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Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices

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ABSTRACT

Human cord blood (CB) is enriched in circulating endothelial colony forming cells (ECFCs) that display high proliferative potential and in vivo vessel forming ability. Since diminished ECFC survival is known to dampen the vasculogenic response in vivo, we tested how long implanted ECFC survive and generate vessels in three-dimensional (3D) type I collagen matrices in vitro and in vivo. We hypothesized that human platelet lysate (HPL) would promote cell survival and enhance vasculogenesis in the 3D collagen matrices. We report that the percentage of ECFC co-cultured with HPL that were alive was significantly enhanced on days 1 and 3 post-matrix formation, compared to ECFC alone containing matrices. Also, co-culture of ECFC with HPL displayed significantly more vasculogenic activity compared to ECFC alone and expressed significantly more pro-survival molecules (pAkt, p-Bad and Bcl-xL) in the 3D collagen matrices in vitro. Treatment with Akt1 inhibitor (A-674563), Akt2 inhibitor (CCT128930) and Bcl-xL inhibitor (ABT-263/Navitoclax) significantly decreased the cell survival and vasculogenesis of ECFC co-cultured with or without HPL and implicated activation of the Akt1 pathway as the critical mediator of the HPL effect on ECFC in vitro. A significantly greater average vessel number and total vascular area of human $CD31^+$ vessels were present in implants containing ECFC and HPL, compared to the ECFC alone implants in vivo. We conclude that implantation of ECFC with HPL in vivo promotes vasculogenesis and augments blood vessel formation via diminishing apoptosis of the implanted ECFC.

Introduction

The progenitor cells for the endothelial lineage play critical roles in vascular homeostasis and regeneration in adult subjects (Asahara et al., 1997; Hirschi et al., 2008). Endothelial colony forming cells (ECFCs) are derived from a rare circulating subset that may arise from resident endothelium of established blood vessels in man (Hirschi et al., 2008). We have successfully isolated human peripheral blood (PB) or umbilical cord blood (CB) derived circulating ECFC that display a hierarchy of proliferative potential through the use of single cell clonogenic and functional assays (Ingram et al., 2004; Huang et al., 2011; Yoder et al., 2007). Human CB derived ECFC also display de novo vessel forming ability in nude or non-obese diabetic/severe combined immunodeficient mice (NOD/SCID) in vivo after subcutaneous implantation of ECFC in type I collagen matrices and upon perfusion with host murine vessels, become a part of the systemic host circulation (Cheng et al., 2011; Critser et al., 2010; Melero-Martin et al., 2007, 2008).

Previous studies have reported that human ECFC-derived vessels formed in subcutaneous implants were found as early as day 1 or 2 after implantation in NOD/SCID mice (Allen et al., 2013). However, these premature vessels were not properly perfused with murine red blood cells of the host circulatory system at those early time points. Proof of human ECFC cell-lined vessel connection to host circulation system after 3–4 days of implantation in vivo was subsequently reported (Allen et al., 2013). It has been reported that up to 60% of human umbilical vein endothelial cells (HUVECs) undergo apoptosis in the first 24 h after suspension in collagen gels and there is further loss of vascular structures in 3D type I collagen matrices after 24 h of culture (Ilan et al., 1998). In our previous study, we demonstrated that human CB ECFC containing collagen matrices displayed ECFC undergoing apoptosis on day 1 following implantation (Kim et al., 2015). After 2–3 days, most ECFC died with only 1–3% of the total implanted cells demonstrating viability in the subcutaneous implants (Kim et al., 2015). Since the stability of newly formed vessels requires systemic blood flow, these

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data suggest that implanted endothelial cells must survive at least 3–4 days in vivo to form a stable perfused capillary network connected to the host circulatory system.

The microvascular network has the intrinsic capacity to remodel itself through changes in endothelial cell differentiation, growth, migration, and matrix modification (Risau, 1995; Hanahan and Folkman, 1996). Previous studies have reported that cytokines, matrix proteins, and integrins are important factors in capillary endothelial network formation and remodeling in various matrix models (Matrigel and type I collagen matrix systems) (Critser et al., 2010; Ranta et al., 1998; Hynes, 1992; Fukai et al., 1998; Sacharidou et al., 2012; Stratman et al., 2011; Whittington et al., 2013). The absence of peri-endothelial support cells or lack of endothelial cell adhesion to matrix proteins causes destabilization and regression of capillary structures within Matrigel or 3D type I collagen matrices (Hynes, 1992; Fukai et al., 1998; Pollman et al., 1999; Stratman et al., 2010). Apoptosis of endothelial cells has been observed in the context of dynamic capillary network remodeling in the various matrix models both in vitro and in vivo and is thought to play a necessary role for optimal network formation (Fukai et al., 1998; Pollman et al., 1999). On the other hand, the generation of anti-apoptotic signals is also required to maintain integrity of the vascular network following realignment of certain matrix proteins and cell–cell or cell–matrix interactions during the process (Fukai et al., 1998; Pollman et al., 1999). Thus, understanding the balance between pro-apoptotic and pro-survival signals is a critical point in capillary structure formation and the remodeling process.

Apoptosis is a cellular process in tissue development that generally culminates with the sequential activation of caspases, the cysteine proteases for cleavage of proteins (Duprez et al., 2009; Taylor et al., 2008). There are two pathways that result in apoptosis depending on the apoptotic signal. The extrinsic pathway is initiated by ligand binding to membrane receptors of the death receptor family, whereas the intrinsic pathway is mediated by stress-mediated damage such as alterations in temperature, osmolality, DNA damaging agents, free radical generation compounds, removal of nutrients, and oxygen deprivation (Duprez et al., 2009; Taylor et al., 2008). The activation of the two pathways leads to the same type of apoptotic response, causing release of cytochrome c (Cyt-C) from mitochondria and activation of the executioner caspase-3 (Duprez et al., 2009; Taylor et al., 2008). In the normal state, Akt activation (phosphorylated Akt, pAkt) by various growth factors induces cell survival via protection of mitochondrial integrity and inhibition of Cyt-C release (Gottlob et al., 2001; Uchiyama et al., 2004). Akt has also been reported to stimulate the expression of anti-apoptotic Bcl-2 proteins, such as Bcl-xL that prevents permeabilization of the mitochondrial membrane by inhibiting activation of Bax/Bak (Zong et al., 1999). However, under apoptotic stimulation, pro-apoptotic members of the Bcl-2 family (Bax, Bak and Bad) are activated and induce the release of Cyt-C from mitochondria by binding and inactivating anti-apoptotic proteins (Scorrano and Korsmeyer, 2003). As the penultimate event, caspase-3 is activated to cleaved-caspase-3 to generate all the biochemical and morphologic hallmarks of cell apoptosis.

Platelets regulate the balance between apoptosis and cell survival in numerous cells involved in the repair of injured tissues (Gambim et al., 2007; Mause et al., 2010). Since platelets have a critical role in regulation of cell death and survival and contains various growth factors and cytokines (Gambim et al., 2007; Mause et al., 2010; Shih et al., 2011), we hypothesized that human platelet lysate (HPL) would increase ECFC survival and enhance ECFC vascularization by modulating the balance between apoptosis and cell survival of ECFC in 3D collagen matrices.

Materials and methods

Culture of human umbilical cord blood (UCB) derived ECFC

ECFCs were isolated and cultured as previously described (Ingram et al., 2004). ECFC colonies appeared between 5 and 22 days of culture and were noted to form colonies of adherent cells with cobblestone morphology. After approximately 10 days of culture, the ECFC-derived ECs were released from the culture dish by TrypLE™ Express (Gibco) and replated onto 25/75 cm^2 tissue culture flasks pre-coated with Type I rat-tail collagen (BD Biosciences) for subsequent passage.

Preparation of three-dimensional (3D) collagen matrices

All of the type I collagen oligomers and associated polymerization reagents were purchased from GeniPhys (Bailey et al., 2011). Stock oligomer was diluted in 0.01 N hydrogen chloride (HCl) and neutralized according to the manufacturer's recommendations to achieve a final oligomer concentration of 1.37 mg/ml (200 Pa matrix stiffness). ECFCs (1×10^5/60 μl or 1×10^6/250 μl) were suspended in the collagen solution with or without human platelet lysate (HPL — 10% of final concentration) at 4 °C. The HPL was purchased from Gemeinnützige Salzburger Landeskliniken Betriebsges (SALK) Graz, Austria. The HPL was prepared from pooled platelet-rich plasma derived from a minimum of 40 whole blood donations (Schallmoser and Strunk, 2009). The collagen-cell suspensions were plated on 96-well or 48-well plates and allowed to polymerize at 37 °C for 30 min and covered with complete endothelial cell growth medium (EGM-2, Lonza) with 10% defined fetal bovine serum (Hyclone) for incubation for one to three days at 37 °C, 5% CO_2.

Toluidine blue staining of 3D collagen matrices and quantification of in vitro vascular structures

For analysis of in vitro vascular structure formation in 3D collagen gel, cellularized collagen matrices were fixed with 4% paraformaldehyde for 20 min on Day 1 or Day 3 after culture. The matrices were stained with 0.1% toluidine blue O dye (30% methanol) for 25 min at room temperature and washed with PBS for 30 min for three times at room temperature. Vascular structures including vacuoles, lumens and tube formation were quantified using ImageJ image analysis software (National Institutes of Health (NIH)). Vacuoles and lumens were defined as areas completely surrounded by a toluidine blue labeled endothelial cell membrane. A single image of the entire well was captured at a depth of 10 μm from the surface of the matrix and then additional places 150 μm apart for each of triplicate samples of each group. Images of vascular structures were captured at 10× and 20× levels of magnification.

Assessment of apoptosis of ECFC in 3D collagen matrices

Apoptosis was assessed by examining the percentage of human $CD31^+$ ECFCs that bound Annexin V and propidium iodide and was performed as per the manufacturer's instruction (Apoptosis Detection kit; eBioscience). ECFC (1×10^5/60 μl) were suspended in collagen matrices with/without HPL (10% of final concentration), with dimethyl sulfoxide (DMSO) vehicle (same volume of solvent for each inhibitor) or with signaling pathway inhibitors, plated in 96-well plates and incubated for one to three days as above. As previously described (Kim et al., 2015), matrices were recovered from one to three days after culture and incubated in 250 μl Collagenase Type I (0.25%) (Stemcell technology) for 20 min at 37 °C. Akt1 inhibitor (A-674563) and Bcl-xL inhibitor (ABT-263/Navitoclax) were purchased from Selleckchem and Akt2 inhibitor (CCT128930) was purchased from Santa Cruz biotechnology. Cell dissociation buffer was added to stop the enzymatic reaction (Invitrogen). Cells were centrifuged at 500 *g* for 5 min at room temperature. Cell pellets were suspended in staining buffer and stained with anti-human CD31 antibody conjugated to phycoerythrin (PE) for 15 min (clone WM-59, BD Biosciences Pharmingen). Cells were incubated and stained for Annexin V-allophycocyanin (APC) and propidium iodide (PI) in binding buffer for 10 min at room temperature in the dark. Stained cells were analyzed by FlowJo software.