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Highlights

- Human mature megakaryocytes may gain the pulmonary circulation.
- The endothelial microenvironment favors platelet formation.
- Shear stress and immobilized GPIb ligand are determinant for platelet production.
- Polyploid megakaryocytes have the potential to change ploidy under flow conditions.
- This is a newly recognized environment affecting platelet production.

The physical and cellular conditions of the human pulmonary circulation enable thrombopoesis.

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Normal Hematopoiesis (megakaryocytopoiesis) ; Microenvironment and Niche

SUMMARY

Animal evidence that platelet production occurs in the lungs is growing [1]. We have investigated whether there is evidence to support pulmonary platelet production from studies using human conditions. We documented the presence of MK in the human pulmonary circulation and analysed the role of the vascular microenvironment on MK function. Our results suggest that the endothelial microenvironment favors platelet formation and that von Willebrand factor combined with appropriate physical forces in flowing blood are determinant for platelet release. We also demonstrate that MKs have the potential to change ploidy as they circulate. These findings demonstrate a new pathophysiological environment affecting platelet production. They also provide new targets for therapeutic intervention.

INTRODUCTION

Thrombopoiesis is the biological process dedicated to the production of functional platelets by their precursors, megakaryocytes (MKs). During their maturation in the bone marrow (BM), MKs migrate to the vascular niche [2] but the final maturation step which involves the process of platelet release is not fully documented in human physiology [1].

Several animal studies have tackled this problem [3] and a recent study showed that, in mice, entire MK migrate to the lungs associated with platelet production in the pulmonary circulation (approx. 50%). [1].Historically, in humans and other mammals, the site of platelet production has been thought to be the BM, although microscopic examination has not clearly identified platelet release at that site. Also MK senescent nuclei are not observed in normal BM. It is noteworthy that entire intact MKs can cross the endothelial barrier of the para-sinusoidal membrane and reach the circulation [4,5] and can be retrieved in the pulmonary vasculature [3,4,6]. When in the circulation MKs become exposed to the shear forces of flow. These are critical for the detachment of proplatelets and platelets [7]. The study of the differentiation of MKs in the pulmonary circulation is therefore crucial to understanding the final events in normal platelet production. Importantly, since platelet reactivity is determined within MKs during thrombopoiesis and causally involved in arterial occlusion, the events around MK differentiation and thrombopoiesis might be important determinants of vascular diseases as well as causing pulmonary pathology [8].

METHODS

- ***Patient samples:*** Blood was harvested from the pulmonary artery and pulmonary capillaries using a Swan-Ganz catheter from 9 patients hospitalized in the medical Intensive Care Unit of Georges Pompidou European Hospital. After Ficoll gradient separation , nucleated cells were stained by Romanosky or were processed for electron microscopy (EM) and immuno-EM.

- ***Histochemistry:*** Lung biopsy was stained with anti- von Willebrand factor (vWF) coupled with Immunoperoxidase.

- ***Human MKs***: $CD34^+$ cells were isolated from umbilical cord blood as described previously [7].

- ***Human Endothelial cells (EC)***: Human umbilical EC (HUVEC) were isolated from the same umbilical cords and grown to confluence as reported [7]. Cells from a first to second passage were used.

- ***Flow cytometry*:** MKs were stained with an anti CD41 and platelet identification was performed on blood samples and MK cultures as previously described [7].

- ***Immunofluorescence:*** Mks and platelets were stained with anti-CD41, anti-CD42b and anti-vWF.

- ***EM:*** Adherent cells were gently scraped and treated for EM by standard methods [7]. Observation was performed on a TEM JEOL 1011.

- ***Shear Experiments*** were conducted as previously described [7]. Videomicroscopy used preincubation with Hoechst dye 1‰ (Invitrogen, USA) to visualize nuclear DNA. Ploidy measurements were performed by flow cytometry.

Pulmonary blood and umbilical cords were obtained with the approval from the appropriate Ethics Committee (Assistance Publique des Hôpitaux de Paris) in accordance with the European Society of Cardiology guidelines. The study was approved by the Ethics Committee

of the French Intensive Care Society. Written informed consent was obtained from the patient or from next of kind.

For more details and specific modifications, see ***Supplementary Material.***

RESULTS AND DISCUSSION

MKs are retrieved from human pulmonary artery blood

Flow cytometric analysis of blood taken from human pulmonary arteries and capillaries showed the MK content to be 307 ± 215 (MKs/ 10^5 nucleated cells ± SD). Light and EM images of MKs retrieved from pulmonary vascular blood are shown in fig. 1 a-e. Mks were also observed in situ, in lung capillaries (fig. 1f).

Endothelial cells are supportive of platelet production

Because there is evidence that MKs migrate from the BM to the lungs at the end of their maturation and circulate in the pulmonary vasculature where they must come into contact with EC rich in vWF [9], we investigated the role of EC on proplatelet and platelet formation by performing co-cultures of MKs with HUVEC during the terminal steps of maturation (day 10-13). Under these conditions MKs started to form numerous, long proplatelets at 24 hrs of co-culture (>50% versus <10 % for control MKs) but without forming individual free platelets (fig. 1g-j; suppl.movie M1,M2). Transwell experiments showed that HUVEC secretion alone induced proplatelet formation (not shown). Thus, our experimental conditions demonstrated a permissive effect of the endothelial microenvironment on platelet production, as noted by others [10,11].

In order to know whether the interaction of GpIb-vWF was involved in proplatelet formation, a blocking anti-GpIb antibody was added to the coculture MKs / EC . It completely inhibited proplatelet formation by MKs (control proplatelet bearing MKs / EC: 5-10 %, ; versus proplatelet bearing MKs + anti-GpIb / EC: <1%) (suppl. figure).

Proplatelet formation in contact with EC was completely inhibited by an anti-CD42b antibody demonstrating the central role of this vWF receptor in proplatelet development.

Platelet release occurs under flow conditions enriched in vWF

Pulmonary capillaries are particularly rich in vWF [9]. To investigate further the capacity of MKs cultured with EC to release platelets, they were exposed to shear forces similar to those experienced in the pulmonary artery in a perfusion chamber coated with vWF. Under high shear stress, proplatelets elongated (suppl. movies M1), separated from the cell core, forming many subunits the size of platelets (suppl. movies M2). MKs exposed to shear stress without contact with EC released 1.6 times more platelets than static MKs whereas MKs grown with EC yielded 3.8 (±0.96 SD) times more platelets in shear conditions (fig. 1i-j, suppl. movies M3,4). These platelets exhibited the ultrastructural characteristics of blood platelets including circumferential microtubules (fig. 1k-l). Finally shed platelets expressed P-selectin following thrombin activation indicating that they were functional (fig. 1m).

MKs rarely release platelets in static culture conditions and shear forces are critical to this process [7]. The mechanoreceptor glycoprotein Ibα (GPIbα) interacts with immobilized vWF, inducing a stop-and-go motion of MKs, accompanied by proplatelet formation and platelet release. Human mature MKs infused into mice release platelets within the pulmonary vasculature. In humans, MKs from the pulmonary circulation are exposed to EC, shear stress, vWF and circulating mediators: the necessary conditions for thrombopoesis.

Mature MKs divide in the circulation and remain functional

The circulatory flow system we used induced MK nuclear lobes to separate in parallel with cytoplasmic deformation and further abscission, leading to cell division (fig. 2a-h, suppl. Movie M5). This process generated reduced ploidy MKs as assessed by flow cytometry. They retained their functional ability. Indeed structured proplatelets continued to be produced being released from the distinct cell fractions (fig. 2j-k; suppl. movie M6). The measured

ploidy shift was significant (fig. 2l), but limited for two reasons: firstly, the small size of the perfusion chamber necessarily underestimates the situation of the vascular network; secondly, only a variation in the extreme ploidy classes was visible since the intermediate classes shifted between themselves.

Endomitosis is the main regulatory mechanism of platelet production [12]. Although mature MKs are still able to perform cytokinesis [13], the late cell division under shear that we have demonstrated relies on a different mechanism of cell abscission, with the absence of a visible contractile ring. The fragmentation of large MKs would facilitate their circulation within microvessels and smaller MKs may prolong their maturation in downstream organs such as lungs or spleen (fig. 2m). Since chromosomes segregate into the MK nuclear lobes with an asymmetrical pattern [14], the new cellular entities may have varied genetic patrimony in the late steps of thrombopoiesis.

Our findings offer a new line of investigation into how changes in the mechanism of thrombopoiesis might modulate platelet reactivity and thus vascular risk. Further, pathological changes in the endothelium might influence the nature of the platelets produced, with prothrombotic consequences [15]. In addition, MKs disorders may be involved in causing lung diseases (such as fibrosis due to abnormal release of growth factors) and vice versa.

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FIGURE LEGENDS

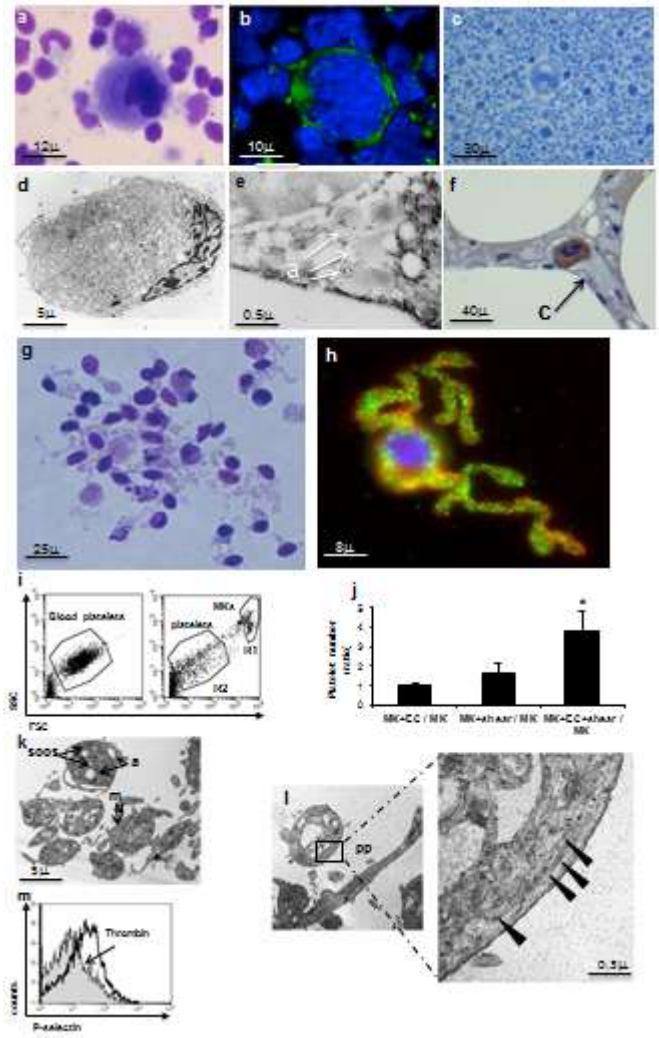

fig. 1: a-f: MKs are commonly observed in human lungs and pulmonary circulation.

(a) Nucleated cell fraction isolated from the pulmonary circulation, displaying a large MK with a polylobulated nucleus - May-Grunwald-Giemsa staining. (b) Immunofluorescent staining of tubulin, surrounding the polyploid nucleus of this circulating MK (same fraction). (c) Semithin section of the nucleated cell fraction isolated from the pulmonary circulation displaying a large MK. (same fraction). (d) Example of the EM aspect of the large MKs isolated from the pulmonary circulation: the cell displays a large nucleus with a well-developed cytoplasm displaying demarcation membranes and numerous alpha-granules. (e)

Immunogold labeling for vWF in a MK from the pulmonary circulation showing characteristic eccentric labeling of alpha-granules (a). (f) Specific immunoperoxidase staining (vWF) of a human lung fragment. A typical MK is located in a capillary (c), among red blood cells.

g-h: EC enhance proplatelet formation. MKs were co-cultured with HUVEC and were harvested for microscopic examination. (g) May Grunwald Giemsa staining shows that MKs have developed individual large proplatelets which tend to interact between themselves, forming clusters in the culture flask. Note that cultured MKs are mostly diploid. (h) Double immunofluorescent labeling for CD42b (red) and vWF (green) shows that individual MKs present in the co-culture are indeed proplatelet bearing. They display peripheral CD42b labeling at the plasma membrane and along the proplatelet with branching structures and punctuate intracytoplasmic labeling for vWF (green), reflecting the homogenous distribution of α-granules along the proplatelet. The nucleus is stained blue with DAPI.

i-n: The combination of EC and shear stress increases platelet production by human MKs. (i) Quantification of platelet release by flow cytometry (CD41 labeling) : Two gates, R2 and R1, were adjusted in size and granularity to that of blood platelets and MKs respectively. The diagram shows the various ratios of platelets released per MK in different conditions: MKs co-cultured with EC in static conditions release only a few platelets similar to control MKs. This ratio reached 3.8 times higher platelet release compared to controls when MKs were cultured with EC and exposed to shear stress. (k) Electron micrograph of platelet-like particles shed in these experimental conditions: the elements visualized are platelet-sized, their cytoplasm contain α-granules (a), surface connected canalicular system (sccs) and mitochondria (m). (l) High magnification of a newly formed platelet, located close to a proplatelet shaft (pp), identifies a characteristic microtubule belt (arrowheads). (m) Flow

cytometry analysis of P-selectin expression in newly shed platelets stimulated by thrombin. When activated by thrombin, P-selectin expression strongly increased on the platelet surface showing that platelets produced from MKs co-cultured with EC are functional .

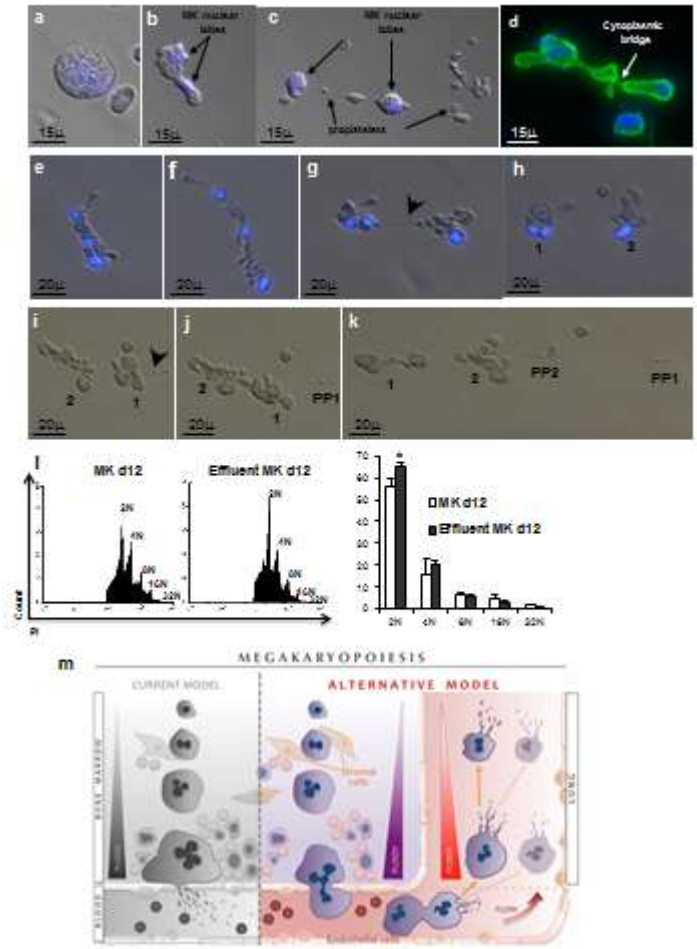

fig. 2: Fragmentation of high ploidy MKs into lower ploidy under flow

a-k: microscopic examination of Human MKs cultured for 12 days, whose DNA was labelled with Hoechst dye and observed in static conditions by DIC fluorescent microscopy. (a) A mature polyploid MK is characterized by a large and compact nucleus fluorescing in blue. (b) During maturation, the cell deforms and elongates. The nucleus tends to separate into two distinct lobes in parallel with proplatelet formation. (c) The cytoplasm

extends and restructures into proplatelets, the nuclear lobes migrate toward two different poles of the cell. (d) CD41 fluorescent labelling (green) definitely identifies this elongated cell with segregated nuclear lobes as a MK. (e,f) During exposure to high shear stress (72 dyne/cm^2) and observed by video microscopy, the cell has elongated in the direction of flow and its cytoplasm has organized in the form of proplatelets with swellings and constrictions. (g) The cytoplasmic elongation forms a thin bridge (arrow) which, after becoming thinner and thinner, (h) eventually breaks yielding two MK units of reduced ploidy (subunits 1 and 2). (i) These two units (one bearing 2 small, diploid nuclear lobes and the other with a larger, possibly 4N nuclear lobe) continue to roll in the direction of the flow, cytoplasm extensions continue to arise from the new cell cores forming structured proplatelets (PP) which get released from the distinct cell fractions (j, k), showing that these subunits are functional.

l: **Flow cytometry**

Ploidy analysis of mature culture MKs shows that the rate of high ploidy MKs decreased from 2%±0.4 to 1.2%±0.1 after exposure to high shear stress in an Ibidi perfusion chamber covered with vWF. However, this process is accompanied by an increase of low ploidy MKs (2N) from 56%±3.2 to 65%±1.9.

m: Schematic representation of the final steps of thrombopoiesis.

The current model of MKs producing proplatelets and platelets into the marrow sinusoids is contrasted with an alternative model arising from the results reported here: MK migrate as a whole into the marrow sinusoids, and reach the pulmonary vasculature, releasing proplatelets and platelets under shear stress. Moreover, MK cells prolong their maturation in the pulmonary circulation, undergoing cell division and continuing platelet production.