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Megakaryocyte polyploidization and proplatelet formation in low-attachment conditions

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ABSTRACT

In vitro-derived platelets (PLTs), which could provide an alternative source of PLTs for patient transfusions, are formed from polyploid megakaryocytes (MKs) that extend long cytoplasmic projections, termed proplatelets (proPLTs). In this study, we compared polyploidization and proPLT formation (PPF) of MKs cultured on surfaces that either promote or inhibit protein adsorption and subsequent cell adhesion. A megakaryoblastic cell line exhibited increased polyploidization and arrested PPF on a low-attachment surface. Primary human MKs also showed low levels of PPF on the same surface, but no difference in ploidy. Importantly, both cell types exhibited accelerated PPF after transfer to a surface that supports attachment, suggesting that pre-culture on a non-adhesive surface may facilitate synchronization of PPF and PLT generation in culture.

1. Introduction

In vitro-derived platelets (PLTs) could provide an alternative source of PLTs for patient transfusions. PLTs are formed when polyploid megakaryocytes (MKs) extend long, cytoplasmic extensions from the bone marrow and into small blood vessels (sinuses). PLT components are shuttled and packaged along these extensions, termed proplatelets (proPLTs), before being sheared off by flowing blood [1]. MKs and their PLT progeny have been successfully derived *in vitro* from $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) from mobilized peripheral blood (mPB) [2,3] and umbilical cord blood [4–8], as well as MK progenitor immortalized cell lines [9], induced pluripotent stem cells [10–12], and embryonic stem cells [13–15].

Although MKs undergo endomitosis and extend proPLTs during *in vitro* culture, the factors responsible for initiating these events are not well understood. MKs express receptors for different extracellular matrix (ECM) components during development [16] and are found near fenestrated endothelium during terminal maturation, making it likely that MKs interact with proteins and/or glycosaminoglycans (GAGs) at this blood-bone marrow interface. Several studies have examined the role that cell-substrate interactions play in polyploidization and proPLT formation (PPF). Chemokine-mediated localization of MKs to the bone marrow vascular niche promotes platelet production [17]. Cultures supplemented with soluble dermatan sulfate show higher MK ploidy [18], and several different covalently immobilized GAGs, including heparan sulfate and heparin, significantly increase the percentage of MKs with PPF and promote PLT release [19]. MKs can also form proPLTs on several immobilized ECM components, including fibronectin, fibrinogen, and von Willebrand factor, although the kinetics of PPF vary across different substrates [20]. Although cell adhesion is important, a number of studies suggest that formation of mature stress fibers and focal adhesions downregulates polyploidization and PPF. Type I collagen supports MK spreading [21,22] and inhibits PPF in human MKs [20,23], while focal adhesion kinase-null mice produce a greater percentage of high-ploidy MKs [24]. Similarly, inhibition of myosin light chain kinase or non-muscle myosin II, by way of blebbistatin treatment or Myh9 knockout, has been shown to increase ploidy and PPF [25–27]. Upstream of myosin II, inhibitors against RhoA and ROCK enhance both ploidy and PPF [26–29].

While several studies have characterized the effect of specific receptor-ligand engagement on MK polyploidization and PPF, the effect of inhibiting MK adhesion has yet to be assessed. In this

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2. Material and methods

Unless otherwise noted, all reagents were from Sigma Aldrich (St. Louis, MO) and all cytokines were from Peprotech (Rocky Hill, NJ).

2.1. Differentiation of human megakaryoblastic cell lines

The human megakaryoblastic CHRF-288-11 (CHRF) and myelogenous leukemia K562 cell lines were cultured in Iscove's Modified Dubelcco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Waltham, MA). On day 0, cells were resuspended in IMDM + 10% FBS to a final concentration of 100,000/mL and seeded in tissue culture-treated (TC) polystyrene, Ultra Low Attachment (ULA; Corning, Tewksbury, MA), or poly(2-hydroxyethyl methacrylate) (polyHEMA)-coated well plates. Cells were seeded such that an entire well could be harvested for each analysis time point. Seeded cells were treated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA; Calbiochem, Whitehouse Station, NJ) to induce MK differentiation [30]. In select experiments, CHRF cells were also treated with various combinations of 12.5 mM nicotinamide (Nic), 0.5 μM H-1152 (Calbiochem) rho-associated protein kinase (ROCK) inhibitor, and 10 μM (−)-blebbistatin (active enantiomer) myosin IIa inhibitor.

2.2. Harvest of PMA-treated CHRF and K562 cells

The supernatant from each well was transferred to conical tubes, then a PBS rinse was performed. Each well was incubated at 37 °C for 15 min with prewarmed Accutase (Millipore, Billerica, MA). The Accutase was pipetted up and down several times to dislodge any loosely-adherent cells before a final PBS rinse was performed. Both rinses and the Accutase were collected in the respective conical tube. Any remaining cell aggregates were easily broken up via repeated pipetting or vortexing.

2.3. Preparation of polyHEMA-coated, non-adhesive culture surfaces

TC well plates and T-flasks were treated with a solution of 10% polyHEMA in 95% ethanol with 10 mM NaOH, such that the bottom and walls were coated. Excess solution was removed and the surfaces were allowed to dry in a biosafety cabinet overnight. Prior to use, the surfaces were rinsed with PBS.

2.4. Primary MK culture

Cryopreserved $CD34^+$ HSPCs from mPB were purchased from the Fred Hutchinson Cancer Research Center with Northwestern University Institutional Review Board approval. Cells were obtained from healthy donors undergoing granulocyte-colony-stimulating-factor (G-CSF) mobilization following informed consent. Cultures of $CD34^+$ cells were initiated in TC T-flasks at 50,000 cells/mL in IMDM + 20% BIT (78% IMDM [Gibco, Carlsbad, CA], 20% BIT 9500 Serum Substitute [STEMCELL, Vancouver, BC, Canada], 1% Glutamax [Gibco], 1 μg/mL low-density lipoproteins [Calbiochem], 100 U/mL Pen/Strep) supplemented with 100 ng/mL thrombopoietin (Tpo), 100 ng/mL stem cell factor (SCF), 2.5 ng/mL interleukin (IL)-3 (R&D Systems, Minneapolis, MN), 10 ng/mL IL-6, and 10 ng/mL IL-11. Cells were cultured in a fully humidified chamber at 37 °C, 5% CO_2, and 5% O_2 for 5 days. On day 5, cells were pelleted and resuspended in fresh IMDM + 20% BIT supplemented with 100 ng/mL Tpo, 100 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-9, and 10 ng/mL IL-11. Cells were cultured at 20% O_2 until day 7. MKs were enriched on day 7 using anti-CD61-conjugated magnetic beads (Miltenyi, Bergisch Gladbach, Germany), then resuspended in fresh IMDM + 20% BIT supplemented with 100 ng/mL Tpo, 100 ng/mL SCF, +/− 6.25 mM Nic. Cells were seeded on TC or polyHEMA-coated surfaces, as described. Cells were transferred from polyHEMA to TC surfaces at day 9 or 11.

2.5. Flow cytometric analysis of MK ploidy

Cells were washed with cold PBS containing 2 mM EDTA and 0.5% BSA (PEB). For primary MKs, FITC-conjugated anti-CD41 antibody (BD Biosciences, San Jose, CA) was added for 30 min at 4 °C. Cells were fixed with 0.5% paraformaldehyde (Polysciences, Warrington, PA) in PBS for 15 min at room temperature, permeabilized with cold 70% methanol for 1 h at 4 °C, treated with RNase for 30 min at 37 °C, then incubated with 50 μg/mL propidium iodide to stain DNA prior to analysis with an LSR II flow cytometer (BD Biosciences).

2.6. Flow cytometric analysis of MK viability

Cells were washed with PEB, then incubated with DAPI (Life, Carlsbad, CA) for 15 min at room temperature prior to analysis with an LSR II flow cytometer.

2.7. Flow cytometric analysis of MK apoptosis

Cells were washed with PBS, then with 1X Annexin V binding buffer (BD Biosciences), incubated with Cy5-conjugated Annexin V for 15 min at room temperature, and washed with 1X Annexin V binding buffer. DAPI was added for 10 min at room temperature prior to analysis with an LSR II flow cytometer.

2.8. Transfer of CHRF cells from ULA to TC surfaces

PMA-treated CHRF cells on a ULA surface were pipetted several times to create a homogeneous suspension. An aliquot of cells was pelleted at 300*g* for 5 min, the supernatant was aspirated, and the cell pellet was resuspended in fresh IMDM + 10% FBS. The cells were reseeded on a TC surface and treated with PMA to a final concentration of 10 ng/mL.

2.9. Quantification of CHRF cell PPF and proPLT length

Supernatant was removed from TC wells with PMA-treated CHRF cells and the wells were washed once with 37 °C PBS + Ca^{2+} + Mg^{2+}. Fresh 37 °C PBS + Ca^{2+} + Mg^{2+} was gently added to each well, being careful not to dislodge any adherent cells. The cells were observed using a DM IL LED inverted microscope (Leica, Wetzlar, Germany), and imaged with a QICAM digital camera (QImaging, Surrey, BC, Canada). The percentage of proPLT forming cells was measured as the number of proPLT-forming adherent cells divided by the total number of adherent cells. ProPLT length was measured using the 'Segmented Line' tracer in ImageJ [31]. To limit error in missing cells and/or double-counting cells, proPLT extensions were measured starting in one corner of the image and working across the image, dividing the image into three rows. The