STEM CELL BIOLOGY

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HUMAN UMBILICAL CORD BLOOD (HUCB) DERIVED STEM CELLS AND THEIR POTENTIAL IN TREATING INHERITED SKIN DISEASES AND PROMOTION OF WOUND HEALING

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Recessive Dystrophic Epidermolysis bullosa (RDEB) is a severe inherited skin-blistering disease caused by mutations in the Col7A1 gene. COL7A1 is synthesized by keratinocytes and fibroblasts and is a major component of anchoring fibrils that extend from basement membrane to papillary dermis. A recent animal study suggested that the hematopoietic enriched cells ameliorated the blistering phenotype in the RDEB mice. However, only limited donor epithelial engraftment and COL7A1 protein were observed and the possibility of rare stem cells co-purifying with enriched cells cannot be excluded. As multiple populations of primitive stem cells with multi-lineage differentiation potential have been identified from HUCB, including unrestricted somatic stem cells (USSCs), they may offer potential for the treatment of RDEB.

Goal: To isolate USSCs from HUCB and determine the potential in treating RDEB and promoting wound healing.

Methods: USSCs were initiated from HUCB in 30% FBS and 10^{-7}M dexamethasone. Q-RT-PCR was utilized to assess gene expression of Nanog, Oct4, Sox2 and Col7A1. DNA methylation at the enhancer and promoter of both Oct4 and Nanog genes was analyzed by bisulfate sequencing. The expression of CCR2 was analyzed by immunocytochemistry.

Results: HUCB-USSCs are lineage negative and share overlapping but distinct surface markers with MSCs. They express a low but consistent level of Nanog, Oct4 and Sox2. Significantly, the Nanog and Oct4 expression in USSCs is about 20- and 400- fold higher than that in human fibroblasts. Their level was further increased 10 fold following the treatment with DNA methylation inhibitor, 5-azacytidine. An average of 65% and 47% of the CpGs were unmethylated in the enhancer and promoter of the Nanog gene respectively, while 56% and 80% were unmethylated at those of the Oct4 gene. We also showed that USSCs express Col7A1, at a level comparable to human keratinocytes, suggesting that USSCs, once engraft in the skin, could rescue the defective anchoring fiber formation by secreting COL7A1. In addition, we demonstrated that USSCs express the CCR2, a receptor for several chemoattractant proteins, such as MCP-1, that have been shown to be highly expressed in the dermal wounds, implying a migratory ability of USSCs under wound conditions. We are now investigating the role of USSCs in promoting wound healing in immunodeficient mice with excisional wounds and comparing the effects of routes of injection on their long-term engraftment.

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ASSESSMENT OF THE BONE MARROW COMPARTMENT VOLUME AND EFFECT OF CHEMORADIOTHERAPY USING 18F-FLUOROTHYMIDINE

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Evaluation of the bone marrow compartment currently consists of a combination of bone marrow histology obtained by bone marrow biopsy, assessment of bone marrow with molecular analysis, and karotype genetics. Despite the recent excitement of additional modalities such and fluorodeoxyglucose (FDG) PET imaging and MRI, neither have proven to be accurate nor are clinically reproducible. We describe the use of Fluorothymidine-PET imaging to quantitate marrow volume in seven patients with normal marrow diagnosed with brain tumors (n = 4) and lymphoma with negative marrow (n = 3). Marrow volume was calculated by three-dimensional assessment of SUV uptake in marrow sites at pelvis, cervical, thoracic, and lumbar vertebrae, sternum, bilateral humeri and femurs. Average marrow volume for seven patients noted to be 2308 ml. Marrow distribution shows the following: pelvis 65%, cervical 1%, thoracic 11%, lumbar 10%, humeri 4%, femurs 4%, sternal 5%. We further describe the reproducibility of imaging using this agent post chemotherapy and post radiotherapy in patients with testicular (n = 9) and laryngeal carcinoma (n = 10). Radiation therapy(RT) to cervical spine was consistent with clinical observation with a decline in marrow SUV from 3.0 ±1.34 before RT to 1.94± 0.60 (P0.013) after RT. Chemotherapy resulted in no significant change in SUV when tested 6 months after last dose of chemotherapy with a mean SUVmax of 4.99 ±1.15 pre-chemotherapy and mean SUVmax of 5.28 ±1.0 post-chemotherapy (P0.21). We suggest that FLT-PET imaging may be a useful tool for the assessment of the marrow compartment and may be useful for assessment of marrow injury following radiotherapy.

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WNT/β-CATENIN SIGNALING COOPERATES WITH PTEN/PI3K/AKT SIGNALING *IN VIVO* AND *EX VIVO* TO PROMOTE HEMATOPOIETIC STEM CELL SELF-RENEWAL AND EXPANSION

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Although self-renewal is the central property of stem cells, the underlying mechanism remains inadequately defined. Separately, the PTEN/PI3K/Akt and Wnt/β-catenin signaling pathways have been implicated in regulating hematopoietic stem cells (HSCs), but neither pathway is sufficient for self-renewal. Using a HSC and progenitor (HSPC)-specific conditional mutant mouse model, we studied *Pten* deletion combined with *β-catenin* activation. In contrast to single mutants, double mutant mice exhibit a novel phenotype including expansion of phenotypic HSCs without extensive differentiation. Together, the PTEN/Akt and Wnt/β-catenin pathways interact to drive HSC expansion by inducing proliferation while simultaneously inhibiting apoptosis and blocking differentiation—demonstrating the necessity of complementary cooperation between the two pathways in promoting self-renewal. Mechanistically, this is accomplished by upregulation of the HSC self-renewal related gene *HoxB4* and *Inhibitor of differentiation 2 (Id2)* combined with downregulation of the apoptosis inducer *s100A8/A9* in double mutants. Informed by this genetic model, we developed an *ex vivo* HSC expansion protocol. In serial transplantation experiments, simultaneous activation of both PTEN/Akt and Wnt/β-catenin pathways achieves long-term engraftment equivalent to a one-hundred fold greater dosage of uncultured HSCs—demonstrating unprecedented expansion of long-term repopulating HSCs. This expansion is achieved using serum-free media with low doses of cytokines but without using feeder-cell layers or permanent genetic manipulation, making our protocol ideal for potential translation into the clinic.

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LOWER VIABLE CD34 RECOVERY IN CRYOPRESERVED ALLOGENEIC PBSC COMPARED TO AUTOLOGOUS PBSC

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Allogeneic peripheral blood stem cells (PBSC) are cryopreserved less often than autologous harvests. The use of cryopreserved allogeneic HPC is becoming increasingly common for storage of excess HPC or T cells for donor lymphocyte infusions, in addition to circumventing issues of donor availability particularly in light of recent worldwide events (Sept 11, swine flu and flight disruptions due to volcanic ash).

During 2006 to 2009, our cell processing laboratory cryopreserved 30 allogeneic and 350 autologous PBSC. It was noted that the post-thaw viable $CD34^+$ recovery was lower in allogeneic PBSC products (median63%, range 16-92) than autologous (72%, 13-151; $p < 0.0004$). Hence this study aimed to determine factors that influence post-thaw CD34 recovery.

We analysed data from all cryopreserved allogeneic and autologous PBSC, with the aim of determining the effect of cryopreserved nucleated cell concentration (NCC), neutrophil content, and time from collection to cryopreservation.

Univariate analysis demonstrated weak inverse correlations were between viable CD34 recovery and NCC (Spearman r = -0.20, $p < 0.0001$), collection to freeze time interval (r = -0.10, p = 0.048),

neutrophil content ($r = -0.20$, $p < 0.0001$). Multiple regression analysis demonstrated that collection to freeze interval ($p = 0.006$), neutrophil content ($p < 0.0001$) and allogeneic donors ($p < 0.001$) significantly affected viable CD34 recovery, but NCC did not ($p = 0.14$).

Of the 35 cryopreserved allogeneic products, 11 have been infused with no significant difference in terms of engraftment of neutrophils (median 18; range 11-31) and platelets (median 27; range 14-58), when compared to infusion of 179 fresh allogeneic products (neutrophils: median 17, range 4-55, $p = 0.9$; platelets: median 19, range1-101, $p = 0.2$).

This data indicates that the lower post-thaw viable CD34 recovery in PBSC may be due to intrinsic properties of allogeneic donor in addition to the neutrophil content and prolonged storage periods prior to cryopreservation. Post thaw analysis of viable $CD34^+$ content is recommended to ensure sufficient viable CD34+ to facilitate engraftment.

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CREATION OF A SEGMENT-BASED ALDEHYDE DEHYDROGENASE ASSAY AS A BIOMARKER FOR UMBILICAL CORD BLOOD POTENCY

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Banked, unrelated umbilical cord blood units (CBU) provide an option for transplantation for patients lacking matched donors. Current methods for selecting a suitable CBU for transplantation include examining the human leukocyte antigen (HLA) matching, total nucleated cell (TNC) count, and, in some situations, viable CD34 counts on the unit prior to cryopreservation. However, 20% of patients experience primary graft failure following UCB transplantation (UCBT), suggesting a better assay to assess potency on a thawed CBU prior to the release from the cord blood bank is needed. Preliminary clinical studies demonstrated that neutrophil and platelet engraftment as well as overall survival can be predicted with post-thaw colony forming units (CFU). The usefulness of post-thaw CFU as a potency assay is limited both by time (14 days) and variability. However, CFUs in fresh cord blood are highly correlated with the number of hematopoietic progenitor cells expressing high levels of the enzyme aldehyde dehydrogenase ($ALDH^{br}$ cells). Retrospective studies of transplanted CBUs indicated that engraftment success was accurately predicted by the number of $ALDH^{br}$ cells infused per kilogram. Therefore, we developed an $ALDH^{br}$ assay for segments attached to CBUs to measure potency for UCBT on segments used for HLA confirmatory typing (CT). We measured CFUs and flow cytometric analysis on 334 segments requested for CT. Each segment was thawed and blood was spotted on a FTA card for CT. The remaining blood was washed with 5% dextran/albumin to remove the DMSO. Following removal of 100,000 white blood cells for CFU, the content of $ALDH^{br}$ [Aldecount®], $CD34^+$, $CD45^+$, glycophorin A^+ and viable ($7\text{-}AAD^+$) cells was measured by flow. Comparisons of the data from the CT segments show that $ALDH^{br}$ correlates well with CFUs ($R^2 = 0.809$), at a ratio approximately 4 ALDHbr cells per 1 CFU. In contrast, CFU and $ALDH^{br}$ counts do not correlate well with viable $CD45^+$ or viable $CD34^+$ count. Of the 334 units assayed, 82 have been administered to patients.

Several graft parameters of potential use in measuring potency of a CBU can be measured prospectively on a segment at the time of CT and can provide information for unit selection. Using unit values calculated from the CT segment, we will compare clinical outcomes to the graft parameters in order to determine which provide predictive information concerning neutrophil and platelet engraftment and overall survival after UCBT.

SUPPORTIVE CARE

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COLLECTION OF G-CSF-MOBILIZED GRANULOCYTES FROM RELATED DONORS TO SUPPORT HEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENTS AT HIGH RISK OF INFECTION IS SAFE AND FEASIBLE

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Background: Granulocyte transfusions may be useful for patients undergoing hematopoietic stem cell transplantation (HSCT) at high risk for the development of life-threatening infections during the neutropenic period. However, their usefulness depends on feasibility and safety of repeated collections of GCSF mobilized granulocytes from related donors.

Methods: A total of 1,536 leucophereses to collect granulocytes from 148 GCSF-mobilized related donors were performed between 1999 and 2009 at Duke to treat 139 HSCT recipients. Donors were screened by a rigourous health history questionnaire and by blood tests for a number of infectious diseases. Central venous line (CVL) placement was performed under sedation or anesthesia. Subcutaneous G-CSF (10mcg/kg) was given the night before pheresis, twice weekly. Each collection was divided into 3 aliquots and infused daily to provide dosing for 6 days per week. Donors received oral iron, vitamin K, and calcium supplements. They were carefully monitored with regular clinical and laboratory tests for any adverse effects. All products were regularly assayed for total nucleated cell counts (TNC), viability and sterility.

Results: Of 148 donors, 74 were male and 62 CMV seropositive. Their median age was 36.8 years (range: 17-58) and median weight was 79.3 kg (50.9 - 183.5). Donors were fathers (n = 72), mothers (n = 57), siblings (n = 8), or other relatives (n = 11). Past medical history was negative in all but 13 donors (asthma, n = 5; hypertension, n = 3; diabetes, n = 1; others, n = 4). Donors underwent a median of 8 phereses (range, 1-71) over a median of 25 days (range, 1-266). Complications included pruritis (n = 8), fever (n = 3), bacteremia (n = 15), CVL tract infection (n = 1), exit site related minor bleeding (n = 3), local discharge (n = 7), and erythema (n = 8). Five required 2 or more CVLs. Laboratory abnormalities included LFT elevation (n = 1), PT > 16 sec (n = 2), and fibrinogen < 100 mg/dL (n = 1). Between the first and the last pheresis, the median drop in Hemoglobin was 2.5 gm/dL (range, 0-6.8) and the drop in serum albumin was 0.6 gm/dL (range, 2-2.2). Ten of 1536 granulocyte products were contaminated with bacteria. Pheresis was stopped for all fevers and positive line cultures.

Conclusions: When performed at an experienced center using well defined guidelines and good supportive care, granulocyte collection from family donors following GCSF stimulation is safe and feasible.

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ERYTHROPOIETIN-ASSISTED PHLEBOTOMY IN STEM CELL TRANSPLANT PATIENTS WITH IRON OVERLOAD

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Stem cell transplantation often necessitates repeated transfusions raising the risk of secondary hemochromatosis. Erythropoietin-assisted phlebotomy (EAP) is a potential treatment to alleviate iron overload in these patients.

Methods: We carried out a retrospective review of 372 stem cell transplants at our hospital from 2000 to 2008. We evaluated the maximum ferritin level for each patient and used a level of 1,000 ug/mL to identify patients at increased risk for complications of iron overload. 65 patients had ferritin levels < 1,000ug/mL and served as controls. Groups were further subdivided into those who received therapeutic EAP and those who did not. Records were also reviewed for transplant related infections.

Results: Among the 56 patients with ferritin levels over 1,000ug/mL, the average ferritin level was 2,965ug/mL. These patients received an average of 26.6 units of transfused PRBCs during their transplant course. Ten of these patients underwent EAP of 500cc of blood approximately every two weeks to decrease their ferritin level. The non-phlebotomized group had an increased rate of infections and trended toward a higher mortality. The non-phlebotomy, hyperferritinemia group had the highest rates of infection within our three groups at both three and six months ($p < 0.004$ and $p < 0.008$) but not at 1 year ($p < 0.086$). There was no significant difference in infection rates in the EAP group and the controls at either 3 or 6 months. ($p < 0.17$, $p < 0.107$) Mortality differences between the two main groups (ferritin > 1,000ng/ml and ferritin < 1,000 ng/ml) were significant at both six months and one year ($p < 0.0004$ and $p < 0.005$ respectively). There was also a significant difference between the hyperferritinemia group who did not receive EAP and controls at both six months and one year ($p < 0.00002$ and $p < 0.0004$ respectively). Two-tailed Fishers exact test showed that the mortality rate between the two subsets in the hyperferritinemia group was significant at six months ($p = 0.009$ two sided; $p =$