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Polyunsaturated fatty acids confer cryoresistance on megakaryocytes generated from cord blood and also enhance megakaryocyte production from cryopreserved cord blood cells

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Abstract

Background aims. Previous data have shown that the addition of docosahexanoic acid (DHA)/arachidonic acid (AA) has a beneficial effect on cytokine-mediated *in vitro* generation of megakaryocytes (MK) from umbilical cord blood (UCB). Cryopreservation forms an inherent part of UCB banking and MK progenitors are known to be very sensitive to the stresses of freezing. It is therefore imperative to generate functional cells from cryopreserved cells, and the generated cells need to be cryopreserved until used. In the present study, cryopreservation of *ex vivo*-expanded MK as well as MK generation from cryopreserved UCB samples was investigated. *Methods.* MK generated with or without DHA/AA were cryopreserved in freezing medium containing 10% dimethyl sulfoxide (DMSO). Freezing efficacy was tested by quantitating MK after revival. Cryopreserved $CD34^+$ cells were cultured with stem cell factor (SCF) and thrombopoietin (TPO), in the presence and absence of DHA/AA for 10 days, and then quantitated for MK. *Results.* We observed a 1.5–3-fold increase in MK numbers, their progenitor content and their expression of phenotypic markers and MK-related transcription factors. DHA/AA sets showed a 2–5-fold improved engraftment in NOD/SCID mice. These data showed that the beneficial effect of DHA/AA obtained during MK expansion was not altered after freezing stress. The enhancement in MK generation obtained from fresh cord blood (CB) cells was reproduced with comparable efficiency when we used cryopreserved CB samples. *Conclusions*. Taken together, our data suggest that *in vitro*-generated DHA/AA MK survive cryoinjuries in a functionally better state. DHA/AA support a more efficient generation of MK from cryopreserved UCB.

Key Words: *arachidonic acid, cryopreservation, cryopreserved umbilical cord blood $CD34^+$ cells, docosahexanoic acid, megakaryocytes*

Introduction

Related and unrelated umbilical cord blood (UCB) has emerged as an alternative source of hematopoietic stem cells (HSC) for the majority of patients who are unable to identify a fully matched donor. The advantages and disadvantages of UCB in transplantation and their stem cell regenerative potential have been reviewed recently by Liao *et al.* (1). In order to overcome the thrombocytopenia period that follows HSC transplantation, a combined infusion of expanded megakaryocyte (MK) progenitors and unexpanded cells has been advocated by a number of investigators, in clinics (2–6) as well as in experimental models (7–10). The adverse effect of cryopreservation on MK progenitors has been reported by many researchers (11–14). Xu *et al.* (14) and Pick *et al.* (12) have shown that, in UCB samples, the recovery rate of MK progenitors after thawing is very low, indicating that these cells are more sensitive to the stresses of freezing. They concluded that the thrombocytopenia observed in patients receiving placental cord blood transplantation (PCBT) can be explained, at least in part, by the disappearance of colony-forming unit–megakaryocytes (CFU-MK) during cryopreservation. Therefore optimal cryopreservation of *in vitro* generated MK is an important aspect that needs to be looked into for their future use in clinical transplantations. In UCB banking and transplantations, long-term cryopreservation of hematopoietic stem and progenitor cells is a unique requirement compared with bone marrow transplantation (BMT) and mobilized peripheral blood (mPBL) transplantations for unrelated transplants (14–16). Thus there is a need for the development and improvement of culture

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methods to obtain sufficient megakaryocytes from readily available cryopreserved hematopoietic cells.

Generation of MK from cryopreserved UCB cells has been attempted by several investigators (13,15,17,18). Lazzari *et al.* (15) evaluated the number of MK progenitors in cultures expanded from both fresh and cryopreserved cord blood (CB) units. They observed 3–5 log expansions of MK precursors both in fresh and cryopreserved CB samples. Mwamtemi *et al.* (18) observed higher numbers of MK progenitors in cultures expanded from cryopreserved $CD34^+$ CB cells compared with cryopreserved bone marrow (BM) $CD34^+$ cells.

Polyunsaturated fatty acids (PUFA) are important structural and functional components of cell-membrane phospholipids (19,20). Their beneficial effects have been observed in humans and in animal models of diabetes, obesity, cancer, hypertension, arthritis, osteoporosis, inflammation, autoimmune disorders, mental health, gene expression and cardiovascular diseases (19,21–23). The *n*-3 and *n*-6 PUFA have different, often antagonistic, effects on inflammation, and their effects vary according to the type of cells and target organs involved, as well as their respective amounts in the diet (24). Thus the balance of *n*-3 and *n*-6 fatty acids is very important for homeostasis, in coronary heart disease and in mental health and immune/inflammatory disorders. In the secondary prevention of cardiovascular disease, a ratio of 4/1 of omega-6/omega-3 PUFA was associated with a 70% decrease in total mortality (23). We have shown previously that two PUFA such as docosahexanoic acid (DHA) and arachidonic acid (AA) have a favorable effect on the *in vitro* generation of MK (25). These studies were carried out with $CD34^+$ cells isolated from fresh CB samples. We have now examined two further issues, namely cryopreservation of MK generated with PUFA, and the generation of MK from cryopreserved UCB. MK generated in the presence of DHA/AA were sturdier and exhibited a higher ability to survive the cryopreservation stress. The beneficial effect of DHA/AA was also observed during MK generation from cryopreserved cells.

Methods

Chemicals, media and cytokines

Heparin sodium salt was from Sisco Research Laboratories (Mumbai, Maharashtra, India). Heta Sep and 35-mm culture dishes for colony assay were from Stem Cell Technologies (Vancouver, Canada). Histopaque (density 1.077 g/mL), DHA (algal vegetable oil), AA (porcine liver), Wright's and Giemsa stain, dimethly sulfoxide (DMSO) and methyl cellulose for colony assay were purchased from Sigma (Sigma-Aldrich Inc., St Louis, MO, USA). The serum-free medium Stem-pro, Trizol reagent, 2′7′dichlorodihydroflurescein diacetate (DCHFDA), 4′,6-diamidino-2-phenylindole (DAPI) and MITOSOX red™ were from Invitrogen (Gibco, Grand Island, NY, USA). Tissue culture-grade plastic ware was from Nunc (Naperville, IL, USA). Human recombinant growth factors such as thrombopoietin (TPO), stem cell factor (SCF), interleukin (IL)-3, IL-6 and fetal liver tyrosine kinase-3 (Flt3) were purchased from Peprotech Inc. (Rocky Hill, NJ, USA). Flow cytometry-qualified antibodies such as Phycoerythrin (PE), Allophycocyanin (APC) -conjugated anti-human CD41a, CD61 and active caspase 3 (PE), purified mouse monoclonal immunoglobulin IgG anti-CD41a/CD61, Cyanine (Cy) 3/fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, purified rabbit monoclonal immunoglobulin IgG anti-caspase 9, FITC-conjugated goat anti-rabbit IgG, Annexin V–FITC, cytofix/Cytoperm™ fixation/permeabilization kit and Cycle test™ plus DNA reagent kit were purchased from BD Pharmingen (Beckton Dickinson, San Jose, CA, USA). Purified rabbit monoclonal immunoglobulin IgG anti-cytoplasmic phospholipase A_2 (cPLA2), cyclooxygenase-1/cyclooxygenase-2 (COX-1/COX-2), thromboxane synthase-1 (Tx synthase-1), catalase, glutathione peroxidase-1 (GPx-1) and copper–zinc-containing superoxide dismutase (CU-ZN SOD), FITC-conjugated goat anti-rabbit IgG antibodies were purchased from Abcam (Cambridge, UK). The $CD34^+$ progenitor selection kit (Dynabeads M-450 $CD34^+$) and mRNA isolation kit were from Dynal (Dynal ASA, Oslo, Norway).

Human CB collection and isolation of $CD34^+$ from CB

UCB samples were obtained from full-term deliveries with informed consent obtained from the mothers. Institutional review board [institutional ethical committee (IEC) National Center for Cell Science (NCCS), Pune, India]-approved protocols for the use of human samples was followed. CB samples obtained from local hospitals were collected in sterile heparinized containers and processed within 24 h of collection. Mononuclear cells were separated from CB and $CD34^+$ cells were isolated through positive selection using Dynal magnetic beads according to the manufacturer's instructions.

Cells and culture conditions

Freshly isolated/cryopreserved UCB $CD34^+$ cells were seeded at a density of 5×10^4 cells/well/mL in Stem-pro medium in 24-well plates. The cytokines SCF and TPO were used at a final concentration of