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Current status of blood “pharming”: Megakaryocyte transfusions as a source of platelets

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Abstract

Purpose of review—Donor-derived platelets have proven to be of hemostatic value in many clinical settings. There is a fear that the need for platelets may outgrow the donor pool in first-world countries. Moreover, there are other challenges with donor platelets that add to the impetus to find an alternative platelet source, especially after the megakaryocyte cytokine thrombopoietin was identified. Megakaryocytes have since been differentiated from numerous cell sources and the observed released platelet-like particles (PLPs) have led to calls to develop such products for clinical use. The development of megakaryocytes from embryonic stem cell also supported the concept of developing non-donor-based platelets.

Recent findings—Several groups have claimed that non-donor-based platelets derived from in vitro-grown megakaryocytes may soon become available to supplement or replace donor-derived products, but their number and quality has been wanting. A possible alternative of directly infusing megakaryocytes that release platelets in the lungs – similar to that recently shown for endogenous megakaryocytes – has been proposed.

Summary—This review will describe the present state-of-the-art in generating and delivering non-donor-derived platelets. Progress has been slow, but advances in our ability to generate human megakaryocytes in culture, generate PLPs from these cells, and test the functionality of the resultant platelets in vitro and in vivo have identified important remaining challenges and raised alternative potential solutions.

Keywords

Non-donor-derived platelets; induced-pluripotent stem cell; platelet bioreactor; pulmonary vasculature

Introduction

Platelet transfusions have proven useful to improve clinical outcomes both prophylactically, such as in patients with chemotherapy-induced thrombocytopenia (CIT), and emergently,

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Conflicts of interest

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such as in post-trauma bleeding. Over the years, the level of platelet counts at which one would give a platelet transfusion to a patient with CIT has decreased, in large part because of little change in the risk of significant hemorrhage [1]. In spite of this decreasing trigger point, the number of transfusions in the United States and other first world countries has steadily increased, so that ~1.9 million platelet units were transfused in the US in 2015 [2]. This demand may outstrip supply, due to the aging of the United States' population, a rise in hematologic malignancies and the development of new, invasive therapeutic interventions, such as extracorporeal membrane oxygenation and ventricular assistant devices [2,3]. These trends have provided the drive to develop non-donor sources of platelets for transfusions.

Non-donor platelets also offer the possibility of generating a more uniform product with known consistent clinical outcomes per transfused unit [4]. Donor-derived platelets units have a marked variability in response to agonists [5] as well as different yields per unit [6] (see Table 1 comparing donor-derived versus in vitro-derived platelets). Moreover, as with all donor-derived products, these platelets have the risk of carrying various infectious agents [7,8]. Because they need to be stored at room temperature to optimize retained functionality, donor-derived platelets also have the highest risk of bacterial contamination of any blood product [9].

Non-donor-derived platelets could potentially offer advantages. If the cell line from which they are derived is self-renewing, it would allow targeted genetic manipulation for the generation of a universal line (or several lines to cover major histocompatibility groups), limiting concerns of developing HLA-based platelet refractoriness [10]. Units can also be generated for individual patients as a form of personalized medicine. Additionally, with the development of easier gene targeting via CRISPR/Cas 9 technology or related genetic manipulation techniques can be done to correct an inherited platelet disorder for particular patients [11]. Targeted protein expression or genetic manipulation of starter cell lines can be used to correct a genetic defect, such as correcting expression of α IIb expression in patients with Glanzmann thrombasthenia [12] or for the megakaryocyte-specific transcription factor Fli1 in patients with Jacobsen syndrome [13]. Alternatively, the expressed protein may be one not normally expressed in developing megakaryocytes or in platelets with the expectation that the ectopically expressed protein is stored in the platelet alpha-granules and released at sites of vascular injury. So far, a number of proteins have been ectopically expressed in megakaryocytes during terminal differentiation, and Factor VIII [14], urokinase [15], and ADAMTS13 [16] appear to be predominantly or exclusively stored in alpha granules. Platelet-expressed Factor VIII may be useful to treat patients with inhibitors, platelet urokinase may be a useful thromboprophylaxis agent [15], while platelet ADAMTS13 may be useful in thrombotic microangiopathy with each product showing limited systemic effects.

Videos of megakaryopoiesis: Its influence on strategies to generate non-donor-derived platelets

The identification and availability of thrombopoietin beginning in the mid 1990s as a megakaryocyte-specific cytokine [17] was important developing in vitro culture conditions for the growth of megakaryocytes. Beginning with CD34⁺-derived hematopoietic progenitor

cells, it was recognized that megakaryocytes shed platelet-like particles (PLPs) in culture. These PLPs share both morphologic features on electron microscopy and functionality, such as adherence and spread on fibrinogen, with donor-derived platelets [18]. Video studies of mature megakaryocytes undergoing thrombopoiesis suggested that megakaryocytes extend multiple processes each undergoing several rounds of bifurcation in a dynamic fashion, although these structures did not undergo terminal platelet release (Figure 1A and [19]). These studies still dominate the field of non-donor-derived platelets, focusing attention on the released particles. However, other studies showed that the PLPs differed considerably from donor-derived platelets [20,21]: the size range of the PLPs, which included many microparticles and did not show a Gaussian distribution as seen with donor-derived platelets, their overall agonist responsiveness was relatively poor, and PLPs showed signs of apoptosis or injury and/or lacked surface CD41 expression. While a subset of PLPs may be platelet-like in nature, the majority of PLPs may represent terminal events in cultured cells without proper niche support or access to the medullary sinuses, and may not be true platelets.

Subsequent two-photon microscopy of murine skulls elegantly showed intramedullary megakaryocytes migrating to and traversing the endothelial lining into the medullary sinus. This was interpreted as showing proplatelet shedding into the flowing blood, supporting the *in vitro* release of proplatelets ([22, 23] and Figure 1B). However, these movies and those of others, both published [23] or unpublished, either show the above or megakaryocytes extending only one process, extending that process across the endothelial lining and shedding large cytoplasmic fragments. In fact, in a number of videos, one can see a cytoplasmic strand connecting the released cytoplasmic fragments like beads-on-a-string (Figure 1C). These studies suggest that either megakaryocytes release platelets in several different ways in the marrow space or that slightly different technologies lead to alternative visualization and interpretation of events.

One alternative interpretation of the events in the medullary space is that the entire megakaryocyte traverses the endothelial lining in bones, much like a migrating neutrophil. The stretched-out megakaryocyte then regroups before reaching the lung, where it is “entrapped”, releasing platelets. Such a model was first proposed in the mid-1930s after measurements of differences in platelet counts in pulmonary arteriolar and venular blood [24]. Recent two-photon microscopy of the pulmonary bed confirms that at least some whole megakaryocytes migrate out of the marrow space and reach the lungs, where they shed platelets by becoming entrapped and extend several proplatelet arms in a process that takes under an hour to complete [25]. Calculation of the number of megakaryocytes seen entrapped in the lung suggests that approximately half of the platelets in the body are released in the lungs. Since there are few data to definitively calculate number of platelets released per megakaryocyte, the actual number of platelets released in the lungs may be only a fraction of this number or may include almost all platelets. Presumably, the remaining platelets are released by the alternative pathways shown in Figure 1.

Cell lines to generate in-vitro megakaryocytes

Early *in vitro* megakaryopoiesis studies, adult- or cord-blood-derived CD34⁺ megakaryocytes [18]. These cells have limited self-proliferation features except perhaps in

the setting of valproic-acid treated cord blood [26], so they were not a practical cell source for developing a non-donor-derived platelet transfusion product. Nonetheless, they provided thrombopoietin-focused protocols for generating megakaryocytes as well as protocols for analyzing the final PLPs. With the development of the self-proliferating induced pluripotent stem cells (iPSCs) and the ability to differentiate these into megakaryocytes, these have become the focus of most group's attention to make platelets in vitro. iPSCs are embryonic-like cells derived from various adult tissues reprogrammed to express Oct4 (Pou5f1), Sox2, cMyc, and Klf4 [27]. Such cells can be differentiated into mesodermal tissue, then hematopoietic progenitor cells, and finally, megakaryocytes, as described [22,23]. The process is not very efficient: one iPSC yields 1–100 megakaryocytes, and the subsequent yield of in vitro-released PLPs is perhaps 10–1000 per initial iPSC. With these characteristics, for a platelet unit of $\sim 3 \times 10^{11}$ platelets one would need to begin with $\sim 10^8$ iPSCs, and the costs (in time and resources) of iPSC tissue culture media and cytokines may be prohibitively high.

To try to overcome this limitation in efficiency, several groups proposed alternative strategies for establishing a self-proliferative intermediate line from human iPSCs that can be grown to large numbers before undergoing terminal differentiation into functional megakaryocytes. The first approach involved overexpression of BMI1 and BCL-XL to suppress cell senescence and apoptosis, respectively [20]. These cells needed the “constrained overexpression” of c-MYC to promote proliferation. Alternatively, overexpression of GATA1, FLI1, and TAL1 has been reported to result in a self-proliferative intermediate cell line that could be terminally differentiated into megakaryocytes [28]. The ability of the resultant megakaryocytes to release in vitro functional platelets, or more importantly, platelets that have the half-life and functionality of donor-derived platelets has not been demonstrated for either approach. Reproducibility of these approaches has yet to be confirmed by independent laboratories.

Another major challenge with iPSC-derived megakaryocytes is that these cells are likely to be of a primitive or embryonic nature [29]. Blood originally forms in the yolk sac of a developing embryo, and includes recognizable megakaryocytes [30]. Analysis of iPSC hematopoietic cells suggest that they represent this stage of development. It is unclear, however, whether the function of these primitive megakaryocytes is hemostatic or perhaps relates to vasculogenesis [31]. Supporting the primitive nature of these cells, iPSC-derived megakaryocytes are small and have low ploidy compared to primary megakaryocytes and CD34⁺-derived in vitro cultured megakaryocytes [20]. Whether the released platelets from these cells would be of clinical benefit in adults is unclear as biological properties of neonatal platelets and megakaryocytes are demonstrably different from adult cells [32]. This concern serves as an impetus to develop more definitive megakaryocytes from iPSCs, although even cord blood-derived platelets have limited hemostatic function and agonist responsiveness compared to adult platelets [33]. Clearly iPSC-derived platelets would need careful evaluation of function before being used in place of donor-derived platelets.

Alternative sources of cells for hematopoietic differentiation have been proposed. Present-day efforts include studies using cord blood-derived hematopoietic progenitor cells. Such cells can be expanded 10–100-fold by exposure to valproic acid, which results in histone

deacetylase epigenetic chromatin modification [26]. Whether the resulting megakaryocytes would release functional platelets needs to be shown and whether these platelets demonstrate fetal or adult features is unknown. Finally, alternative adult tissues have been used to generate megakaryocytes and platelets. While endothelial cells may give rise to megakaryocytes under appropriate stimulation [34], the most successful approach begins with liposuction-derived adipocytes that can be grown in an adipocyte-induction medium followed by differentiation in a megakaryocyte-induction media [35]. The advantages of the latter approach are that the necessary beginning cells are readily available and induction to produce megakaryocytes requires no genetic manipulation. Whether these megakaryocytes release sufficient numbers of functional platelets for therapeutic application still needs to be convincingly demonstrated.

Platelet bioreactors

After megakaryocytes are generated in vitro, the challenge is to generate large numbers of functional platelets from these cells. Above, we already discussed the natural release of PLPs from in vitro-grown megakaryocytes, and the fact that the yield and function of these PLPs does not fully recapitulate donor-derived platelets (Figure 2). In addition to the two-photon microscopy videos discussed above, studies have shown that shear can increase the release of PLPs in culture from megakaryocytes [36,37]. Half-life and functionality of these PLPs, especially in an in vivo model, have yet to be reported, although current data demonstrate near normal platelet receptor expression levels and demonstrable in vitro platelet function.

Subsequently several groups have presented data on plastic-based bioreactors [4,38,39] (Figure 2). While acknowledging the potential value of an endothelial lined system for physiologic platelet generation [39], none of the actual systems tested used to generate PLPs were coated with endothelial cells. All appear to use significant shear and a plastic sieve to force the megakaryocytes to shed their cytoplasm. Videos show that most megakaryocytes appear to traverse these sieves without shedding cytoplasm [39]. Comparative analysis to well-prepared, donor-derived platelets in vitro and in vivo were not included for the PLPs harvested from this system. It is unclear whether the yield and functionality is any better than spontaneously released PLPs from a similar number of megakaryocytes. A silk-fiber-based three-dimensional matrix system wherein the megakaryocytes were directly grown within the device or added after more standard growth and PLPs released downstream were gathered and characterized [40] (Figure 2) was also described. The flow through a protein matrix was felt to be more physiologic than forcing flow through a plastic device; however, the in vivo functionality of the resultant particles was not measured; thus, whether it has advantages compared to other devices needs to be tested.

The final bioreactor tested for releasing platelets from megakaryocytes was to infuse human megakaryocytes into immunodeficient mice and using the pulmonary bed as a natural “platelet bioreactor” [21] (Figure 2). The infused megakaryocytes released platelets over a timeframe similar to endogenous megakaryocytes shedding of platelets [25]. These released platelets had a Gaussian-size distribution much like donor-derived platelets and comparable half-life. Functionality of these released human platelets studied by response to agonist

within extracted whole murine blood was nearly identical to infused donor-derived platelets as was their incorporation into thrombi in the mice. These studies suggest that in vitro-generated megakaryocytes can release platelets of similar function and half-life as donor-derived platelets. They, as well as the studies of endogenous megakaryocyte shedding platelets in the lungs, support that megakaryocyte infusions might be an alternative strategy to developing an in vitro bioreactor, but additional studies of the clinical impact of such transfusions, especially the implications of infusing so many cells that transiently obstruct the pulmonary vascular system and the risk of tumorigenesis need to be addressed. In 1997, however, preliminary studies in human recipients suggested that infusion of ex-vivo generated megakaryocytes is probably safe in adults (PMID: 9108385). Additionally, these studies may offer insights into how to design a platelet bioreactor including the three-dimensional organization of the endothelial-lined pulmonary channels and the nature of its lining. Finally besides shear, changes in oxygen and carbon dioxide tensions may be important factors that regulate shedding of platelets from megakaryocytes, as previously suggested [37].

Conclusions

The replacement of donor-derived platelets with a product derived from in vitro-grown megakaryocytes would ensure the continued availability of platelet transfusions as the United States population ages, while enhancing product quality and expanding its potential uses. Significant progress has been made in understanding the biology of megakaryocyte formation and platelet release, but much remains to be done to enhance yield efficiency and to ensure that the final released platelet product is of clinical utility for prophylactic or emergent therapy. So far, the best platelet bioreactor appears to be a natural one, the pulmonary vascular bed with its ability to shed platelets from intravenously infused megakaryocytes.

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Keypoints

- Non-donor-derived platelets will be key to expanding available platelets for transfusions as demand increases to support new, life-saving technologies.
- Induced pluripotent stem cells are one potential source for developing such platelets, but are expensive to support and are hampered by their embryonic nature.
- Alternative cell sources, such as expanded cord blood progenitor cells, and endothelial cell- and adipocyte-derived megakaryocytes, may overcome issues of primitive hematopoiesis and efficacy.
- Bioreactors to release platelets from megakaryocytes have so far had modest results in producing a platelet transfusable product, and the final product should be held to a high standard, comparable to donor-derived platelets when infused into appropriate in vivo models.
- At present, none of these bioreactors are as efficient in animal models as intravenous infusion of the megakaryocytes and allowing their release of platelets within the pulmonary vasculature, although whether that strategy can be translated to clinical application needs further testing.

Figure 1. Schematic of platelet release from megakaryocytes: three potential sites of release
 Megakaryocytes are thought to migrate (red arrows) from a periosteum niche to a perivascular niche in the bone marrow space (depicted at the left). At the perivascular space shown near the center, megakaryocytes can shed platelets or small proplatelets intramedullary (A), or as they transverse the endothelial lining (B), or release large cytoplasmic fragments or complete megakaryocytes (C). In (C), circulating megakaryocytes are entrapped in the pulmonary vasculature where they release platelets over an ~40-minute window.

Figure 2. Schematic of various potential platelet bioreactors

(A) Megakaryocytes (megs) in culture release PLPs that can be harvested. Physical conditions such as oxygen tension and shear can be modified to enhance PLP shedding. (B) Silk fibers have been used to establish a protein-based architecture for entrapment of megakaryocytes with subsequent platelet yield from an applied shear force. (C) Platelet bioreactors of various design have been described, but all are designed from plastic and involve shear forces to entrap megakaryocytes on a sieve and a second shear to produce PLPs. (D) Intravenously infused megakaryocytes are predominantly entrapped in the lungs where they shed platelets in the microcirculation.

Table 1

Comparative analysis of a donor-derived platelet product and a potential, non-donor derived platelet product.

Feature	Donor-derived platelets	Non-donor derived platelets
Platelet availability:	Limited by availability of donors. Limited availability of appropriate HLA-matched donors.	Unlimited availability of universal applicable platelets.
Platelet yield:	Target: 3×10^{11} platelets per unit. Significant variation per unit.	Unlimited and consistent number of platelets per unit.
Platelet reactivity:	Significant variation per unit.	Consistent agonist responsiveness of each unit.
Platelet half-life:	Significant variation per unit.	Consistent half-life of each platelet unit with anticipated longer half-life due to the young average platelet age.
Infectious concerns with platelets:	Bacterial contamination risk of ~1:50,000 units infused. Concerns of known and unknown infections transmitted from donor.	Less shelf storage time, less bacterial contamination concerns likely. Likely low to no risk of novel infections from cell line.
Tumorigenesis/graft versus host (GVHD) risk:	Not a known cause of tumorigenesis. Irradiated units or leukocyte-reduced units have decreased GVHD risk.	Concern over remaining embryonic stem cells in preparation may necessitate product irradiation.
Corrected platelet defect:	Not applicable.	Individualized medicine providing corrected platelets for an inherited defect.
Novel therapeutic applications:	Not applicable.	Platelets can be altered to provide targeted therapy via platelets of hemostatic or fibrinolytic or anti-angiogenic value, for example.
Cost per unit:	Volunteered platelets which limits expense to processing and distribution costs.	Likely to be high secondary to the costs of growing megakaryocytes and isolating final products.