukaemia recurrence following UCB transplantation are similar to other cell sources (Eapen, *et al* 2007, Rocha, *et al* 2004). Considering that GVL probably occurs early after allo-HCT and that natural killer (NK) cells recover rapidly (Abu-Ghosh, *et al* 1999), such cells may be instrumental in post-UCB transplant GVL reactions. The purpose of this review is to highlight both the differences and similarities between UCB and PB NK cells to understand both their strengths and weaknesses as they are applied to clinical medicine.

Immunophenotype of UCB NK cells

Comparison of the immunophenotype of NK cells from UCB and PB demonstrates many similarities. For instance, the major NK cell subsets, $CD56^{bright}$ and $CD56^{dim}$ cells, are present at the same proportions in both UCB and PB (Dalle, *et al* 2005, Tanaka, *et al* 2003). Some studies suggested that expression of NK cell triggering receptors, including CD94, killer-cell immunoglobulin-like receptors (KIR; CD158a/h and CD158b/j), NKp46 and NKG2D do not differ between UCB and PB NK cells, while other studies showed that a higher percentage of UCB NK cells express the inhibitory receptor complex of CD94/NKG2A and CD158b/j (Dalle, *et al* 2005, Wang, *et al* 2007). Other activating receptors, including NKp30 and DNAM-1, are also expressed by UCB NK cells (unpublished observations), but it is not known whether the percentage (or the level of expression) differs between UCB and PB NK cells.

There are also significant differences in the immunophenotype of UCB and PB NK cells. A lower percentage of NK cells from UCB display L-selectin (CD62L), perhaps suggesting a reduced capacity for lymph node homing (Dalle, *et al* 2005). Likewise, a number of adhesion molecules, including CD2, CD11a, CD18 and CD54, are present on a lower percentage of UCB NK cells (Dalle, *et al* 2005, Lin and Yan 2000, Tanaka, *et al* 2003). Compared to PB NK cells, more UCB NK cells express toll-like receptor 4 (TLR4) which mediates innate inflammatory responses to lipopolysaccharide (Lopez, *et al* 2008), although no studies have compared the responses of these two NK cell sources to lipopolysaccharide. Some investigators have also found that fewer UCB NK cells express receptors associated with terminal NK cell maturation, including CD8 and CD57 (Dalle, *et al* 2005). Based on this, it is tempting to speculate that UCB NK cells might be less mature than PB NK cells. However, caution is warranted because UCB-derived NK cells also express equivalent or higher levels of effector molecules, including perforin and granzyme B (Dalle, *et al* 2005, Fan, *et al* 2008, Wang, *et al* 2007), which have also been linked to NK maturation.

Within the lymphocyte gate of UCB, two fractions of cells can be observed based on CD45 expression. More specifically, UCB contains both a $CD45^{dim}$ and $CD45^{high}$ lymphocyte population, whereas only the latter is found in adult PB (Lopez, *et al* 2008). While the $CD45^{high}$ fraction from both UCB and PB contain NK cells, the $CD45^{dim}$ population in UCB also contains a high percentage of NK cells (~50% of the total $CD45^{dim}$ cells) (Lopez, *et al* 2008). The significance of this reduction in CD45 expression is not known, but may be explained by increased cell proliferation or immaturity, since both are associated with a $CD45^{dim}$ phenotype. Clearly, more work is needed to define the differences between these two populations.

A number of NK progenitor populations can be found in UCB that are not typically present in PB. Such populations include $CD34^{-}CD133^{-}CD7^{-}CD45^{+}lin^{-}$ cells that can differentiate into NK cells following culture with interleukinIL-15 and stromal cells (Rutella, *et al* 2003). Further advanced, $CD34^{+}CD7^{+}$ and $CD34^{-}CD7^{+}$ progenitors are also more abundant in UCB and these cells also develop into NK cells (McCullar, *et al* 2008, Miller and McCullar

2001, Theilgaard-Monch, *et al* 2001). Still other progenitor cell populations present in UCB have been described, including a $CD34^{-}CD56^{-}$ adherent cell fraction that shows heterogeneous expression of myeloid antigens (CD14, CD11b, CD13 and CD33). When cultured with FLT-3 ligand (FLT-3L) and IL-15, these cells showed progressive loss of CD14, followed by NK cell differentiation defined by the acquisition of CD56, NK cell receptor expression, cytotoxicity and cytokine production (Perez, *et al* 2003).

While the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets are present at about the same proportions in UCB and PB (Dalle, *et al* 2005, Tanaka, *et al* 2003), other NK cell populations, such as the $CD56^{-}CD16^{+}$ subset are more abundant in UCB (Gaddy and Broxmeyer 1997). $CD56^{-}CD16^{+}$ cells are found at higher frequencies in immune compromised hosts, including those with chronic viral infections (human immunodeficeincy virus (Mavilio, *et al* 2005) and hepatitis C (Gonzalez, *et al* 2008, Zarife, *et al* 2009)). Culture of resting UCB-derived $CD56^{-}CD16^{+}$ NK cells with IL-2 and/or IL-15 resulted in CD56 acquisition, possibly suggesting that they may be NK progenitors (Gaddy and Broxmeyer 1997). In line with this, UCB-derived $CD56^{-}CD16^{+}$ NK cells have poor cytotoxicity, which is enhanced following IL-2 or IL-15 stimulation (Gaddy and Broxmeyer 1997). Interestingly, $CD56^{-}CD16^{+}$ cells have also been identified after allo-HCT using UCB, but not after BM or PB transplants (Lu, *et al* 2008).

Cytotoxicity of UCB NK cells

Regarding their capacity to kill malignant cells, a number of studies showed that resting UCB mononuclear cells (MNCs) have less cytotoxicity compared to PB MNCs (Hoshina, *et al* 1999, Suen, *et al* 1998, Tanaka, *et al* 2003). However, stimulation of UCB MNCs with either lL-2, IL-12 or IL-15 resulted in an enhancement in killing that was comparable to similarly treated PB MNCs (Hoshina, *et al* 1999, Nomura, *et al* 2001). While these early studies did not directly implicate NK cells as having reduced killing capacity, recent studies using purified NK populations confirm these findings. Like the unseparated MNC studies, purified UCB NK cell cytotoxicity could be significantly augmented following IL-2 and IL-15 stimulation (Choi, *et al* 2004, Dalle, *et al* 2005, Gaddy and Broxmeyer 1997). Experiments using other cytokines, including IL-12 or IL-18 either alone or in combination with other cytokines, showed a similar effects (augmentation of UCB cytotoxicity) (Nomura, *et al* 2001, Satwani, *et al* 2005). Thus, while there is little question that resting UCB-derived NK cells have less *in vitro* cytotoxicity, they rapidly respond to cytokine stimulation, resulting in killing that is not different from similarly treated NK cells from adult PB.

It is well established that the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets differ functionally (reviewed in (Cooper, *et al* 2001)). While the percentages of $CD56^{bright}$ and $CD56^{dim}$ NK cells in UCB and PB are similar (Dalle, *et al* 2005, Tanaka, *et al* 2003), studies comparing the cytotoxicity of these NK subpopulations reveal considerable differences. Not surprisingly, purified $CD56^{bright}$ NK cells from both cells sources had low cytotoxicity, which was not different. In contrast, purified $CD56^{dim}$ NK cells from UCB showed significantly inferior target cell killing compared to PB $CD56^{dim}$ NK cells (Tanaka, *et al* 2003). Thus, UCB $CD56^{dim}$ NK cells have less cytotoxic capacity compared to their PB counterparts.

While the causes of this reduction in killing of UCB NK cells are not well understood, there are some differences between UCB and PB NK cells that may account for these findings. In assays testing the binding of NK cells to K562 cells, approximately 3-fold fewer conjugates to target cells were made by UCB NK cells compared to those from PB (Tanaka, *et al* 2003). These findings may be explained by the relatively lower expression of adhesion molecules

(CD2, CD11a, CD18 and CD54) by UCB NKs (Dalle, *et al* 2005, Lin and Yan 2000, Tanaka, *et al* 2003). As described above, UCB NK cells rapidly acquire cytotoxicity after cytokine stimulation (Choi, *et al* 2004, Dalle, *et al* 2005, Gaddy and Broxmeyer 1997), and Lin & Yan (2000) found that UCB NK cells rapidly acquired CD54 following IL-12 or IL-15 stimulation. These findings possibly provide a mechanistic explanation for the increase in cytotoxicity following cytokine stimulation of UCB NK cells.

Other possible mechanistic explanations for the reduction in killing by UCB NK cells include a relatively higher expression of inhibitory receptor complexes including CD94/NKG2A and/or KIR (Wang, *et al* 2007). Still other surface receptors might also account for the reduced killing by resting UCB NK cells. For example, a significant proportion of UCB NK cells, but not PB NK cells, express the glucocorticoid-induced tumor necrosis factor receptor (GITR) (Lopez, *et al* 2008). Recent studies using PB NK cells showed that soluble GITR-ligand (shed from malignant cells) significantly attenuates NK cell cytotoxicity (Baessler, *et al* 2009, Baltz, *et al* 2008). What impact cytokine stimulation has on the GITR expression of UCB NK cells is not known. Still other explanations for the lower cytotoxicity may be found in the higher content of regulatory T cells in UCB compared to PB (Godfrey, *et al* 2005).

Cytokine Elaboration of UCB NK cells

Prior studies comparing UCB and PB T cells showed a reduction in cytokine elaboration (including granulocyte-macrophage colony-stimulating factor, tumour necrosis factor α [TNF-α], interferon γ [IFN-γ]) in UCB T cells following mitogen stimulation (Kadereit, *et al* 1999, Kaminski, *et al* 2003). This has been attributed to less NFAT1 expression in UCB T cells. While no studies have addressed the NFAT levels in UCB NK cells, NK cells from UCB also show a lower percentage of TNF-α producing cells compared to PB NK cells (Krampera, *et al* 2000). In contrast, NK cell IFN-γ production in response to mitogen was similar between the two sources (Dalle, *et al* 2005, Krampera, *et al* 2000).

$CD56^{bright}$ NK cells are an important source of IFN-γ and this cytokine then feeds back to other elements of the adaptive immune system, including dendritic cells (Gerosa, *et al* 2002, Vitale, *et al* 2004). One established method to induce IFN-γ production in NK cells is the stimulation with IL-12 and IL-18 (Fehniger, *et al* 1999). Comparison of the IL-12 and IL-18 induced IFN-γ responses of UCB and PB NK cells showed that NK cells from UCB produced significantly more IFN-γ (Nomura, *et al* 2001, Satwani, *et al* 2005). Similarly, UCB NK cells showed more CD69 expression following IL-12 and IL-18 stimulation, indicative of activation (Nomura, *et al* 2001).

Adoptive Immune Therapy With UCB NK cells

One major obstacle to NK cell adoptive transfer studies is the challenge of obtaining sufficient cells to obtaining meaningful antitumour responses. Considering the fixed number of cells present in a UCB unit, the issue of cell number is especially important in the context of UCB. However, UCB contains many progenitor cell populations that might expand significantly *in vivo* and this justifies exploring UCB as a NK cell source. Moreover, with the advent of UCB freezing bags that contain a partition, it is conceivable that hematopoietic cell transplant studies (or adoptive immune therapy with isolated UCB cell populations) could be incorporated into current treatment protocols where one portion of the unit is used for hematopoietic cell transplantation and the other portion is used for expansion of the desired cell population, in this case, NK cells.

With the goal of devising strategies for augmenting NK cell dose, a number of investigators have attempted to expand either mature or immature NK cells from UCB. As reviewed

above, resting UCB NK cells rapidly respond to cytokine stimulation by increasing cytotoxicity. The expression of the IL-2 receptor complex on PB and UCB NK cells has been compared. Relatively few NK cells from both sources express the IL-2α chain (CD25), while significantly more display the IL-2β (CD122) and IL-2γ (CD132) chains. There were no significant differences in the percentage of cells expressing these between UCB and adult PB NK cells (Dalle, *et al* 2005). Likewise, using the membrane dye carboxyfluorescein diacetate succinimidyl ester (CFSE) to compare the proliferative response to IL-2, Dalle *et al* (2005) found no differences in the early rates of proliferation between the two cell sources. Studies that focused only on UCB NK cells showed that combinations of low dose IL-15 and FLT-3L expand UCB NK cells ~20-fold within 2 weeks of culture (Nagamura-Inoue, *et al* 2004). Still others have shown that the addition of IL-21 and hydrocortisone to the above cytokine combination (of IL-15 and FLT-3L) results in significantly better UCB NK expansion, however, the effect was observed over 30 days in culture (Perez, *et al* 2006).

In general, studies using feeder cells yield superior UCB NK cell expansion, supporting the concept that surface receptor ligation augments proliferation and maintains viability. It was recently reported that a genetically modified K562 cell line expressing membrane bound IL-15 and 4-1BBL (K562-mbIL15-41BBL (Imai, *et al* 2005)) resulted in a 35-fold expansion of UCB NK cells over 7 days in culture (Hochberg, *et al* 2008). Positively selected UCB NK cells cultured with high dose IL-2 (1,000 IU/ml) and irradiated autologous feeder cells could expand up to 156-fold over a month in culture. Using this technique, UCB NK cell expansion was significantly better than PB suggesting either more robust proliferation of mature UCB NK cells or perhaps the differentiation of progenitors into NK cells (Condiotti, *et al* 2001). Harada *et al* (2004) used a Wilm's tumour the cell line, HFWT, to expand UCB NK cells. This cell line was found to support dramatic expansion of both mature NK cells, as well as the differentiation of NK progenitors into mature, functional NK cells (Harada, *et al* 2004).

Regarding progenitor expansion, we have previously used a number of murine fetal liver cell lines to demonstrate that a single stem cell ($CD34^{+}Lin^{-}$) can give rise to ~3,000 NK cells (Grzywacz, *et al* 2006, McCullar, *et al* 2008, Miller and McCullar 2001). Other studies have used UCB-derived mesenchymal stem cells as feeder cells to culture CD3-depleted UCB MNCs. In the presence of a cytokine cocktail of IL-2, IL-3, IL-15 and FLT-3L, UCB NK cells expand ~65-fold over the course of 2 weeks. Expansion was significantly reduced in the absence of either MSCs or the early acting cytokines (IL-3 and FLT-3L), probably suggesting that these conditions were inducing NK differentiation from progenitors, as well as more mature NK cell expansion (Boissel, *et al* 2008).

Our prior studies have established that high dose chemotherapy and infusions of NK cell-enriched products from adult haploidentical PB cell are safe and do not cause GVHD despite post-infusion IL-2 administration (Miller, *et al* 2005). Moreover, this therapy resulted in remissions for ~30% of patients with chemotherapy-refractory acute myeloid leukaemia (AML) (Miller, *et al* 2005). We observed that remissions were frequently accompanied by *in vivo* expansion of the NK cell-enriched product. As described above, UCB is rich in NK cell progenitors. If these progenitors can expand and differentiate into mature, functional NK cells, then perhaps UCB could ultimately be more useful than PB in mediating GVL reactions. We are currently testing this hypothesis in chemotherapy-refractory AML patients. Briefly, in this study patients receive a myeloablative conditioning regimen followed by an infusion of an IL-2 activated, NK cell-enriched UCB unit that was HLA 3/6 or 4/6 matched to the patient. Following the NK cell-enriched UCB infusion, IL-2 is administered every other day for 6 doses. After this, patients are “rescued” with a double UCB cord transplant with UCB units that contain sufficient numbers of stem cells for hematopoietic engraftment. We have previously reported the results for the first three

patients treated on this trial (Miller, *et al* 2006). In two of the three patients, the T cell-depleted, NK cell-enriched haploidentical UCB unit resulted in sustained hematopoietic cell engraftment. This finding is remarkable because the numbers of progenitor cells in these "NK units" were not expected to be sufficient for sustained hematopoietic cell engraftment based on prior UCB transplant studies (Barker, *et al* 2005, Wagner, *et al* 2002). Thus, IL-2 activated, T cell-depleted UCB units can engraft. Whether this is in some way related to a graft facilitating effect of the NK cells in this unit and whether this approach is successful in chemotherapy-refractory leukaemia requires further study.

Caveats, Considerations and Conclusions

There is considerable variability in the reports describing the phenotypic and functional characteristics of UCB NK cells. While differences in reagents and laboratory techniques probably account for some of this variation, other factors typically not available to laboratory investigators (and specific to UCB) should also be considered. For instance, a number of issues influence the NK cell content of UCB, including the race and sex of the donor. UCB units from Caucasians and males tend to have a higher NK cell content compared to UCB from non-Caucasians and females (Cairo, *et al* 2005). There are also more NK cells in UCB obtained at caesarean section compared to vaginal deliveries (Cairo, *et al* 2005).

Parameters associated with mode and timing of delivery also influences NK cell functionality. For instance, the NK cells from premature infants are less cytotoxic than those from full term infants (Georgeson, *et al* 2001, McDonald, *et al* 1992, Sancho, *et al* 1991) and thus, the gestation age of the UCB donor may impact NK function. The mode of delivery and the medications used also affects UCB NK cell function. More specifically, De Amici *et al* (1999) compared the NK cell function from UCB units obtained after vaginal delivery and caesarean section with either general anaesthesia or epidural anaesthesia. They showed that after caesarean section with general anaesthesia UCB NK cells had the highest cytotoxicity. In the vaginal deliveries, there were significantly higher concentrations of cortisol, and lidocaine could be detected in UCB units obtained by caesarean section with epidural anaesthesia. While both cortisol and lidocaine have been implicated in NK dysfunction, neither were statistically correlated with a reduction in killing in this relatively small series. Similarly, NK cells from the UCB of neonates with sepsis were considerably less cytotoxic than healthy newborn or adult blood (Georgeson, *et al* 2001). Thus, the mode of delivery (vaginal vs. caesarean section), the anaesthetic used and other clinical parameters may significantly influence NK cell numbers and function in UCB units. Moreover, whether these same variables influence UCB transplant outcomes is not known.

In summary, UCB NK cells have both similarities and differences to PB NK cells. The data reporting on surface phenotype, cytokine production and cytotoxicity supports the concept that UCB NK cells differ from adult PB NK cells. While resting UCB shows a clear attenuation in killing, this is rapidly correctable with cytokine stimulation. The higher content of NK cell progenitors may be highly beneficial both for allo-HCT, as well as *in vitro* expansion studies. Collectively, the differences between UCB and PB NK cells cannot be explained as a simple issue of immaturity. Rather, the differences between these two cell sources may be accounted for by their different environments. Understanding these differences and developing methods of expanding NK cells has the potential to harness the full GVL potential of this cell population.

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