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In vitro generation of platelets: Where do we stand?:

Génération des plaquettes in vitro : où en sommes-nous ?

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Abstract

Millions of platelets, specialized cells that participate in haemostatic and inflammatory functions, are transfused each year worldwide, but their supply is limited. Platelets are produced by megakaryocytes by extending proplatelets, directly into the bloodstream. Bone marrow structure and extracellular matrix composition together with soluble factors (e.g. Thrombopoietin) are key regulators of megakaryopoiesis by supporting cell differentiation and platelet release. Despite this knowledge, the scarcity of clinical cures for life threatening platelet diseases is in a large part due to limited insight into the mechanisms that control the developmental process of megakaryocytes and the mechanisms that govern the production of platelets within the bone marrow. To overcome these limitations, functional human tissue models have been developed and studied to extrapolate *ex vivo* outcomes for new insight on bone marrow functions *in vivo*. There are many challenges that these models must overcome, from faithfully mimicking the physiological composition and functions of bone marrow, to the collection of the platelets generated and validation of their viability and function for human use. The overall goal is to identify innovative instruments to study mechanisms of platelet release, diseases related to platelet production and new therapeutic targets starting from human progenitor cells.

Abstract

Des millions de plaquettes, cellules spécialisées qui participent aux fonctions hémostatiques et inflammatoires, sont transfusées chaque année dans le monde entier, mais leur offre est limitée. Les plaquettes sont produites par les mégacaryocytes en émettant les pro-plaquettes directement dans la circulation sanguine. La structure de la moelle osseuse et la composition de la matrice extracellulaire ainsi que les facteurs solubles (par exemple la thrombopoïétine) sont des régulateurs clés de la mégacaryopoïèse en favorisant la différenciation cellulaire et la libération de plaquettes. Malgré cette connaissance, la rareté des traitements cliniques à vie des maladies des plaquettes est en grande partie due à une connaissance limitée quant aux mécanismes qui contrôlent le processus de développement des mégacaryocytes et des mécanismes qui régissent la production de plaquettes dans la moelle osseuse. Pour surmonter ces limites, des modèles de tissus

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Disclosure of interest

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humains ont été développés et étudiés pour extrapoler ex vivo des résultats pour une nouvelle vision sur les fonctions de la moelle osseuse in vivo. Il y a beaucoup de défis que ces modèles doivent surmonter, notamment : imiter fidèlement la composition physiologique et les fonctions de la moelle osseuse, collecter les plaquettes générées et valider de leur viabilité et fonctions pour l'usage humain. L'objectif global est d'identifier des instruments innovants pour étudier les mécanismes de libération de plaquettes, les maladies liées à la production de plaquettes et de nouvelles cibles thérapeutiques à partir de cellules progénitrices humaines.

Keywords

Megakaryocytes; Platelets; Transfusion; Bioreactor; 3D models; Thrombocytopenia; Mégacaryocytes; Plaquettes; Transfusion; Bioréacteur; Modèles 3D; Thrombopénie

1. Introduction

Millions of platelet transfusions are conducted each year, yet the supply of this blood component is limited, thus patient access to treat disorders is problematic. There are also many diseases where platelet production or function are impaired, resulting in severe consequences and where there are limited clinical options available. To address these current limitations, new modes to generate functional platelets *in vitro* would provide a major benefit to many patients, as well as provide an approach to permit the systematic investigation of mechanisms involved in functional platelet formation. The current model of platelet biogenesis provides that maturing megakaryocytes (Mks) migrate from the osteoblastic niche to the bone marrow (BM) vessels, where they extend proplatelets in order to release nascent platelets directly into the bloodstream [1,2]. This process is finely regulated by the interaction of Mks with the different adhesive proteins of the BM microenvironment and by soluble factors (e.g. Thrombopoietin [TPO]) [3,4]. The BM microenvironment has a unique protein composition paired with a distinct range of stiffness. The chemical and physical cues provided by the extracellular matrix (ECM) components are key to the proper regulation of platelet production [5–7]. Although the BM has been proposed to be a major site of platelet production [2], Lefrançais et al. recently showed that in mice Mks circulate from the BM to the lungs, where they release platelets [8]. On this basis, it is becoming more and more important to shed light on the mechanisms that govern platelet production *in vivo* especially in humans where threatening platelet diseases are still cureless. Research on the BM has been hindered by technical difficulties in obtaining an intact organ without bone decalcification in mouse models or BM biopsies in humans. Therefore, the scientific community has intensively investigated *ex vivo* BM cell and tissue culture models to extrapolate such structures and functions into new insight on BM function *in vivo*. In addition, such a system would respond to the critical need for improved models to understand diseases and predict efficacy, safety and toxicology outcomes for new candidate therapeutics for treatment of platelet related diseases. Sometimes, animal models are poor predictors of efficacy and toxicology of human drugs because of interspecies differences. Therefore, specialized tools, integrating all physical and physiological elements characterizing the BM niche, are needed to study the mechanisms of action of these drugs

and the overall impact on BM environment to allow future personalization of clinical treatments.

2. Bioreactors for platelet production

A number of studies point to the BM niche as the core of blood cell production but with many interesting complex environmental factors for consideration. Thus, future advancements in the study of megakaryopoiesis will depend on the evolution of bioengineering techniques for reproducing physiologically relevant conditions for mimicking the environment where platelets are released. The first biomaterial BM scaffold was constructed with colloidal crystals as a template for 3D polyacrylamide hydrogels [9]. Next, a biomimetic artificial blood vessel system was used that enabled real-time visualization of Mks during culture [10]. A microfluidic bioreactor was also established and consisted of transparent PDMS bonded to glass slides to ensure efficient gas exchange and to support high-resolution live-cell microscopy [11,12]. This system was connected to a syringe pump to create a differential flow across a 2D plastic surface into which microscopic holes have been made in order to allow platelet release in the fast flowing media. The impact of flow was also explored in a device consisting in a wide array of von Willebrand Factor-coated micropillars. Such pillars acted as anchors on Mks, allowing them to remain trapped in the device and subjected to hydrodynamic shear. The combined effect of anchoring and shear induced proplatelet elongation promote platelet release [13]. The search for a biomaterial that can be chemically and mechanically tailored to entrap bioactive molecules, while retaining bioavailability, has spawned our research into the use of silk fibroin from *Bombyx mori* silkworm cocoons, as scaffold for engineering a physiologically relevant human BM environment [14]. Silk fibroin is a naturally-derived, biocompatible and tunable biomaterial extensively used in cell culture studies, regenerative medicine and biomedical applications [15]. Fundamental characteristics of this protein include low thrombogenicity, non-toxicity and low-immunogenicity, thus making it a useful blood vessel substitute [16]. Moreover, silk can be enhanced through a variety of chemical modifications that affect cell attachment, growth and differentiation and is processed entirely in aqueous systems allowing the incorporation of cells and labile compounds without loss of bioactivity [17]. Our 3D human BM niche tissue model for functional platelet production showed that silk vascular tubes, embedded in a silk sponge mimicking the “spongy” marrow, support Mk growth and the release of platelets into the perfused vascular tube lumen [14]. Importantly, the silk was functionalized with characteristic ECM components [3,18], and Mks could sense these proteins and modify their behavior accordingly. Therefore, silk provides a unique and versatile system for reconstituting all BM features that support platelet production.

3. Strategies for functional platelet production

Future success of a bioreactor system implies the implementation with human cells capable to release safe and functional platelets. So far, the different bioengineering approaches described above have already tested different sources of Mks such as umbilical cord blood, peripheral blood and BM. All these are invaluable cell sources to understand the basic mechanisms of human platelet production both in physiologic and pathologic conditions taking advantage of the possibility to differentiate Mks from patients with platelet related

diseases. The need for cells that could overcome the limits related to donor-derived platelets for transfusions have also prompted several groups to develop efficient culture systems from human induced pluripotent stem cell (hiPSCs). hiPSCs are potentially an inexhaustible source of Mks which can produce human platelets resembling peripheral blood once in many aspects, including ultrastructure, surface antigen expression, and function [11,19,20]. Further, hiPSCs can be used for studying either cell physiology or disease by starting from disease cells for drug and toxicology screening [21]. A major advantage of hiPSCs will be the possibility to use human leukocyte antigen (HLA)-matched hiPSCs or genetically engineered cells lacking HLA antigens to produce platelets able to avoid production of antibodies to transfused cells by the recipient, a serious problem that is currently responsible for unresponsiveness to transfusion therapies in the long run [22]. The yield of platelets is still far below what would be required to generate platelet concentrate and long-term self-replication of Mks still limited. However, increasing the consideration on the instructive role of the environment where platelets are produced will be essential for improving the production of hiPSC-derived blood cells *in vitro* [23]. The hematopoietic differentiation is a dynamic process finely regulated by cell–cell interactions, ECM components, and soluble factors from cell niches, acting either in an autocrine or paracrine fashion. Novel cues on this complex regulation will surely lead to the development of systems that more closely mimic the physiology of platelet production and thus the *in vitro* differentiation outcome.

4. Modeling bone marrow environment for studying disease mechanisms and testing drugs

Thrombocytopenias are a heterogeneous group of hematological diseases, due to mutations in genes relevant for regulation of Mk biology, viral infections or autoimmune pathology, in which a severe platelet deficiency poses the continuous risk of spontaneous fatal hemorrhages. Recently, the opportunity to synthesize *in vitro* molecules able to mimic the physiologic effect of TPO on Mk differentiation and platelet production, opened new perspectives for their treatment [4]. Among these, Eltrombopag, a small non-peptide TPO receptor (c-Mpl) agonist [24], is currently approved to increase platelet count in idiopathic thrombocytopenic purpura (ITP), aplastic anemia (AA) and thrombocytopenia due to hepatitis C infection [25,26]. Further, Eltrombopag has been shown to be efficacious in patients with myelodysplastic syndromes (MDS) and patients affected by inherited thrombocytopenia (IT) due to mutations of MYH9 gene [27]. Despite this evidence, treatment of IT patients with Eltrombopag is still debated due to limited insight into its mechanisms of action, a fundamental aspect to take into account if considering that TPO mimetics may increase the risk of leukemia and BM fibrosis in humans [28]. Unfortunately, at variance with Romiplostim, studies on animal with Eltrombopag are prevented because of its specific affinity only for human and chimpanzee c-Mpl. Therefore, future advancement in testing the efficacy and safety of Eltrombopag, as well as other new TPO mimetics, strongly depends on the opportunity to develop bioengineering techniques for accurately reproducing the physiologic features of the human BM niche, including soluble and surface-bound signaling factors, ECM components, and blood shear, that together provide an ideal habitat to support platelet formation [29]. We have demonstrated that our 3D tool is a suitable tool for understanding mechanisms of diseases or testing drug treatment efficacy [14,30]. When

the silk bioreactor was exploited with progenitor cells derived from peripheral blood of patients with primary myelofibrosis patient, the number of released platelets in the vascular silk microtube reflected the peripheral blood platelet count observed *in vivo* [14]. Moreover, treatment of human cord blood-derived hematopoietic progenitors with Eltrombopag promotes a dose-dependent increase of Mk proliferation paralleled by increased production of functional platelets within the silk sponge system. In the system Eltrombopag, at variance with Romiplostim, induces a concomitant balanced activation of both AKT and ERK1/2 pathways, which in turn promote normal *in vitro* Mk differentiation with a significant dose-dependent increment of both Mk output and proplatelet formation [30]. These data suggest that our model is suitable for *ex vivo* testing of the effect of drugs on human samples.

5. Conclusions

Thrombocytopenia often requires platelet transfusions for patients. The availability of this blood component is limited and varies depending on the availability of donors. Further, the paucity of clinical options for functional platelets is in large part due to the lack of insight into the mechanisms that control the developmental fate of Mks toward the production of platelets. These data highlight the importance of improving the modeling of the complex environment where platelet are produced, in order to better understand and reproduce human thrombopoiesis in both physiologic and pathologic conditions. To achieve this goal, the field is moving towards the reproduction of the characteristic features of the physiologic BM microenvironment *ex vivo* by the use of relevant biomaterials and bioreactors, along with appropriate human cell sources (Fig. 1). Incredible advances have been achieved in the last 20 years on platelet production *ex vivo* suggesting that in the near future novel protocols to fully differentiate functional Mks from available progenitor sources should be expected. Finally, modeling platelet production in the BM will help to investigate in-depth the pathogenic mechanisms leading to defective platelet production, thus allowing the identification of novel potential targets for designing new drugs.

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Fig. 1.

Comparison between *in vivo* and *ex vivo* platelet production. *In vivo*: bone marrow is located within the trabecular bones, near the endosteum, where the hematopoietic stem cells (HSCs) are committed towards the megakaryocytic lineage under control of TPO. Maturing megakaryocyte (MKs) migrate towards bone marrow sinusoids prior to extend proplatelets. Proplatelets elongate through the vascular endothelium, into the lumen of sinusoidal vessels where platelets (PLTs), stemming from their terminal ends, are released into bloodstream thanks to blood shear forces. *Ex vivo*: all most recent bioengineered tissue-models mimicking the bone marrow environment are made of biocompatible porous barriers supporting mature megakaryocyte function. Once in contact with the biomaterial, MKs extend proplatelet into the perfused culture medium mimicking the blood flow. PLT release is prompted by media flow obtained by controlled electronic pumps (hHSC: human hematopoietic stem cell; mHSC: mouse hematopoietic stem cell; hiPSC: human induced pluripotent stem cell; hESC: human embryonic stem cell; SDF-1 α : Stromal Derived Factor-1 α ; PDMS: polydimethylsiloxane; PLCL: poly-L-lactide-co-epsilon-caprolactone; vWF: von Willebrand Factor).