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XXI CICLO

STUDY OF THE ORIGIN OF PLATELETS COAGULATION PROTEIN S BY HUMAN MEGAKARYOCYTE CULTURES AND CHARACTERIZATION OF PLATELETS PROTEIN S IN PATIENTS WITH INHERITED PROTEIN S DEFICIENCY

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SUMMARY

Protein S (PS) is a vitamin K dependent plasma glycoprotein with multiple functions in coagulation, inflammation and apoptosis. The molecular weight of PS is approximately 70 kDa and its concentration in plasma is about 25 mg/L. In human plasma 40% of PS circulates as free form and the remaining 60% is complexes with complement C4b-binding protein, a component of the complement system. PS circulating in plasma is mainly derived from liver synthesis but, in addition, endothelial cells, testicular Leydig cells and a megakaryocytic cell line (MEG 01) can synthesize PS. Platelet contain PS, but whether this is derived from megakaryocytic synthesis or from uptake of plasma PS by megakaryocyte (Mk) is not known. Free PS acts as a cofactor for activated protein C (APC) in the inactivation of procoagulant factors Va and VIIIa. However, PS also has APC-independent anticoagulant functions, probably through direct inhibition of both the prothrombinase and the tenase complexes. It is hypothesized that intra-platelets PS, release upon platelets stimulation, plays a crucial role in regulating thrombin generation and therefore controlling procoagulant activity. PS deficiency is inherited as an autosomal dominant disordered and is classified in three types: I) reduced plasma levels of total and free PS antigen (PS_{Ag}); II) normal concentration of total and free PS_{Ag} but with low PS activity; III) low free $PS_{Ag;}$ and normal total PS_{Ag}.

Inherited PS deficiency is generally associated with increased risk of deep venous thrombosis, pulmonary embolism and some cases of arterial thrombosis. The risk of venous thrombosis in PS deficiency increased if

associated with other genetic or acquired conditions these includes factor V (FV) Leiden, HR2 aplotype of FV and prothrombin mutation. Several factors influence the concentration of plasma PS, pregnancy, oral contraceptive and oral anticoagulant therapy decreased the levels of PS. To clarify the origin of intra-platelets PS, we development an *in vitro* model of human megakaryocyte cell culture. Hematopoietic stem cells were isolated by the histopaque system from whole blood of healthy and PS deficiency subjects. Mononuclear cells have been grown in a serum free medium in presence of thrombopoietin (TPO) and interleuchin-3 (IL-3) to stimulate the differentiation into megakaryocytes lineage. The morphology of differentiated mononuclear cells was similar to MKs, and their positive stain with anti-CD41 antibody allowed us to conclude that these cells were indeed Mk. Mk was labeled with αβ-tubulin and γ-tubulin antibodies and we observed the cytoplasmatic extensions called proplatelets and the release of platelets. In addition, through immunofluorescence techniques, we detected FV in their cytoplasm whereas protein C was not present as expected. As for PS, it was present in the cytoplasm of MKs obtained from healthy and PS deficiency individuals. Our study demonstrated the PS biosynthesis by megakaryocyte. To study the mechanisms that regulate the concentration of plasma and platelets PS we analyzed plasma and platelets PS from normal and PS deficiency subjects. PS contained in platelets have the same immunoblotting pattern respect to plasma PS. Plasma and platelet PS immunoblotting pattern demonstrated different molecular weight of PS in some deficient PS individuals as compared to normal control, suggesting different mutations in PS gene. We analyzed the presence of mutation and the presence of PS Heerlen allele. We investigated platelets PS antigen levels in type I and type III PS deficient patients. In type I subjects total and plasma free PS antigen levels were (PS_{Ag}) 62±7% and 37±12%

respectively. In carries of type III defect total and free PS_{Ag} levels were 85±13% and 41±13% respectively. Platelets PS_{Ag} in type I and type III were 66 ±32% and 80±37%.In a subgroup of healthy individuals total, free and platelet PS_{Ag} levels were 119±17%, 110±17% and 101±30%, respectively.

The results indicate that type I and III subject's total and plasma free PS_{Ag} levels were lower than normal individuals. Intra-platelets PS_{Ag} levels in type I and type III were lower than of healthy individuals. Our analysis demonstrates a strict correlation between total and free plasma PS and Plts PS. The reduction of platelet PS mirrors the reduced levels of free and total PS_{Ag} present in carries of the defect even though PS levels in Plts appears unexpectedly higher than the free PS counterpart. Moreover, we study the interaction of anticoagulant drugs on PS_{Ag} levels on 35 patient treatments with warfarin. The levels of total and free plasma PS decreased during treatment with oral anticoagulant, since PS is a vitamin K-dependent protein. Our study demonstrated significant decreased levels of platelet PS respectively plasma free and total PS. We valuated the effect of anticoagulant drugs (warfarin) and of vitamin K on Mk cells. The Mk were treatment with 1µg/ml of warfarin or 1µg/ml of vitamin K and analyze synthesis of PS. We observed decreased PS synthesis on MKs with warfarin than control MKs; on the contrary, MKs cultured under vitamin K treatment increase PS synthesis.

RIASSUNTO

La proteina S (PS) è una glicoproteina plasmatica, vitamina K-dipendente, con molteplici funzioni nell'ambito della coagulazione, infiammazione e apoptosi. Il suo peso molecolare è di 70 kDa e la sua concentrazione plasmatica di circa 25 mg/L. Nel plasma umano il 40% della PS circola in forma libera, mentre il restante 60% è legato alla C4b-binding-protein, una proteina del sistema del complemento. La PS circolante nel plasma viene sintetizzata principalmente nel fegato ma anche le cellule endoteliali, le cellule di Leydig e una linea cellulare di megacariociti sono in grado di sintetizzarla. Le piastrine contengono PS, anche se la sua origine non è ancora stata chiarita. Si ipotizza che derivi dalla sintesi dei megacariociti o che siano gli stessi megacariociti ad assumerla dal pool plasmatico mediante un meccanismo di endocitosi.

La PS libera agisce da cofattore per la proteina C attivata (APC) nell'inattivazione dei fattori procoagulanti Va (FVa) e VIIIa (FVIIIa). La PS esercita anche un'azione anticoagulante APC-indipendente, probabilmente inibendo direttamente i complessi tenase e protrombinase. Si suppone che la PS rilasciata dalle piastrine in seguito alla loro attivazione regoli la generazione di trombina, controllando perciò l'attività procoagulante.

I difetti di PS sono a trasmissione autosomica dominante e vengono classificati in tre tipi:

- difetto di tipo I, caratterizzato da ridotti livelli plasmatici di PS totale e libera;
- difetto di tipo II, caratterizzato da livelli fisiologici di PS totale e libera associati ad una ridotta attività;

– difetto di tipo III, presenta una PS libera ridotta ed una PS totale nella norma.

I difetti di PS sono generalmente associati ad un aumentato rischio di trombosi venosa profonda, embolismo polmonare ed, in qualche caso, a trombosi arteriosa. Nei deficit di PS il rischio di trombosi venosa aumenta se associato ad altre condizioni di carattere genetico o acquisito quali il FV Leiden, l'aplotipo HR2 del FV e mutazioni a carico del gene che codifica per la protrombina. Molteplici fattori, tra cui la gravidanza, la terapia anticoncezionale e anticoagulante orale, riducono la concentrazione plasmatica della PS.

Al fine di chiarire l'origine della PS piastrinica, abbiamo messo a punto un modello *in vitro* di colture di megacariociti umani. Le cellule staminali ematopoietiche sono state isolate con histopaque da sangue intero di soggetti sani e con difetto di PS. Le cellule mononucleate sono state coltivate in un terreno privo di siero ed in presenza di trombopoietina (TPO) e interleuchina 3 (IL3) per stimolarne la differenziazione in una linea magacariocitaria. Le cellule mononucleate differenziate presentavano una morfologia simile a quella dei megacariociti e risultavano positive all'anticorpo anti-CD41; questi elementi ci hanno permesso di confermare che si trattasse effettivamente di megacariociti. Inoltre, la marcatura dei megacariociti con anticorpi anti αβ-tubulina e γ-tubulina ha evidenziato sia la presenza di estensioni citoplasmatiche denominate "proplatelets" sia il rilascio di piastrine da parte dei megacariociti. In aggiunta, mediante tecniche di immunofluorescenza, abbiamo rilevato la presenza del FV a livello citoplasmatico, mentre la PC era assente. La PS era presente nel citoplasma dei megacariociti isolati da individui sani e con difetto di PS. La nostra ricerca ha così dimostrato la sintesi di PS da parte dei megacariociti.

Per studiare il meccanismo che regola i livelli di PS presenti nel plasma e all'interno delle piastrine, abbiamo determinato la concentrazione di PS plasmatica e piastrinica in soggetti sani e portatori di difetto di PS. La PS piastrinica mostrava lo stesso pattern elettroforetico di quella isolata dal plasma. L'analisi immunologica ha inoltre evidenziato, per alcuni soggetti portatori del difetto, una PS plasmatica con differente peso molecolare rispetto ai controlli sani; questo ci ha suggerito la presenza di mutazioni nel gene della PS. Abbiamo quindi testato la presenza di eventuali mutazioni e dell'allele Heerlen.

In soggetti portatori di difetto di PS di tipo I i livelli di PS totale plasmatici, e libera erano: 62±7% e 37±12% . In soggetti portatori di difetto di PS di tipo III i livelli di PS totale e libera nel plasma erano di 85±13% e 41±13%. I livelli di PS nelle piastrine nei soggetti portatori di difetto di PS di tipo I e di tipo III erano di 66 ±32% e 80±37%. In un gruppo di persone sane i livelli di PS totale, libera e piastrinica erano di 119±17%, 110±17% e 101±30%, rispettivamente.

Dall'analisi dei livelli plasmatici e piastrinici di PS in soggetti portatori del difetto di tipo I e III è emerso che a) nei pazienti con difetto i livelli di PS totale e libera erano più bassi rispetto ai soggetti sani; b) i pazienti con difetto presentavano livelli di PS piastrinica ridotti rispetto agli individui sani utilizzati come controllo.

La nostra analisi ha dimostrato una stretta correlazione tra la PS plasmatica (libera e totale) e quella piastrinica. La diminuzione della concentrazione di PS piastrinica, osservata negli individui portatori del difetto, riflette l'abbassamento del livello di PS plasmatica, sebbene la quota di PS all'interno delle piastrine risulti maggiore rispetto a quella della PS presente nel plasma in forma libera. In seguito abbiamo studiato l'effetto di sostanze anticoagulanti sui livelli plasmatici e piastrinici di PS in pazienti

sani in trattamento con warfarina. E' noto che la warfarina abbassa i livelli plasmatici di PS in quanto quest'ultima è una proteina vitamina K-dipendente. Anche i livelli di PS plasmatica, (totale e libera), e piastrinica dei medesimi soggetti in terapia con warfarina risultano ridotti rispetto alla norma ma l'abbassamento della concentrazione di PS appare molto più marcata all'interno delle piastrine piuttosto che nel plasma.

Infine abbiamo valutato l'effetto della warfarina e della vitamina K sulla sintesi di PS da parte dei megacariociti. Mediante tecniche di immunofluorescenza abbiamo osservato una ridotta sintesi della PS nei megacariociti trattati con warfarina rispetto alle cellule di controllo; al contrario, i megacariociti coltivati in un terreno supplementato con vitamina K mostravano un incremento della sintesi di PS.

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MEGAKARYOCYTOPOIESIS

Introduction

Megakaryocytes are the hematopoietic precursor cells from which platelets are derived (1). It has been estimated that they comprise 0.02% to 0.05% of the bone marrow nucleated cell population with a total number of approximately $4x10^7$ cells. Megakaryocytes in fixed marrow smears have average diameters of approximately 35μm, whereas viable megakaryocytes in suspension cultures have average diameters of approximately 20 μm (2, 3). Their large size and abundant cytoplasm presumably allow them to produce several thousand platelets per cell (4, 5). Cellular enlargement is mediated by multiple rounds of end mitosis, a process that amplifies the DNA by as much as 64-fold (the mean ploidy of normal human megakaryocyte is approximately 16N). This physical characteristic is so striking and unique that early microscopists named the cells for their large (mega) nucleus (karyon) (6).

I MEGAKARYOCYTE DEVELOPMENT

Megacaryocyte progenitor cells

Megakaryocytes, like all other cells in the blood, developed from a master stem cell. Stem cells are the most primitive of these cells (7,8). By definition, they are non cycling and lineage-indifferent and have the capacity to self-renew. Through a series of poorly understood events, stem cells give rise to more differentiated progenitor cells, which are characterized by decreased self-renewal capacity, decreased commitment to development within a given hematopoietic lineage and increased proliferate activity. Progenitor cell proliferation and acquisition of lineage-specific phenotypic markers accompany one another initially, but proliferate activity eventually declines. At this stage of development, the cells are often morphologically identifiable as belonging to a given lineage and are called precursor cells. Precursor cells are capable of one or two divisions as they complete their maturation. In adults, these hematopoietic stem cells reside primarily in the bone marrow. During mammalian development, stem cells also successively populate the embryonic yolk sac, fetal liver, and spleen.

Committed megakaryocytic precursor cells develop from pluripotential hematopoietic progenitors. All hematopoietic progenitors express surface CD34 and CD41 (integrin αIIb, GPIIb) and the development of megakaryocytic lineage is indicated by the expression of CD61 (integrin β3, GPIIIa). Three types of megakaryocytic progenitor cells have been defined on the basis of their physical properties, the type of colonies they

give rise to *in vitro* and how long they take to develop in culture. The committed myeloid progenitor cell (colony-forming unit-granulocyte-erythroid-macrophage-megacaryocyte [CFU-GEMM]) is a bi-potential progenitor cell, which give rise to bi-clonal colonies composed of megakaryocytic and erythroid cells (9). The least mature of the progenitors is the burst-forming unit megacaryocyte (BFU-Meg) and at 21 days in culture, colonies of multiple clusters of megacaryocyte cells are formed (10). Under appropriate culture conditions, the BFU-Meg can develop into 40 to 500 megakaryocytes within one week. The colony-forming unit megacaryocyte (CFU-Meg) is a more mature megakaryocytic progenitor that gives rise to a colony containing from 3 to 50 mature megakaryocytes; the cells of these colonies tend to have high ploidy values (Fig 1).

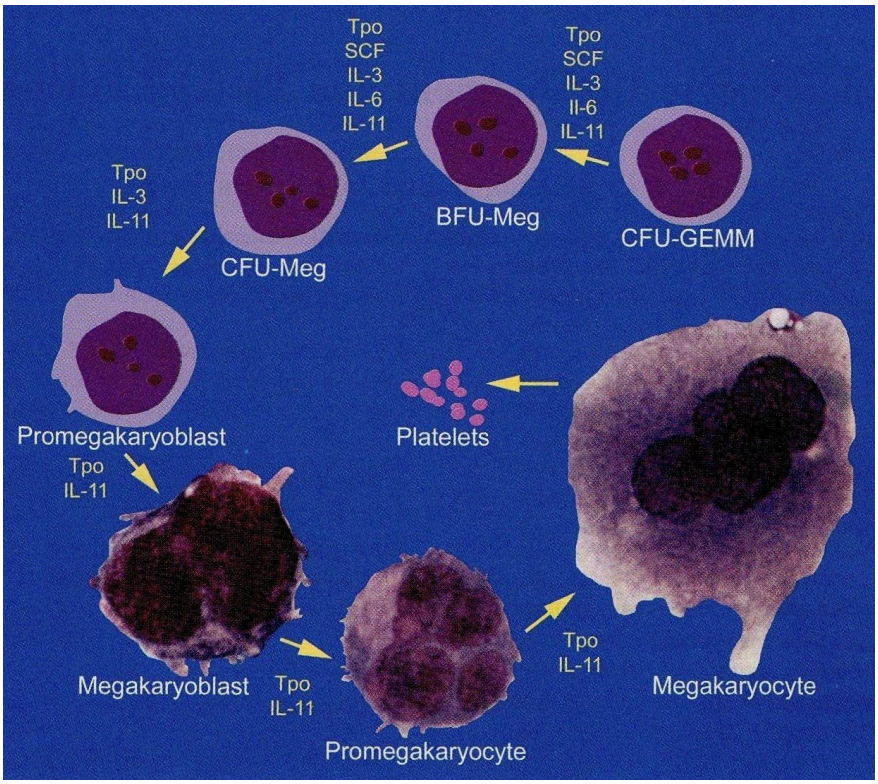


Fig1. Megakaryocytes and platelet development. From a committed myeloid progenitor cell, colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM), is the intermediate cell that differentiates into the megakaryocytic, basophilic and erythroid lineages. The burst–forming unit-megakaryocyte (BFU-Meg) is committed to megakaryocyte differentiation. Both types of cells, BFU-Meg and CFU-Meg, express specifics megakaryocyte antigen. The promegakaryoblast is the first morphologically recognizable megakaryocyte precursor in bone marrow. Megakaryoblasts have a large and oval nucleus. Promegakaryocyte have polychromatic staining cytoplasm and a lobulated nucleus. Megakaryocytes as the largest hematopoietic cells in the bone marrow, and they have a multilobed nucleus. Megakaryocyte undergoes maturation increased polyploidy stage and proplatelets formation. Proplatelets elongation at the final stage released new formed platelets.

Megakaryocytic precursor cells

On the based of morphological features, histochemical staining and biochemical markers have been used to classify different stages of megakaryocytic development. In general, three types of morphologies can be identified in bone marrow. The promegakaryoblast is the first recognizable megakaryocytic precursor. The former cells are small and mononuclear and express megakaryocyte/platelet specific markers. These cells were found to contain acetyl cholinesterase (AchE), a megakaryocytic lineage-specific marker. The megakaryoblast, or stage I megakaryocyte, is a more mature cell. The megakaryoblast has a kidney-shaped nucleus with

two sets of chromosomes (4N). These cells still actively synthesize DNA and undergo endomitosis. The promegakaryocyte, or stage II megakaryocyte, has a horse-shoe shaped nucleus and there is an increased content of platelet organelles. Granular megakaryocytes, or stage III megakaryocyte, have a lobulated nucleus with dense chromatin and with abundant platelet organelles. Megakaryocytes are the largest hematopoietic cells in the bone marrow, with diameters as large as 150μm, and they have a high multilobed nucleus (11).

Megakaryocyte endomitosis and polyploidization

Megakaryocytes are unique because DNA synthesis continues in these cells in the absence of nuclear division, a process called endomitosis. Megakaryocytes undergo endomitosis and become polyploid through repeated cycles of DNA replication without cell division. Mononuclear megakaryocyte precursors exit from the diploid state to differentiate and undergo endomitosis, resulting in a cell that contains multiples of a normal diploid chromosome content (i.e., 4N, 16N, 32N, 64N) (12). The biologic imperative that drives megakaryocytes to become polyploid is mysterious. It is appealing to assume that some evolutionary advantage derives from the ability to make platelet-producing cells in this manner. It is likely that polyploid cells produce more platelets because they have more cytoplasm, but whether actual platelet production and release are more efficient in a single large cell than in smaller ones is unknown. The formation of a polyploid megakaryocyte nucleus clearly requires alterations in its cell cycle. Megakaryocyte polyploidization results in a functional gene amplification, the likely purpose of which is an increase in protein

synthesis and cell enlargement. It was initially postulated that polyploidization might result from a complete absence of mitosis after each round of DNA replication. However, recent studies of primary megakaryocytes in culture indicate endomitosis does not result from a complete absence of mitosis, but rather a prematurely terminated mitosis (13). Megakaryocytes become polyploid through repeated cycles of DNA replication and proceed from prophase to anaphase A but do not enter anaphase B, telophase and they do not undergo cytokinesis (14).

DNA synthesis and mitosis are under the control of large "cell cycle" genes and proteins are involved in the progression of the cell cycle (15). Megakaryocytes appear to be the exception to this rule, indicating that they have managed to deregulate this process. Recent studies have focused on identifying the candidate genes and proteins involved in cell cycle progression. The detection, of c-myc transcripts, in mature human megakaryocytes, using in situ hybridization, has been reported by several investigators (16, 17). It has also, been reported that c-myc expression is inducible in several cell types after activation of either the phosphoinositide signaling system or protein kinase C, regardless of whether DNA synthesis or mitosis has been initiated. (18). Mature megakaryocytes might express c-myc because high levels of protein kinase C in these cells stimulate c-myc gene expression (19). The role of c-myc was not specifically addressed. Cyclins and cyclin-dependet kinases, named for their cyclical synthesis and degradation, play a critical role in regulating cell cycle progression. These proteins have been shown to form a protein-kinase complex in which the catalytic unit is the cyclin-dependet kinase and the regulatory unit is a cyclin. During mitosis, cdc2 and cyclin B form a cdc2/cyclin B kinase complex, also known as maturation promoting factor (MPF) that is necessary for entry of cells into mitosis. This complex

regulates the entrance into mitosis during G2/M phase, as well as spindle fiber formation and cytokinesis (20). Cyclin-dependent kinase cdk2 and its cyclins have been shown to regulate G1/S transit and S phase progression. Cyclin E is involved in G1 progression and G1/S transit, whereas cyclin A is needed in the S phase and initiation of DNA replication (21, 22). Cdk4/cyclin E complex is made in the middle of the G1 phase and its associated kinase activity peaks in late G1 and in the early S phase. Cdk2/cyclin A activity regulate S phase (23, 24) Cdk6 and Cdk4 with cyclin D regulates G1 progression (25). Most groups investigated the cyclin-dependet kinase complexes during the induction of differentiation in human erythroleukemia cells (HEL), a common model for studying megakaryocyte differentiation. They found that during polyploidization, HEL cells undergo a dramatic change in G1/S phase associated Cdk complexes and marked an increase in their specific activities. This phenomenon was facilitated by a differential loss of the p21 and p27 Cdk-inhibitory protein/kinase-inhibitory proteins (CIP/KIP) bound to cyclin/Cdk complexes. It was suggested that the loss of S and M phase in polyploid cells occurs within the context of an up regulated function in those Cdk complexes associated with both G1 and S phase progression. Other studies performed in primary cultures of mouse bone marrow cells showed that mature megakaryocytes express the G1 phase cyclin and cyclin D3, but not mitotic cyclin, cyclin B1. Ravid and colleagues reported that cyclin D3 is required from megakaryopoiesis and polyploidization in mouse megakaryocytes. In addition, it has recently been demonstrated that the molecular programming involved in endomitosis is characterized by the mislocalization or absence of at least two regulators of mitosis: Aurora-B/AIM-1 and survivin. Another hypothesis that endomitosis is driven by the inhibition of microtubule-based forces during anaphase B is based on

the observation that mitosis proceeds normally up to anaphase B, but is then blocked in the spindle pole elongation. Spindle pole separation during anaphase B is believed to be a consequence of the sliding of antiparallel and interdigitating non kinetochore (polar) microtubules that pass one another (26). Further studies are needed, to understand how the megakaryocyte cell cycle occurs in normal cells.

II MEGAKARYOCYTE MATURATION

Introduction

When the endomitosis is completed megakaryocytes begin a maturation stage in which the cytoplasm rapidly fills up with platelets-specific proteins, organelles and membrane systems that package nascent platelets.

Demarcation Membrane System

Mature megakaryocytes elaborate an extensive network of membrane channels composed of flattened cisternae and tubules, called: Demarcation membrane system (DMS). The exact function of this elaborate smooth membrane system has been debated for years. Kautz and De Marsh first reported the sub organization of the megakaryocyte cytoplasm and a detailed description of these membranes was reported by Yamada (27, 28). However, recent studies suggest that the DMS functions primarily as a membrane reserve for proplatelets formation and extension.

Dense tubular system

Megakaryocytic maturation is characterized by the formation of a variety of secretory granules. The principal granules are α granules, which contain proteins essential for platelets adhesion during vascular damage. These

granules have spherical shapes with a dark central core which diameter is approximately 200-500 nm. They originate from the trans-Golgi network at the early stage of megakaryocytes. α granules acquire their molecular contents from both endogenous protein synthesis and endocytosis of plasma proteins (29). Megakaryocytes endogenously synthesized proteins such as von Willebrand factor, platelets factor 4 and β-thromboglobulin. Megakaryocytes can uptake and package plasma proteins by receptor-mediated endocytosis. It has been well documented that uptake and delivery of fibrinogen to α granules is mediated by integrin αIIbβ3 membrane protein (30, 31, 32). Regarding the intracellular tracking of proteins in megakaryocytes, further studies are needed as little is known about this phenomenon.

THROMBOPOIESIS

I MECHANISMS OF PLATELET PRODUCTION

Platelets derive form megakaryocytes cells although the mechanism by which platelets form and release from these precursors remains unclear. Three models have been proposed to explain the mechanism of platelet production: 1) platelet budding, 2) cytoplasmatic fragmentation via the demarcation membrane system (DMS), and 3) proplatelet formation. In past years, scientists attempting to understand the mechanisms of platelets biogenesis as a system have found it hard. The reason why it has been so difficult to investigate this system is because of the lack of *in vitro* systems to create isolation and expand progenitor cells which is considered an obstacle in the understanding of how it works. These is due to the fragility of these type of cells and the low number of bone marrow megakaryocytes. The discovery of thrombopoietin (TPO), which is a cytokine that binds to the megakaryocytes specific receptor c-MPL and promotes the growth and development of megakaryocytes precursors, has provided systems of study that explains the terminal phase of thrombopoiesis (33, 34).

1) Budding from the Mk membrane

Based on scanning electron micrographs of Mk with apparent platelets-size blebs on their surface, it was proposed that platelets shed from the periphery of megakaryocytes cytoplasm (35, 36). However, with the use of

the electron microscopy, it was revealed that these blebs did not contain platelets organelles, an observation which is inconsistent with the concept of platelet budding as a mechanism for platelet release. Under the microscope, platelet buds were probably confused with the formation of the cytoplasmatic prolongation found in mature megakaryocytes called pseudopods.

2) Cytoplasmatic fragmentation via the Demarcation Membrane System

The DMS, described in detail by Yamada in 1957, has been proposed to define preformed "platelets territories" within the cytoplasm of the Mk. Microscopists recognized, that maturing Mk filled up with membranes and platelets organelles, and postulated that these membranes formed a system defined as territories or fields where platelets developed (37, 38). Release of individual platelets was proposed to occur by a massive fragmentation of the megakaryocyte cytoplasm along DMS fracture lines residing between these fields. The DMS model predicts that platelets are formed by an extensive internal membrane reorganization process (39). Tubular membranes, which may originate from invagination of the Mk plasma membrane are predicted to interconnect and branch, forming a continuous network throughout the cytoplasm. The fusion of tubules to generate a flat membrane that surrounds the cytoplasm of the new platelets has been proposed as a mechanism. This model, however, has lost support because of several inconsistent observations. On the basis of this model if platelets are delineated within the megakaryocyte cytoplasm by the DMS, then platelets fields should exhibit structural characteristics of platelets, which is

not the case. Platelets territories within the megakaryocyte cytoplasm lack marginal microtubule coils, the most characteristic feature of the resting platelet structure. In addiction there are no studies demonstrating that these platelets fields when shattered give mature functional platelets (40).

3) Proplatelet formation

Wright at 1906 recognized that platelets derived by megakaryocytes and described "the detachment of plate-like fragments or segments from pseudopods" of megakaryocytes (41). Thiery and Bessis and Behnke later described in more detail the structure of these cytoplasmic prolongations from megakaryocyte during platelet formation. This model proposed that the DMS subdivide the megakaryocyte cytoplasm into platelets areas (42, 43). The term proplatelet is generally used to describe the long thin cytoplasmatic processes emanating from Mk. These extensions are characterized by multiple platelet-size swellings linked together by thin cytoplasmic bridges which represent intermediated structures megakaryocytes to platelet transition. The classic "proplatelet theory" was introduced by Becker and DeBruyn who proposed that megakaryocytes formed long pseudopodilike processes that subsequently fragment to generate individual platelets (44). Radley and Haller later developed the "flow model" which postulated that platelets derived exclusively from the interconnected platelet-size beads along the shaft of the proplatelet and suggested that the DMS did not function to define platelets fields, instead as a reservoir in the surface of the membrane that is invaginated during proplatelet formation (45). Proplatelet have been observed a) both *in vivo*

and *in vitro* and maturation of proplatelets yield platelets that have functional structures similar to blood platelets (46 , 47, 48); b) in a wide range of mammalian species, rats, guinea pigs, dogs cows and humans (49-53). The proplatelet processes, in the bone marrow, are extended into the lumen of myeloid sinusoids, where it has been hypothesized that they are released into the circulation and after fragmentation into individuals platelets (54). In mice the lack of two distinct hematopoietic transcription factors, as such GATA-1 and FOG-1, proplatelets have still not been observed. Such mice fail to produce proplatelets in culture and exhibit severe thrombocytopenia (55, 56, 57). All these finding, underscoring the important role, of proplatelet formation in thrombopoiesis (Fig 2).

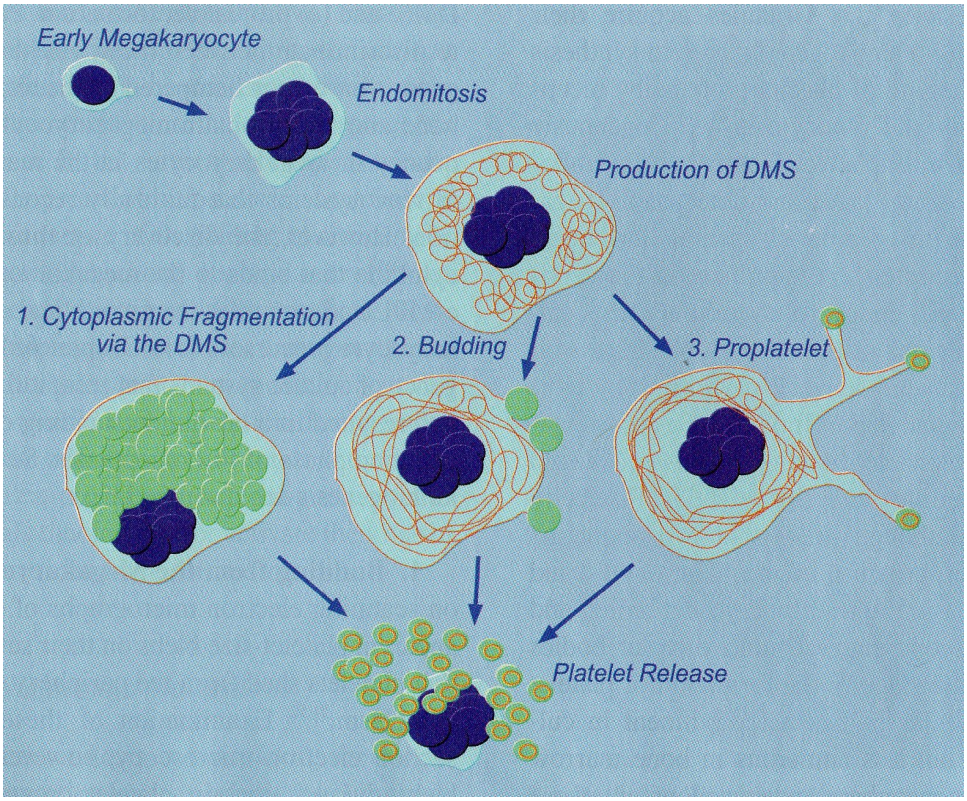


Fig 2. Schematic representations of the three models have been proposed to explain the mechanisms of platelets production. The first model is cytoplasmic fragmentation, platelets derived through cytoplasm fragments along the DMS .The second model is platelet budding. Platelets derived by protrusion formation of periphery membrane megakaryocytes. The third model is proplatelets formation. Platelets derived by proplatelets intermediated formation from cytoplasmatic membrane extensions, characterized by multiple platelet size swellings (Italiano JR et al. 2007).

II CYTOSKELETAL MECHANISMS OF PROPLATELET FORMATION

1) Mechanism of proplatelet elongation

The discovery of thrombopoietin (TPO) gives the opportunity to develop megakaryocytes culture *in vitro* and to see the proplatelets formation. Platelets formation by mice megakaryocyte cultured produce a long processes called proplatelets that are extended in a complex manner of which only certain aspects are clear. Mice megakaryocyte during the initial stages of proplatelet formation the megakaryocyte spreads and its cortical cytoplasm begins to unravel at one pole. Thick pseudopodia initially form and subsequently elongate into thin tubules of 2 to 4 μm in diameter. Proplatelet extensions frequently bend and bending sites subsequently bifurcate to generate new proplatelets processes. These slender tubules, in turn, undergo a dynamic bending and branching process, and develop

periodic densities along their length. The process of proplatelets elaboration ends in a rapid retraction that separates strands of proplatelets from the residual naked nucleus and releases individual platelets into the circulation (58).

2) Structure of the proplatelet's formation

Proplatelet formation is a dynamic process that is complicated by a repetitive dynamic bending and branching that bifurcates the shaft multiple times and thereby increases the number of free proplatelet ends. In the proplatelet tips, a single microtubule, derived from the microtubule bundles of the proplatelet shaft, rolls up into a circumferential coil that defines the territory of an individual platelets. When the coil has been formed the new platelet fills with its content of granules organelles and coagulation proteins. Sliding movements by microtubules in the shaft elongate and separate the ends from the of shaft; mediating platelets release (59) (Fig 3).

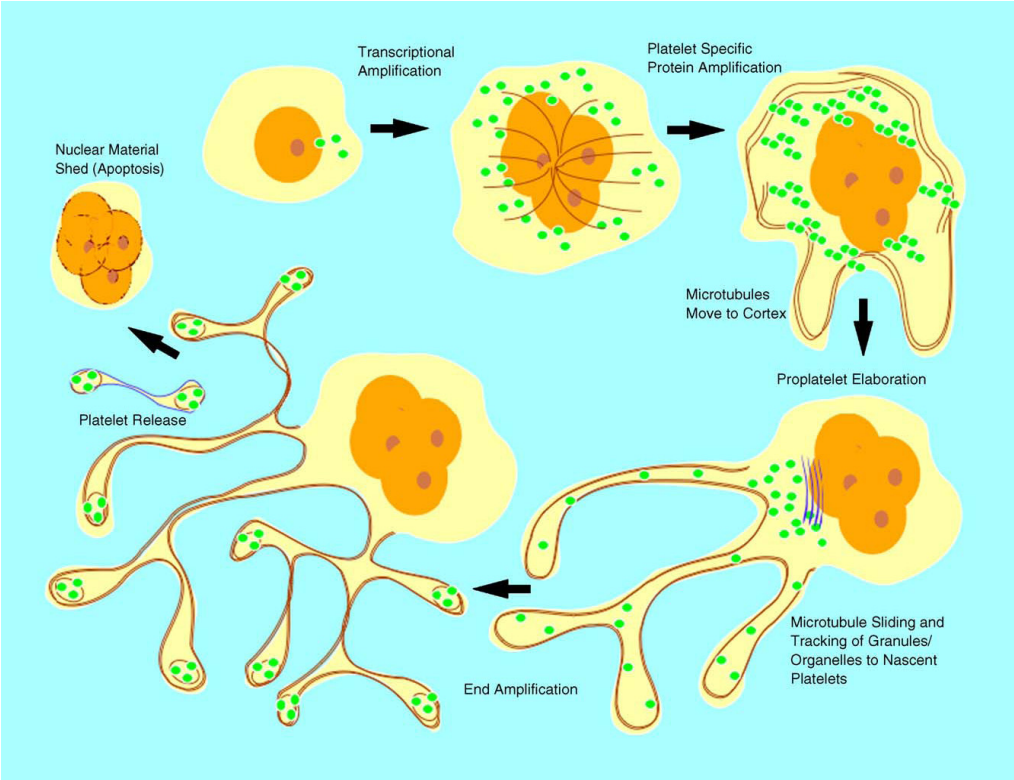


Fig 3. Hypothesizes model for platelets biogenesis. Immature megakaryocytes cells undergo endomitosis and transcriptional activation. During cytoplasmic maturation and expansion, the megakaryocyte undergoes cytoskeleton and membrane proteins synthesis. Proplatelets formation initiates with the formation of cytoplasmatic elongations, called pseudopodia, that elongates and form thin proplatelet with bulbous and give rise to new platelets formation. The extruded naked nucleus remaining undergoes apoptosis (Italiano JR. 2007).

3) Cytoskeleton mechanism of proplatelet formation

The cytoskeleton of the mature platelet plays a crucial role in maintaining the discoid shape of the resting platelet and is responsible for the shape change associated with platelet activation. The same proteins provide the force to bring megakaryocyte maturation. Recent studies support a model of platelet formation in which microtubules and actin play a crucial role.

The first insights into the cytoskeletal mechanics of platelets formation date from the work of Tablin and Leven who found that microtubules poisons, such as colchicine, prevent proplatelet formation (60). The observation that mice lacking the hematopoietic specific β-tubulin show profound thrombocytopenia and fail to produce proplatelets *in vitro* which adds molecular support for the essential role for microtubules in platelet assembly (61, 62). Proplatelet formation is dependent on the microtubule's function, because treatment of megakaryocytes with drugs which depolymerize microtubules, such as nocodazole or vincristine, blocks proplatelets formation (63). The principal cytoskeletons polymers for power proplatelet elongation are αβ-tubulin. Antitubulin immunofluorescence and high resolution microscopic studies have delineated the changes in microtubule organization that lead to the elaboration of proplatelet (64). The microtubules cytoskeleton in megakaryocytes undergo a considerable reorganization during proplatelet production. In immature megakaryocytes without proplatelets, microtubules spiral out from the center to the cortex. As blunt pseudopodia form during the initial stage of proplatelets formation, cortical microtubules consolidated into thick bundles situated just beneath the plasma membrane of these structures. When pseudopodia begin to elongate microtubules, they

form a linear arrays and entire in the proplatelet extension. The distal end of has a platelets size enlargement contain a microtubule bundle to form a drop shape structure and after break release the platelets enter in to circulation. While microtubules are used to propel proplatelet elongation, an actin dependent reaction is used to form the proplatelet's shaft. The branching mechanism, in mice, is mediated by actin forces and becomes quiescent after treatment mouse megakaryocyte and to stop this mechanism with drugs such as cytochalasin B and D. Cytoplasmatic dynein is another protein contributed to proplatelets formation, inhibition of dynein, through disassembly of the dynactin complex, prevents proplatelet formation (65, 66). These protein are important for the microtubule based motor that elongates proplatelets, actin-based force is used to bend the proplatelet in the end amplification. Megakaryocyte treated with the actin toxins cytochalasin or latrunculin can only extend long, unbranched proplatelets that are decorated with few swellings along their length. Electron microscopy and phalloidin staining of Mk undergoing proplatelets formation indicate that actin filaments are distributed throughout the proplatelet and particularly abundant within swellings and at proplatelet branch points (67)

Interestingly, a mutation in the non muscle heavy chain-A gene in humans results in a disease called May-Hegglin anomaly, characterized by thrombocytopenia with grant platelets. This suggesting proplatelet bending and branching is powered by actin-based molecular motor myosin. However the role of actin filament dynamics in platelet biogenesis remains unclear. Of the two major microtubule motors kinesin and dynein only kinesin is situated in a pattern similar to organelles and granules, and is likely responsible for transporting these elements along microtubules. It appears that a twofold mechanism of organelles and granules movement

occurs in platelet assembly. Cytoplasmatic dynein is responsible for sliding proplatelet microtubules relative to one another in proplatelets. Dynein remains associated with detergent-treated proplatelets that can be reactivated by adenosine triphosphate (ATP) to elongate via microtubule sliding. ATP supports the enzymatic activity of microtubule based molecular motors (68, 69 70).

4) Proplatelet Release

In vivo proplatelets extend into bone marrow vascular sinusoids, where they may be released and enter the bloodstream. The actual events surrounding platelet release in vivo have not been identified because of the rarity of megakaryocytes within the bone marrow. Only in mice, megakaryocytes platelet release has been documented. After complete conversion of the megakaryocytes into a network of proplatelets, a retraction event occurs that release individual proplatelet from the proplatelet mass (71). Maturation of platelets occurs at the end of pseudopodia. Microtubules filling the shaft of proplatelets are reorganized into microtubule coils as platelets release from the end of each extension. At the end of proplatelet a single microtubule can roll into a coil; the same microtubule coil structure observed in circulation. The mechanism of microtubule coiling formation remains to be elucidated, but is likely to involve microtubule motor proteins such as dynein or kinesin and tubulin. However platelet maturation is limited to these sites and efficient platelets production requires the generation of a large number of proplatelet which ends during megakaryocyte development (Fig 4).

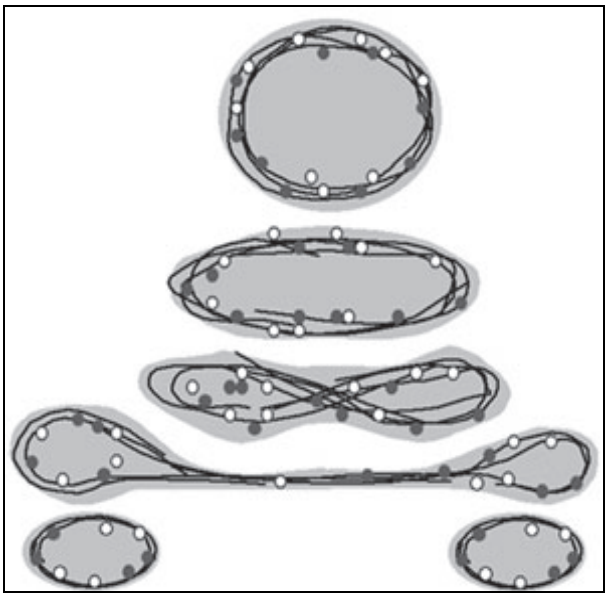


Fig 4. Mechanistic model for proplatelet elongation. Isolated proplatelets can mature into platelets. Proplatelets develop by cytoplasmatic processes emanating from megakaryocytes. As proplatelets elongate, their microtubule bundles twist. This brings opposite bundles in contact, allowing them to become zippered together in the proplatelet shaft and form loops at the end of proplatelet elongation. Cytoskeleton movement by microtubules in the shaft elongate and separate the ends from the shaft, mediating platelet release. Once the microtubule coil has been established, the nascent platelet fills with its content of granules.

5) Megakaryocyte and platelets apoptosis

The process of platelet formation in megakaryocyte exhibits some characteristics related to apoptosis, including cytoskeletal reorganization, membrane condensation and ruffling. Apoptosis is a programmed cell death and is responsible for destruction of the nucleus in senescent megakaryocytes. Apoptosis has been described in mature megakaryocytes after the release of proplatelets (72, 73, 74). Apoptosis inhibitor proteins such as Bcl-2 and Bcl-x_L is expressed at the early stage of megacaryopoiesis. When over-expressed in megakaryocytes both factors inhibit proplatelets formation (75). Caspase activation has been established as a requirement from proplatelet formation. Caspase 3 and 9 are active in mature megakaryocytes and inhibition of these proteins blocks proplatelet formation (76, 77). Interesting is the distinct expression of apoptotic factors in mature megakaryocytes and platelets. Caspase 3 is present in platelets and caspase 12 in differentiated megakaryocytes. These suggest differential regulation mechanism for apoptosis in platelets and in megakaryocytes (78).

6) The sites of platelet formation *in vivo*

Megakaryocytes arisen in the bone marrow, they can migrate into the bloodstream and as a consequence e platelet formation may also occur at non marrow sites. Platelet biogenesis has been proposed to take place in different tissues including bone marrow, lungs and blood.

Megakaryocytes cultured *in vitro* outside the confines of the bone marrow can form proplatelet, this indicates that direct interaction with the bone marrow is not a requirement for platelets productions (79). Scanning electron micrographs of bone marrow, one can see the formation of megakaryocytes of the extending proplatelet's junctions occurring in the endothelial lining which is on the sinusoidal lumen suggesting platelet productions occurs in the bone marrow (80). Bone marrow megakaryocytes are located in the extravascular space on the albuminal side of sinus endothelial cells and appear to send beaded proplatelets projections into the lumen sinusoids (81). Studies demonstrated that thrombopoiesis is dependent on the direct interaction of megakaryocytes with bone marrow endothelial cells specific adhesion molecules (82). Important molecules for this interaction are the chemokines such as stromal cell derived factor 1 (SDF-1) and fibroblast growth factor (FGF-4) which induce the productions of adhesion molecules including very late antigen (VLA) on megakaryocytes and VCAM-1 on bone marrow endothelial cells (83, 84).

In the bloodstream it is unclear whether individual platelet are released from proplatelets into the sinus lumen or whether megakaryocytes release large cytoplasmic processes into the sinus lumen that later fragment into individual platelets within the circulation. Behnke and Forer have proposed that the final stage of platelet maturation occurs exclusively in the circulation. In this model proplatelets released into the blood by megakaryocytes are transformed into platelets while in circulation (85). This theory is supported by several observations:

a) megakaryocytes fragments are observed in blood and when isolated from platelets rich plasma, this suggests that megakaryocytes fragments elongate and undergo to form disk shape structure resembling chains of platelets b) human and mouse megakaryocytes can form functional platelets *in vitro*,

neither the bone marrow environment nor the pulmonary circulation is essential from platelets formation and release (86-90).

Megakaryocytes have been identified in intravascular sites within the lung, suggesting that platelets are formed from pulmonary cells (91, 92, 93). In 1893, Aschoff first described pulmonary megakaryocytes and suggested that megakaryocytes originated in the bone marrow and then migrated through the bloodstream to the lung where they release the platelets (94). The size of megakaryocytes would seem limiting, to the migration of megakaryocytes through endothelial apertures into the circulation (95, 96, 97). However megakaryocytes are present in the lung and pulmonary circulation. In addiction platelet counts are higher in the pulmonary vein than in the pulmonary artery this is evidence that pulmonary contributes to platelet formation (98-100). Kaufman and colleagues studies the platelets formation in the lung by rearranging the pulmonary vessels in a dog so that the blood from the right heart perfuses first the right lung and then was directed to the left lung (101-103). The majority of megakaryocytes were found in the right lung suggesting filtration of megakaryocytes by the pulmonary circulation. Despite these observations the estimated contribution of pulmonary megakaryocytes to total platelet production remains unclear. Experiments using accelerated model of thrombopoiesis in mice suggest that the fraction of platelet production in the mice lung is insignificant (104). In theory proplatelets as well as megakaryocytes may also reach the pulmonary circulation and complete their development into platelets in lung capillaries.

III CYTOKINES OF MEGAKARYOCYTE DEVELOPMENT

Megakaryocytes development and platelet formation are regulated at multiple levels by many different cytokines (105, 106). Interleukin 3 (IL-3) a cytokine produced by both mast cells and T lymphocytes can stimulated the early stage of megakaryocyte development up to the endomitosis. The most important cytokine for megakaryocytes development was the discovery of trombopoietin (TPO) in 1995. TPO regulates all stages of megakaryopoiesis from the hematopoietic stem cell through to cytoplasmatic maturation (107). The receptor for TPO (c-Mpl) is constitutively expressed in hematopoietic tissues. Ligation of c-Mpl by TPO initiates signaling through the Janus kinase family that leads to activation of phosphoinositide 3 kinase (PI3K) and mitogen activated protein kinase. Kit ligant also known as stem cell factor, steel factor, or mast cell growth factor, is a cytokine that exist in both soluble and membrane-bounds form and influence primitive stage of hematopoietic stem cell. Interleukin 6 (IL6) and interleukin 11 (IL11) regulate megakaryocyte development at multiple levels, but appear to function only in concert with TPO and IL3 (108). Transforming growth factor β1, platelet factor-4 and IL-4 have all been deemed regulators of megakaryocytopoiesis.

IV TRANSCRIPTIONAL REGULATION OF MEGAKARYPOIESIS AND DISEASES

Transcription factors that regulate megakaryocyte development are well established. Expression of GATA-1 and its binding protein FOG-1 is important for many stages of megakaryopoiesis. In mice lacking GATA-1 leads to severe thrombocytopenia, in these mice megakaryocytes are small with reduced polyploidization. FOG-1 knockout mice resulted delayed megakaryocytic development through its interaction with GATA-1 (109). A family with X-linked dyserythropoitic anemia and thrombocytopenia resulting for as mutation in GATA-1 gene has been described (110). Megakaryocytes of this family are small and with several abnormal features and a lack of granules.

Nuclear factor-Erythroid 2 (NF-2) regulates many genes involved in cytoplasmatic maturation and platelet formation. Megakaryocytes from NF2 knockout mice have important thrombocytopenia.

Reorganization of the megakaryocyte cytoskeleton and assembly of the microtubule coil are important steps in platelets formation. β1 tubulin deficient mice result in thrombocytopenia. In these mice megakaryocytes lack the capacity to form proplatelets. A human β1 tubulin mutation (AG-CC substitution) inducing both structural and functional platelet alteration has been described. This β1 tubulin mutation was found in 10% of the general population and in 24% of 33 unrelated patients with undefined congenital macrothrombocytopenia (111). A more than doubled prevalence of the β1 tubulin variant was observed in healthy subjects not undergoing

ischemic events, suggesting it could confer an evolutionary advantage and protective cardiovascular role.

Resting blood platelets express the GPIb-IX-V complex, also called von Willebrand factor receptor that, by binding to activated von Willebrand factor, initiates hemostasis by causing rolling of platelets over the vascular surface (112). In addition to this role, the GPIb-IX-V complex is an important structural component of platelet cytoskeleton that functions as the principally membrane-actin filament linkage in platelets. The important role of this interaction is demonstrated by the abnormal morphology and extreme fragility of platelets observed in Bernard-Soulier, witch lack the GPIb-IX-V complex (113). Patients with this syndrome have increased megakaryocytes cytoplasmatic volume and ploidy and abnormal distribution of DMS, granules and microtubules. Bernard–Soulier syndrome is characterized by severe bleeding, giant spherical platelets and thrombocytopenia.

May-Hegglin anomaly (MHA) is the most common inherited giant platelets disorder was first described by May in 1909 , and later by Hegglin in 1945 (114). This syndrome is an autosomal dominant platelet disorder is characterized by giant platelets, thrtombocytopenia, leukocyte inclusions and middle bleeding tendency. The disease appears to be the result of the mutation in the gene encoding non muscle heavy chain 9 (MYH9) (115). Myosin II is an ATPase motors molecule that binds to actin filaments and generate force for the contraction. The major role of myosin II is to permit the molecules to assemble into bipolar formation. MYH mutation result block the polymerization of myosin II during megakaryocytes development and platelet formation. In rod MYH deficiency animals results, on defects in nonmuscle myosin IIA assembly *in vitro* (116). Mutation in MYH are also responsabile for Fechtnr and Sebastian syndromes which are

characterized by thrombocytopenia, leukocyte inclusion and giant platelets. Gray platelets syndrome is an autosomal dominant disease that presents with macrothrombocytopenia. The large platelets appear gray due to a reduction in α-granule content (117). Alpha-granule normally contains a number of proteins including von Willebrand factor and fibrinogen. In Gray platelets syndrome, platelets inadequately package these proteins within α-granule. As consequence, a number of factors fail to be released upon platelets activation, which increases risk of bleeding. Although, the precise genetic defects, responsible for this syndrome, are unknown.

COAGULATION PROTEIN S

Introduction

Protein S is a vitamin K dependent protein with anticoagulant properties. Blood coagulation proceeds as a cascade of proteolytic events. Tight regulation is required to ensure efficient haemostatic responses to vascular injury while preventing thrombus formation. Protein S is one of the molecules involved in the down regulation of the coagulation process. Protein S was discovered in 1979 by DiScipio and Davie as a sixth vitamin K plasma protein that they isolated from bovine and human plasma (118). PS is a cofactor of the anticoagulant enzyme activated protein C (APC) in the inactivation of activated factor V (FVa) and factor VIII (FVIIIa), as a cofactor for the tissue factor pathway inhibitor (TFPI) in the inactivation of activated factor Xa (FXa) and tissue factor (TF)/factor VIIa (FVIIa) (119, 120). Inherited deficiency of protein S constitutes an important risk factor for venous thrombosis. Protein S has multiple functions such as inflammation and apoptosis (121).

Structure of Protein S

Protein S (PS) is a single plasma glycoprotein with a molecular weight of approximately 70kD. PS normally circulates in plasma at a concentration of 25mg/L. In human plasma 40% of PS circulates as a free form and functionally active anticoagulant protein, while the remaining 60% of the complexes attach to the complement C4b-binding. PS is composed of multiple domains composed of a NH_2 terminal domain, rich in γ-

carboxyglutamic acid (Gla domain), a thrombin-sensitive region (TSR; amino acids 46-74), a short stretch of aromatic residues, four epidermal growth factor (EGF) like domains, and a C terminal sex hormone-binding globulin (SHBG) like region comprising two laminin G (LamG)-type domains (122, 123).The Gla domain has a high affinity for negatively charged phospholipid membranes and it also interacts with APC. The Gla domain is pivotal for Ca^{2+}-dependent binding of PS to phospholipid membranes, this is where it exerts its biological function and is also associated with the expression of APC cofactor activity. (124, 125). Chemical modification of the Gla residues of PS lead to a loss of both binding capability of the phospholipids and the anticoagulant cofactor activity. Protein S is destroyed and looses its cofactor activity when in the thrombin-sensitive region cleaves at Arg49 and Arg70 and when factor Xa is at Arg60. This is shown by the mutation of these three sites which generate a thrombin-resistant form of PS (126, 127). The EGF domains function is to cooperatively bind Ca^{2+}; the combination of the EGF domains binding Ca^{2+} increases their activity in comparison to the isolated domain (128). EGF1 and EGF2 are important for the expression of APC cofactor activity, EGF1 interacting directly with APC. The N-terminal of the EGF domain is critical for the functioning of the cofactor (129). Antibodies directed towards the EGF domains of protein S, inhibit the cofactor's activity (130). The two LamG-like domains of PS, comprised of the SHBG-like region, interact with the complement C4BP; it has been demonstrated that several regions are importance in this binding process, however, the stages of these interactions still remain unclear. Moreover the second LamG-domain has been shown to play a role in the APC mediated cleavages of both FVa and FVIIIa. When PS is bound to C4BP the function of PS as a cofactor is abolished (131, 132). In plasma, C4BP and PS,

interact reversibly and with high affinity, this interaction is substantially tightened by the presence of Ca^{2+} (133, 134). C4BP is a large protein (540kD) containing six or seven identical α chains of approximately 70kD each (135). Using electron microscopy, C4BP appears as a spider-like structure with long arms corresponding to the α-chains (136). PS sits at the base of the α-chains and interacts with the single β-chain that forms the PS binding site (137). Free PS and the S-C4BP complex bind to the cells surface via the Gla domain of PS. This provides the potential for the regulation of both the coagulation and the complement systems found on the surface of apoptotic cells. In addition the bound PS stimulates phagocytosis of the apoptotic cells. C4BP is an acute phase reactant, but only the C4BP α-chains seem to be elevated in the acute phase response (138) (Fig 5).

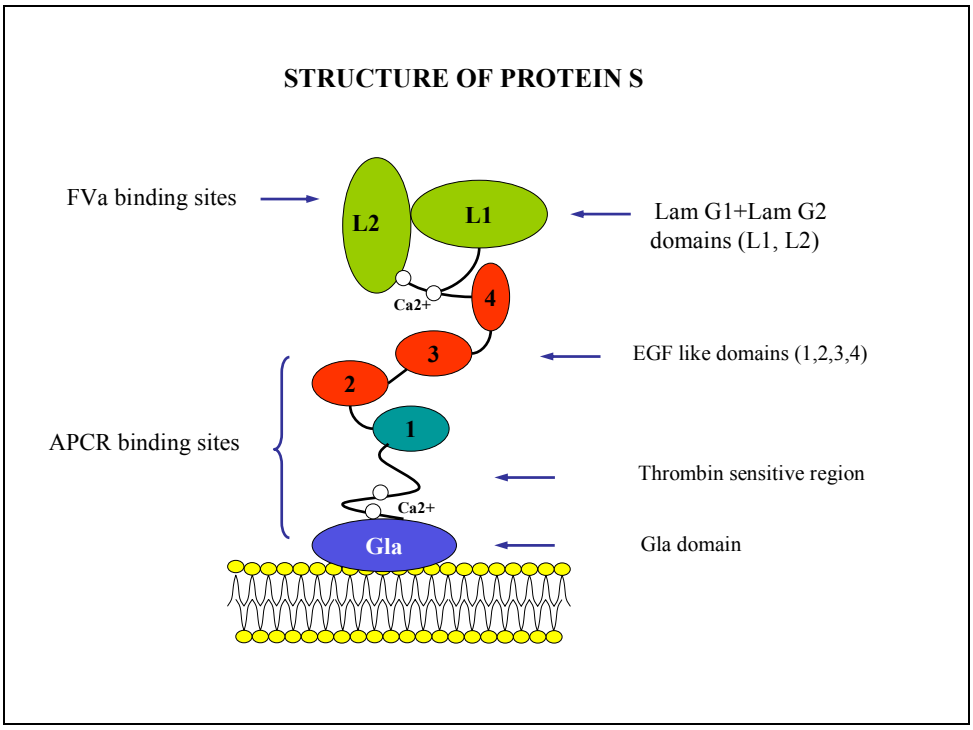


Fig. 5. Schematic model of human protein S. Protein S is a single chain molecule and is a vitamin K dependent protein. The Gla domain, contains eleven Gla residues, and has high affinity for the negatively charged phospholipids membrane. Gla domains interact with activated protein C (APC). The thrombin sensitive region (TSR) interacts with APC. Thrombin and activator factor X (FXa) interacts with TSR region resulting in the loss of APC–cofactor activity. EGF1-EGF2 are important for the interaction with APC.

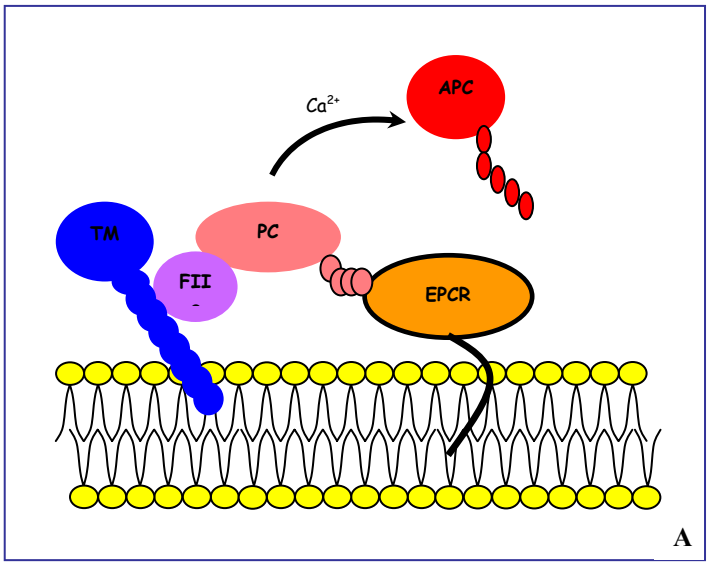
Protein S gene and synthesis

PS is expressed in liver hepatocytes, osteoclasts, testicular Leydig cells and endothelial cells. Schwarz and colleagues identified PS in α granules of human platelets, which suggests that the released PS may bind to stimulated platelets and thereby promotes and localizes the anticoagulant activity on the platelet surface (139). Platelets contain PS but whether this is derived from megakaryocytic synthesis or from uptake of plasma PS is not known. Only a megakaryoblastic cell line (MEG-01) has been demonstrated to synthesize PS, suggesting that megakaryocytes probably have the capacity to make PS (140). In the human genome there are two homologous PS genes (PROS1 and PROSP), although only PROS1, is the active gene whereas PROSP is a pseudogene. Both genes are located on chromosome 3, close to the centromer, except on different sites of, PROS 1 at q11.2 and PROSP at p21-cen. PROS1 is approximately 80kb long and contain 15 exons and 14 introns. PROSP contains multiple base changes, a

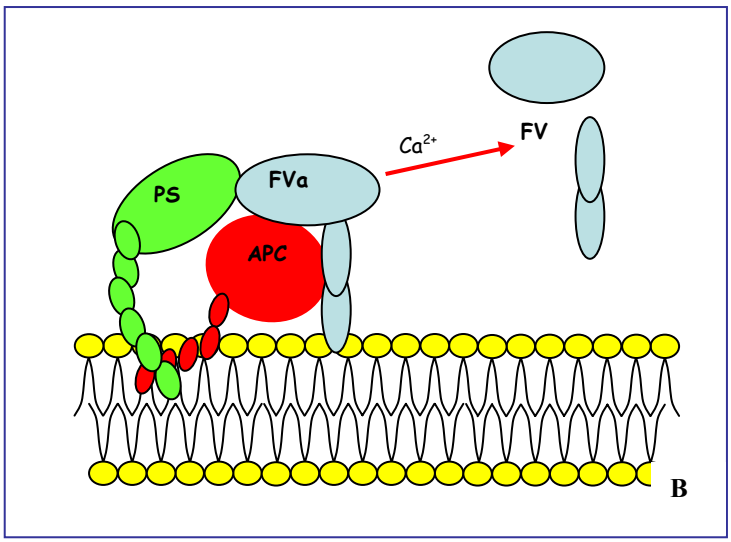
variety of mutations and lacks exon 1. The pseudogene hampers the genetic analysis of the PROS1 gene. There is a high degree of sequence identity between the exons of the two genes (97%). Mutations in the S gene lead to PS deficiency.

Anticoagulant APC-dependent activity of protein S

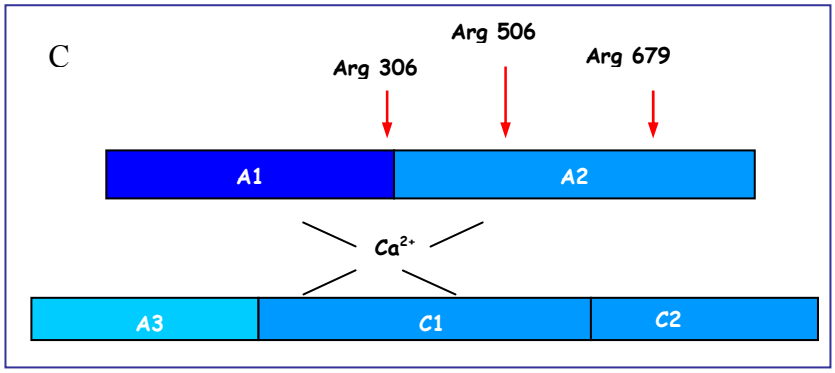
PS plays an important role in regulating thrombin generation and therefore controlling procoagulant activity. PS. works as a cofactor for APC in the inactivation of FVa and FVIIIa. PS and APC form a complex and interact with the negatively charged phospholipids membrane. PS has been suggested to decrease the distance between the active site of APC and the phospholipids membrane (141). APC cleaves FVa at three sites Arg506, Arg306 and Arg679. The APC-mediated cleavages at Arg506 and Arg 306 in FVa have a different dependence on PS. Thus, even though PS provides stimulation to both cleavages, the APC cleavage at Arg506 in FVa is less dependent on the presence of PS than the cleavage at Arg306 . The cleavage at Arg506 results in a partial loss of FVa activity, whereas the cleavage at Arg 306 results in total inactivation of FVa. FV has been found to act as a cofactor for APC in the FVIIIa degradation (142, 143). The LamG domains of PS are also important for the interaction with FV during the degradation of FVIIIa (Fig 6).



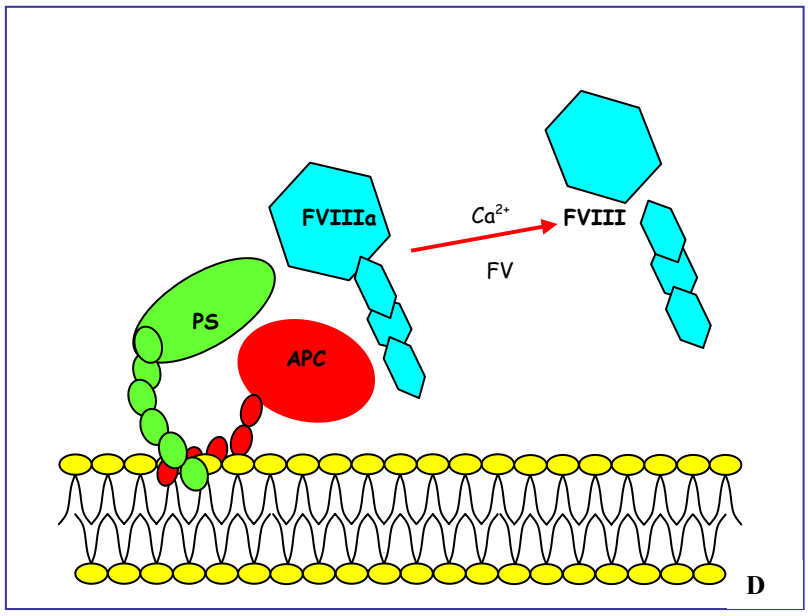
A) Schematic model of the protein C anticoagulant protein. Thrombin and thrombomodulin, make a complex, with endothelial protein C receptor activate protein C.



B) Degradation of FVa by APC and its cofactor protein S. APC/PS complex cleaves FVa at three sites, Arg306, Arg506 and Arg679.



C) Degradation of FVa at three sites (Arg306, Arg506 and Arg 679) by APC/PS complex.



D) Inactivation of FVIIIa by APC/PS complex in the presence of FV.

Fig 6. Schematic model of anticoagulant APC-dependent activity of protein S. A) Activation of protein C (anticoagulant protein C pathway). B) Degradation of activated FV (FVa). C) Specific sites of inactivation FVa

by APC/PS complex. D) Inactivation of activated factor VIIIa by APC/PS/FV complexes.

APC independent anticoagulant activity of protein S

PS exhibits APC-independent anticoagulant activities in vitro by directly inhibiting both the prothrombinase and tenase complexes. The APC independent activity has been suggested to be due to direct interactions between PS and FVa/FVIIIa and/or FXa. It has also been shown in a flow system using endothelial cells and plasma; where protein S-depleted plasma gave considerably higher prothrombin activation than plasma containing PS (144). Recent studies have suggested that PS specifically inhibits tissue factor (TF). TF activity promotes the interaction between the full-length TF pathway, the inhibitor and FXa (145).

Interaction between protein S and C4b-Bindig Protein

The protein S-C4BP complex in human plasma has a 1:1 stoichiometry, the interaction being non covalent and of high affinity (Kd 0.1nM -0.6nM) . C4BP is an important cofactor to the serine protease factor I in the degradation of C4b in the classic complement pathway. C4b degradation inhibits the formation of the C4b2a complex (C3 convertase) and accelerates its decay, an important step to the regulation of an inflammatory response (146). C4BP acts as the cofactors for FI (a complement regulatory enzyme in the blood) and in the inactivation of C3b and C4b (complement factors). This could be important in avoiding deposition of inflammatory complexes on tissue and maintaining tolerance for self-antigens, thereby providing protection against inflammation and autoimmunity (147).

C4BP in human plasma exists in several forms, which are formed by different combinations of its constituent α and β chains and with a molecular weight of approximately 570kD. C4BP is composed of seven identical α-chains (70kD) and a single β-chain (45kD), disulphide bridges linking the chains. C4BP has a spider or octopus shape seen under the electron microscope. The β chain of C4BP contains the PS binding site, and therefore binds only–containing isoforms (C4BPβ+) (148, 149). In normal human plasma, at least 80% of the C4BP contains the β-chain (C4BPβ+). The molar concentration of C4BPβ+ is approximately 30-40% lower than that of PS, because the protein S-C4BP interaction is of very high affinity; free PS is equivalent to the molar surplus of PS over the C4BPβ+. During inflammatory disorders the levels of C4BP increase up to 400% and the

synthesis of α-chains increase to more than that of β-chain. This is due to the differential regulation of α and β chain gene expression given by cytokines. During inflammation, C4BP is consisted only of α chains which are unable to bind free PS. The protein S synthesis does not increase during inflammation and therefore ensures stable levels of free PS. During these inflammatory states, the plasma's C4BP level may be much higher than normal. The protein S-C4BP interaction provides a link between the regulation of blood coagulation and the complement system. The binding of protein S-C4BP to apoptotic cells can provide local regulation of the complement system and inhibition of inflammation in the near dying cells (150).

Apoptosis and protein S

Apoptosis is a very complex process involving a number of molecules present in the apoptotic cell, in the macrophage as well as in molecules recruited from serum. The macrophages silently engulf the apoptotic cells and prevent leakage of contents from the dying cells that would otherwise lead to inflammation.

Apoptotic cells expose in the membrane, have many marker molecules, such as; phosphatidylserine, differentially glycosylated molecules, oxidized low density lipoprotein-like sites and pathogen-like apoptotic cell-associated molecular patterns which are all recognized by the macrophage. The negative phospholipids, phosphatidylserina, under normal condition are exposed in the inner leaflet of the cell membrane. During apoptosis these phospholipids are exposed in the external leaflet of the cell's membrane. PS do not bind to the surface of normal cells but only when the

membrane surface is exposed to phosphatidylserina, which is found during apoptosis or platelets activation. This explains why PS can bind to apoptotic cells and on platelet microparticles (151). The binding of PS is calcium dependent and mediated by the Gla domain. PS binds apoptotic cells and stimulates phagocytosis; however, these mechanisms are yet to be defined (152). In contrast, binding of protein S-C4BP complex on the apoptotic cells counteracts the phagocyte process, suggesting that the bound of C4BP inhibits the interaction between PS and its receptors on the macrophages (153). PS and protein S-C4BP complexes have additional functions on the apoptotic cell surface, e.g. PS may be anticoagulant and control phagocytosis, whereas protein S-C4BP complex may be involved in the regulation of the complement system (Fig 7).

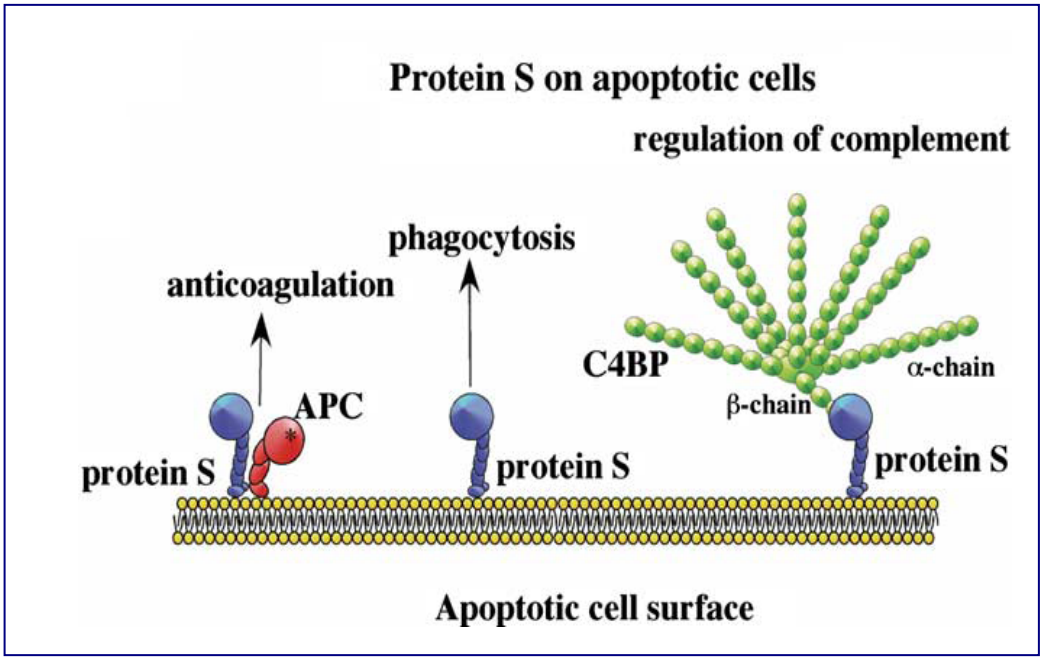


Fig 7. Protein S and the proteinS-C4BP complex bind to apoptotic cells. The apoptotic cells expose, in the negatively charged membrane, phosphatidylserine, and which is a signal for professional phagocytes as a macrophage for the elimination of these cells. The Gla-domain of protein S mediates the binding of both free protein S and the protein S complex to the apoptotic cells, providing the potential for regulation of both coagulation and the complement systems on the surface of apoptotic cells. In addiction protein S stimulates phagocytosis on the apoptotic cells, whereas the protein S-C4BP complexes have the opposite effects (Dahlbäck B, 2007).

Platelets Protein S

PS is a granule platelet constituent that is present in platelet lysates and released on the platelet upon thrombin stimulation. APC inactivates FVa on the platelet surface in a PS dependent reaction and reduces the number of FXa binding sites, blocks the platelet prothrombin converting its activity (154, 155). Since stimulated platelets in contrast to non stimulated platelets do not require exogenous PS to inactivated FVa by APC, this suggests that PS is released from platelets in its active form (156). The pathophysiological role of the participation of platelets in the protein C-protein S pathway remains to be clarified. Plasma derived from protein S is mainly synthesized in the liver; however the origin of the platelet PS has not been unambiguously defined. Evidence suggests that platelets derived from PS might originate from the plasma through endocytosis, as well as from fibrinogen, albumin, or by synthesized precursors of platelets.

Megakaryocyte synthesized molecules such as platelet factor 4, von Willebrand factor and FV but it still unknown if megakaryocytes can synthesize PS (157).

Protein S deficiency

Hereditary PS deficiency is a rare but serious autosomal dominant disorder. Individuals, with heterozygous PS deficiency most commonly present deep venous thrombosis (DVT), pulmonary embolism (PE), superficial thrombophlebitis and in some cases arterial thrombosis.

The first cases of heterozygous PS deficiency were described in 1984 (158). Familial PS deficiency is an inherited risk factor of venous thrombosis present in 2-4% of thrombosis patients and 0.03-0.13% of the general population (159). Heterozygous PS deficiency increases the risk of thrombosis 5-10 fold. Homozygous deficiency is extremely rare and without treatment, incompatible with life, because it leads to extensive microvascular thrombotization. Homozygous PS deficiency, in the neonatal period, is usually associated to severe *purpura fulminans*. Protein S deficiency if associated with other genetic or acquired conditions, have a greater risk of venous thrombosis. These conditions include factor V Leiden, HR2 aplotype of factor V and prothrombin G20210A mutation (160, 161). Inherited PS deficiency has been classified into three types I, II and III. Type I patients have decreased, free and total PS, type II patients have normal levels of free and total PS although with a decrease of PS activity denoting functional deficiency, and type III patients are characterized by low levels of free PS, however, with a normal concentration of total PS. In several family pedigrees, types I and III were

found to coexist suggesting the two types to be phenotypic variants of the same disease . The plasma levels of PS and C4-BPβ+ are approximately equimolar in PS deficiency patients, which explains the low plasma levels of free PS in type I patients. This shows why the affinity of the protein S-C4BP interaction is so high and furthermore this analysis clarifies that free PS has a higher predictive value for PS deficiency than total PS (162).

Mutations within PROS gene that are associated with PS deficiency are collected and published in an online database by the ISTH (International Society of Thrombosis and Haemostasis). According to the database, 95% of patients had a quantitative (type I or type II) deficiency PS and 5% had a qualitative (type II) deficiency. In type I most of the gene defects are missense or nonsense mutations (163). In type II, mutations have been found in the Gla domain resulting in defective γ carboxylation, and presumably provoke folding problems resulting in an unstable structure. AThr103 to Asn substitution in EGF1 causes a low interaction of PS with APC. In EGF2, a Lys155 to Glu mutation results in a functional defect in PS with low cofactor activity (PS Tokushima), suggesting that the EGF domain is important for the interaction of PS with APC. Type III deficiency is caused by mutations leading to increased affinity of the resultant PS molecules for C4BPβ+ with consequently reduce levels of free PS. Exons 12,13 and 14, that encoding the LGR domain, contain the C4-BPβ+ binding site, mutations in these exons increase PS affinity for C4-BPβ+ (type III PS deficiency). A single substitution Ser 460 Pro (commonly known as PS Heerlen) has been reported to have this effect. PS Heerlen, resulting in the loss of the N-glycosylation consensus sequence (Asn-Xxx-Ser) and thus leading to N-linked glycosylation at Asn458. PS Heerlen allele has been found in almost 40% of probands with type III PS deficiency and in only 0.5% of the normal population. Initially it was considered a neutral

polymorphism, later it was shown that this mutation is associated with low lives of free PS. The Ser460Pro mutation has been found in 22% of PS deficient patients with venous thrombosis (<65%), although in about a half of the carriers another risk factor was present (164, 165). PS Heerlen displayed a normal APC cofactor activity in the inactivation of normal FVa and a poor cofactor activity in the inactivation of FVa Leiden, suggesting that a FV Leiden and PS Ser460Pro mutation may have a synergistic thrombophilic effect (166). However as yet, no clear correlation between the Ser460Pro mutation and venous thrombosis has been established.

Acquired PS deficiency

Several factors influence the concentration of PS in plasma; these include things such as, pregnancy and oral contraceptive which decrease PS levels. In pregnancy, the levels of both plasma total and free PS may reach levels similar to those found in patients with inherited deficiency. Plasma levels of PS decrease in patients during treatment with oral anticoagulants (warfarin and $_{L}$-asparaginase), as PS is a vitamin K dependent protein (167). Acquired PS deficiency can be associated with Nephrotic Syndrome, this is because the high molecular weight of C4BP does not allow its glomerular filtration, whereas free PS is lost in urine resulting in decreased levels of functionally active PS. This may contribute to the thrombotic risk associated with nephrotic syndrome. Acquired protein S deficiency, with autoimmune diseases or HIV infection may be the result of immunological mechanisms yet to be defined. Acquired PS deficiency with autoantibodies against PS and severe thrombosis is observed after varicella infections in children. Autoantibodies against PS can also be found in antiphospholipid

syndrome, and these seem to be associated with the increased risk of venous thrombosis. Indeed anti-PS antibodies are common finding in patients with Systemic Lupus Erythematosus syndrome.(SLE) (168).

AIM OF THE STUDY

- To develop a model *in vitro*, of human megakaryocytes in culture taken from haematopoietic stem cells.
- To analyze the basic mechanism of platelet production and release, in human and *in vitro* megakaryocytic culture.
-To determine the origins of endocytosis or synthesis, of platelets PS using the model of *in vitro* megakaryocytes in culture
- To understand the mechanisms that regulates the concentration of plasma and PS platelets.
- To study the influence of anticoagulant drugs (warfarin) in megakaryocytic culture.
- To determine the amount of plasma and platelets PS in normal and inherited or acquired PS deficit subjects.
-To study the immunological phenotype of plasma PS and platelets PS in normal individuals and in patients with inherited PS deficiency.
-To investigate the pathophysiological role and participation of platelets PS in the anticoagulant protein C pathway.
- To analyze the possible interference of the vitamin K antagonist in plasma PS and platelets PS concentration.

MATERIALS AND METHODS

Subjects

Twenty families with type I and/or type III protein S deficiencies (80 individuals) were analyzed. Families were ascertained through a proband who underwent thrombophilia screening after a first episode of venous thrombosis (deep-vein thrombosis, pulmonary embolism or superficial-vein thrombosis) or arterial thrombosis (myocardial infraction or stroke). Probands and relatives were invited to participate to the study after informed consent. Protein S deficiency was defined on the basis of free protein S and protein S activity applying a cut-off of 70%. The type of protein S deficiency was assigned according to total protein S levels, using a cut off of 70%. Normal ranges were determined in 100 healthy blood donors who had no family history of thromboembolism, who were not on anticoagulant therapy and had not used oral contraceptives. Thirty five patients on anticoagulant therapy (warfarin) with 2-3 Prothrombin Time (PT)/International Normalized Ratio (INR) were enrolled after informed consent to study protein S levels.

Blood collection and plasma preparation

Whole blood was drawn in 129 mM sodium citrate (1:9 vol/vol) and platelet poor plasma (PPP) was prepared by centrifugation at 3000 rpm for

10 minutes. Plasma was separated and stored in aliquots at -80^{o}C until use. Buffy coats were stored at -20^{o}C for DNA extraction.

Protein S platelet isolation

Whole blood was centrifuged at 1000 rpm for 10 minutes (min), brake off, and at 22°C to separate the platelet-rich plasma (PRP) from the cellular component. PRP was diluted 5:1 with phosphate buffer (137 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO, pH 7.4) (PBS) containing 10mM EDTA (PBS/EDTA) and centrifuged at 14000 rpm for one min to yield PPP and a platelet (Plt) pellet. The Plt pellet was gently re-suspended with 200µl PBS/EDTA and centrifuged at 14000 rpm. To eliminate residual erythrocytes the re-suspended Plts were centrifuged at 3500 rpm for 10 seconds and the supernatant transferred to a fresh tube. The re-suspended platelets were washed twice with PBS/EDTA. Washed Plts were re-suspended in Tyrode's buffer (140 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 2 mM $CaCl_2$ and 5 mM dextrose, pH 7.4) at a concentration of $1x10^9$ Plts/ml. Plts lysates were prepared by the addition of 1% Triton X-100, frozen in small aliquots at -80^{o}C and thawed until analysis.

Coagulation assays

Determination of PS concentration.

Assay of PS antigen

The concentration of total, free plasma and Plts PS was determined by enzyme-linked immunoassorbent assay (ELISA). Plate wells were coated with 2µg /ml rabbit anti-human PS antibody (DAKO, Milan, Italy) in 0.05M sodium carbonate buffer pH 9, were left overnight at 4^{o}C. The wells were washed five times with 0.05M TEA, 0.15 M NaCl, 2.5mM $CaCl_2$, 2.5 U heparin and 0.1% Tween 20, pH 7.5 (TEA/NaCl-Tw). Then100 µl of sample solution, diluted appropriately quenched in TEA/NaCl-Tw and 0.1% ovalbumin was added and incubated for two hours. Assays plates then were washed five times before 0.8 µl /ml polyclonal anti-human PS peroxidase conjugated was added (DAKO, Milan, Italy). After two hours incubation at room temperature plates were washed five times. Subsequently 100 µl /well of substrate solution (2mM 3,3',5,5'-Tetramethylbenzidine (TMB), 0.12 mM 30%H_2O_2, 1.2 mM sodium acetate, pH 5) was added (Sigma Aldrich, Milan, Italy). After a 10 min incubation at RT the reaction was stopped with 100µl of 4N of H_2SO_4 and the absorbance at 450nm was measured using a multilabel counter analyzer (Victor ^{3}. Pekin Elmer Finland) The assay was calibrated with dilutions of pooled normal plasma and Plts (1/200, 1/300, 1/400, 1/800, 1/1000 and buffer). The same assay was used to detect free PS antigen, after polyethylene glycol (PEG) 6000 precipitation of plasma PS-C4b-BP complex. Plasma was mixed with 20% of PEG-6000 and incubated on ice

for 30 minutes. After centrifugation at 14000 rpm for 10 minutes kept the supernatan for assays of free PS. Normal plasma and platelets lysates for the making of a normal pool were obtained from 20 healthy individuals, who were not on anticoagulant and oral contraceptives therapy.

Assay of PS activity

Protein S activity was measured by the ability of PS to function as a cofactor for the anticoagulant activity of activated protein C (APC). Factor clotting activities were assessed by measuring the degree of prolongation of a prothrombin time (PT) in the presence of the tissue factor; deplete PS plasma, phospholipids, calcium ions and APC. The protein S activity is proportional to the prolongation of the clotting type of a PS deficient plasma (HemosIL, Instrumentation Laboratories, Milan, Italy) to which a diluted sample has been added (according to manufacture's instructions). Clotting time was measured in an automatic coagulation timer (ACL 9000, Instrumentation Laboratories, and Milan, Italy). Standard curves were constructed with normal human plasma dilutions.

Genetic analysis

DNA amplification and analysis of the PROS1 Heerlen (S/P460) genotype

The genotype for the S/P460 polymorfism was determined by electrophoresis restriction length polymorfism fragment (RFLP) analysis. Genomic DNA was isolated from buffy coats using a Genomic purification kit (Qiagen, Milan, Italy). The PROS1 mutation screening was perfomed by polymerase chain reaction (PCR) of exon 13 using the forward primer PSf: (GTGCATTGATCATGCTTCTG) and the reverse primer PSr: (CTGTATTTTTCAGAGGTGG). The amplification was performed in 30 cycles consisting; 30 second denaturation at 95^{o}C, a 40 second annealing at 50^{o}C and one minute extension at 65^{o}C. The amplified DNA was incubated with 2.5U of RsaI enzyme (New England BioLabs USA) for two hours at 37^{o}C and the digestion products were analysed by electrophoresis on 2% agarose gel.

Plasma free PS purification for immunoblotting

PS was purified from plasma using barium citrate absorption. 100 µl of citrate plasma was mixed with 8 µl of 1M $BaCl_2$ and 2 µl of 1 M benzamidine was put for 30 minutes in ice. After centrifugation at 14000 rpm the pellet was washed twice with 80 mM $BaCl_2$, 20 mM benzamidine and then centrifuged. The pellet was then re-suspended in 10 mM Tris-HCl, 100 mM EDTA, 20 mM benzamidine and centrifuged at 14000 rpm and the

supernatant was kept. To the supernatant was added 16 µl of 40% TCA mix and was left to stand in ice for 10 minutes and centrifuged. The pellet was then dissolved and washed twice with 50 µl of absolute ethanol and after centrifuged. The pellet were dissolved in 20 µl of sample buffer 2x prior to electrophoresis.

Immunoblotting

Plts and plasma PS were analyzed by discontinuous sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), 5%-15% slab gells using cooling coils to keep the temperature at approximately 14^{o}C. After electrophoresis separated proteins were transferred from the polyacrylamide gel onto the polyvinylidenefluoride (PVDF) membranes (Sigma Aldrich, Milan, Italy) in the transfer buffer (25mM Tris-HCl, pH 7.5, 192mM glycine, 20% methanol) at 75Volts for two hours in a Trans Blot apparatus (Bio.Rad, Milan, Italy). After transfer, the PVDF sheets were placed into 150mM NaCl, 20mM Tris-HCl, pH 7.4, 0.1% Tween-20 buffer (TBS-Tw) supplemented with 0.01% (w/v) ovalbumin (Sigma Aldrich, Milan, Italy) for 30 minutes to block nonspecific binding. Before labeling, the membrane was washed twice in TBS-Tw. Human plasma and Plts protein S were visualized on PVDF membrane by incubating the membrane with a rabbit anti-human PS peroxidase (HRP) conjugated (Dako, Milan Italy) for two hours at RT on a shaker. The antibody was diluted 1:500 in a TBS-Tw buffer. The PVDF membrane was then washed twice with TBS-Tw buffer. Peroxidase activity was visualized with 3,3'-diaminobenzidine (DAB) (Fluka, Milan Italy). Standard proteins, which included myosin heavy chain (Mr 200000), phosphorylase B (Mr 97000), BSA (Mr 68000), ovalbumin

(Mr 43000) and α-chymotrypsin (Mr 25700) (Bio-Rad, Milan Italy), were run simultaneously.

Megakaryocytes in culture

Following informed consent, whole blood samples from patients with PS deficiency as well as normal individuals were collected in a 1:10 volume of 3.8 % (vl/vol) trisodium citrate. The syringe was slowly churned therefore to mix blood and anticoagulant homogeneously. All passages described below were executed under complete sterility. Whole blood was centrifuged at 1000 rpm for 10 min; brake off, and at 22°C to separate the platelet-rich plasma (PRP) from the cellular component.

The cellular component was diluted 1:3 in phosphate buffered saline pH 7.4 (PBS) supplemented with EDTA 50 mM, stratified on 15 ml of Histopaque-1077 (Sigma Aldrich, Milan, Italy) density gradient and centrifuged at 1800 rpm for 30 min at 22°C, brake off, to obtain a "cellular ring" containing mononuclear cells. This layer was carefully aspirated, diluted with PBS-EDTA 1:3 and centrifuged at 1500 rpm for 15 minutes at 22°C. The supernatant was aspirated and discarded while the cell pellet was re-suspended with PBS and centrifuged twice at 1300 rpm for 10 minutes. Cells were re-suspended in Iscove's modified Dulbecco's Medium (Euroclone, Celbio, Milan, Italy), serum-free. The surviving cell numbers were counted with Trypan Blue (Sigma Aldrich, Milan, Italy) exclusion assay and cells suspension was seeded into 24-well culture plates (8 x10^5 cells/well) on glass coverslips. Medium was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml Streptomycin (Gibco, Invitrogen, Milan, Italy). Cells were grown at 37°C under 5% CO_2 in a

humidified incubator and the medium was changed twice a week. Thrombopoietin (TPO) (Pepro Thec EC, London, United Kingdom) 50 ng/ml and interleukin 3 (IL3) (Pepro Thec EC, London, United Kingdom) 10 ng/ml were added to the medium to stimulate the differentiation of mononuclear cells towards megakaryocytic (MK) lineage, since the day of seed and subsequently every three days the medium was changed. In selected experiments, the cells were treated, since the day of seed or after 3 days in culture, with warfarin derivative (3(α-acetonylbenzyl)-4-hydroxycoumarin) or with Vitamin K_2 at different concentration (1, 5 μg/ml) (Sigma Aldrich, Milan, Italy).

Immunocytochemistry

Cells were fixed in ice-cold acetone for 10 minutes at 4^0C at days 5, 8, 10 and 15 the slides were air-dried. To block the endogen peroxides the sample was quenched by the addition of 30% H_2O_2 in methanol for 15 minutes and washed twice in PBS. The slides were stained with May-Grünwald-Giemsa and with modified haematoxylin eosin (Sigma Aldrich, Milan, Italy) for morphological analysis. The immunocytochemical analysis for detection of PS within MKs was leaded incubating the cells with rabbit anti human PS conjugated with horseradish peroxidase (HRP) antibody (The Binding Site, Birmingham, England) diluted 1:100 in a buffer containing 20 mM Tris-HCl pH 7.4 and 150 mM NaCl for two hours at RT. Polyclonal anti human protein C HRP conjugated antibody as a negative control and sheep anti human factor V HRP conjugated antibody as a positive control were used (DAKO Cytomation, Denmark). The antibodies developed with 3, 3'-diaminobenzidine (DAB, Fluka, Milan

Italy) and slides were mounted with mowiol mounting solution (Sigma Aldrich, Milan, Italy). The glasses were observed by phase-contrast microscope (Leica DM 5000, BM Medical, Padua, Italy).

For immunofluorescent analysis cells were fixed in 2% paraformaldeyde (PF) in PBS for 20 minutes at room temperature (RT) and treatment with 50mM NH_4Cl and permeabilized with 0.5% Triton X-100 in PBS for 15 minutes. Cells were incubated with rabbit anti human PS diluted 1:50 (DAKO Cytomation, Denmark), monoclonal mouse anti human CD41 (BioLegend, San Diego, CA) diluted 1:20 and mouse anti human factor V 4156 (Haematologic Thecnologies Inc., USA) diluted 1:100. After two washes in PBS, cells were incubated with the appropriate secondary antibodies; goat anti rabbit IgG fluorescein conjugated diluted 1:150 and goat anti mouse IgG fluorescein isothiocyanate conjugated (FITC) diluted 1:100 (Chemicon International, Milan Italy). The primary and secondary antibodies were diluted in 0.5% bovine serum albumin (BSA) in PBS and incubated for one hour at 37^{o}C in a humidified incubator. After two washes in PBS, DNA was then labeled with 1.5 μg/ml Höechst 33258 (Aldrich Sigma, Milan, Italy) for ten minutes at RT and slides were mounted with mowiol antifading solution (Sigma Aldrich, Milan, Italy).

For the detection of cytoskeleton structure cells were incubated with primary antibodies mouse anti α-tubulin and mouse anti γ-tubulin (1:100 dilution in PBS contained 0.5% BSA) for one hour at 37 ^{0}C in a humid chamber for one hour. Followed two washes in PBS the cells were incubated with goat anti mouse IgG fluorescein conjugated for one hour at 37^{0}C. Nuclei were finally counterstained with 1.5 μg/ml propidium iodide in PBS contained 10μg/ml RNAse A (Sigma Aldrich, Milan, Italy) for 15 minutes at 37^{0} C, slides were mounted with an antifading medium. In order

to assess nonspecific binding of the secondary antibodies, the same procedure was carried out in the absence of the primary antibody.
Examination of the samples was performed with a fluorescent microscope Leica DM 5000 (Leica, BM Medical, Padua Italy). Höechst fluorescence of nuclei was visualized by excitation at 330-385 nm with a 450 nm barrier filter. FITC fluorescence was visualized by excitation at 475-490 and emission at 530 nm. Propidium iodide was visualized by ecxitation at 545-565 nm and emission at 590 nm. All samples were analysed by differential interference contrast (DIC) objective. For this analysis all imagines were viewed and capture at 40x, 60x, 100x oil magnification.

Statistics

Results of experimental points obtained from multiple experiments were reporter as the mean ±SD. The Student t test was used to test the significance of any differences between the experimental conditions.

RESULTS

Morphology of cells derived from peripheral blood

Isolated hematopoietic mononuclear cells were grown under serum free conditions (IMDM) in the presence of TPO and IL3 to differentiate in megakaryocytic lineage. At different days of culture (5,8,10 and 15 day) cells were fixed and staining with haematoxylin eosin. At the early stages of cultures, we observed many small immature cells with unilobet nuclei and cells with large kidney-shape nucleus. As maturation proceeds the nucleus indents and forms lobed, and the cells gradually enlarge. The cells undergo nuclear endomitosis, resulting contains bilobed nucleus or different number of nuclei. Mature cells resulting with highly multilobed nuclei, completing cytoplasm maturation and showing long cytoplasmic protrusions, similar to the form of megakaryocytes (Fig 8).

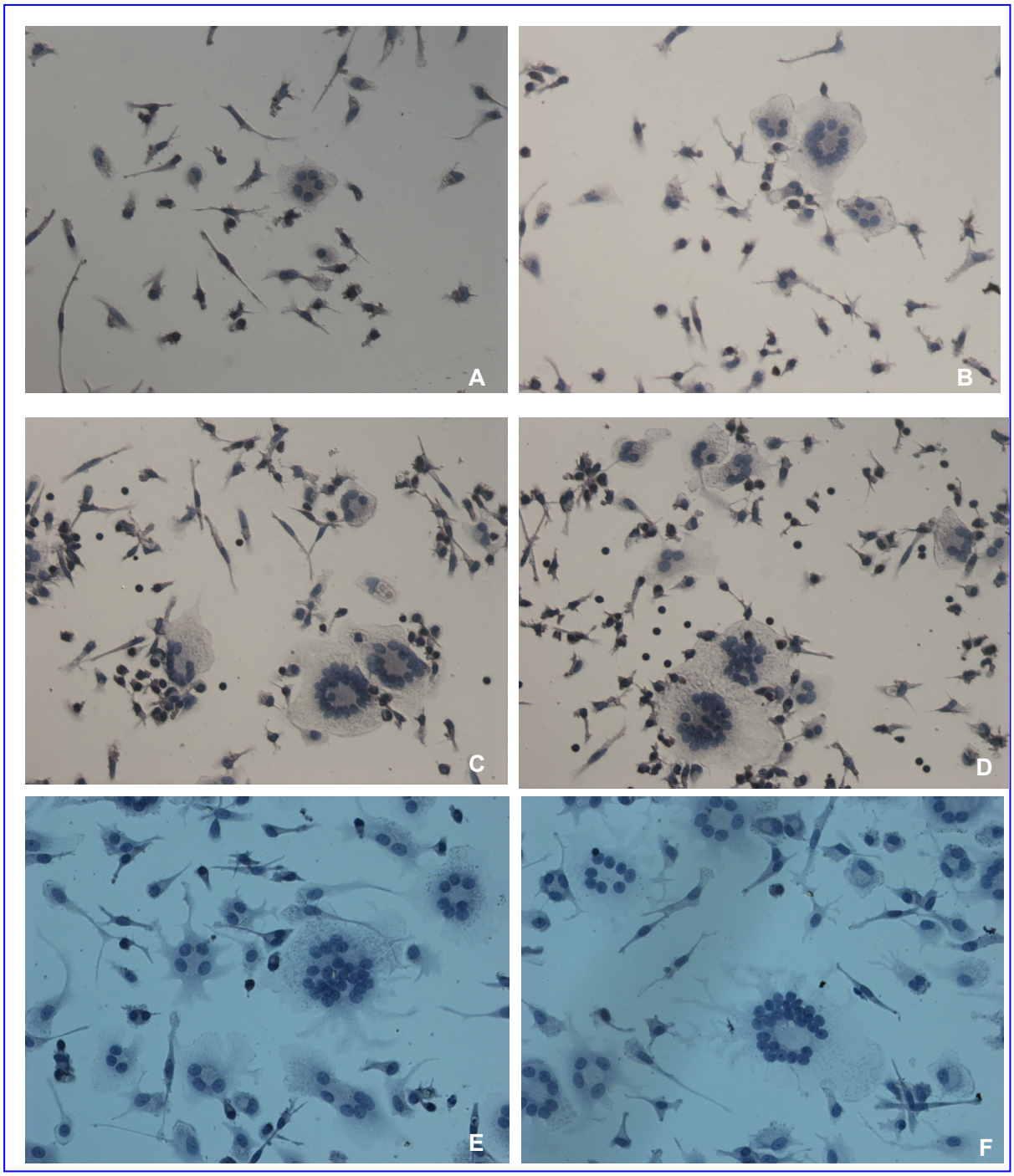


Fig 8. Interference contrast light microscopy of expanded cells (objective 40X). Modified haematoxylin eosin staining. A: small cells with scant cytoplasm and unilobed nucleus. B: Cells with multilobed nucleus. C and D: High multinucleated cells. E and F: Cells with multilobed nucleus and cytoplasmatic extensions.

INDIRECT IMMUNOFLUORESCENCE CD41

The expression of CD41 was analyzed by immunofluorescent staining. CD41 is a specific megakaryocytes marker. The results indicated the presence of this marker in the polynucleated cells as well as on mononucleated cells suggesting that these cells were indeed MKs. Platelets expressing CD41. In cell culture, we observed the present of numerous platelet-like particles among the cells and there is positive for CD41 (Fig 9).

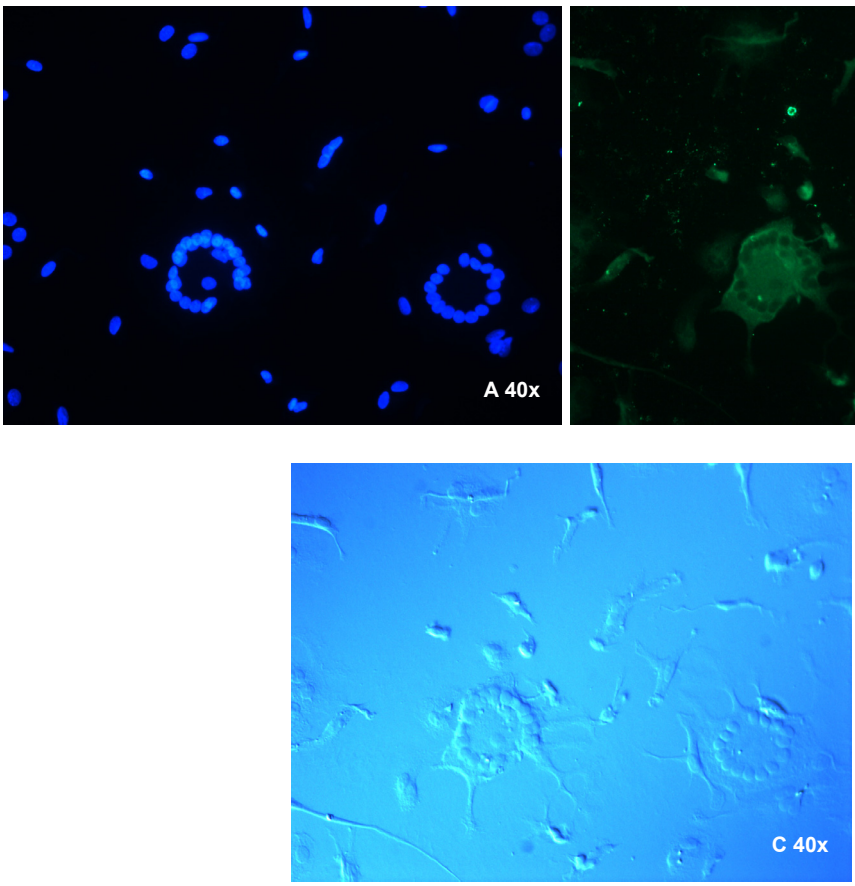


Fig 9. A: Monoclonal anti human CD41 immunofluorescent labeling of megakaryocytes and platelets. B: The nucleus was labeled with Höechst. C: interference contrast micrograph of cell labeled in panel A.

Megakaryocytes characterization and platelets biogenesis

Isolated hematopoietic stem cells from peripheral blood at day 5 cells were essentially mononuclear, representing Mk precursors. After day 5 Mk undergo at cytoplasmic maturation and expansion. First Mk undergoes nuclear endomitosis increased cell ploidy (2N, 4N, 8N, 16N). Mk maturation is characterized with proplatelet formation, initiated by cytoplasmatic expansion and elongation. These extensions are typically characterized by multiple cytoplasmatic swellings and are thought to represent intermediate structures in the formation of platelets. In the final stages proplatelet appear to be released by the fragmentation of proplatelet elongation (Fig 10, 11). Proplatelets morphogenesis depends on the elaboration of a dense and highly organized array of microtubules. Microtubules, which are formed when tubulin molecules assemble into linear filaments, are a major component of this cytoskeletal network and function as the primary motor for proplatelets expansion. The principally proteins involved in this cytoskeletal mechanism are α, β and γ tubulin dimers. As shown in fluorescently labeled tubulin incorporates into length of proplatelet β and γ tubulin is the most important protein involve in the chromosome segregation during megakaryocyte polyploidization. The nucleus after complete platelets release undergoes apoptosis (Fig 12). In the culture are observed the present of microtubule coils, similar to blood resting platelets, which form in the nascent platelets (Fig 13).

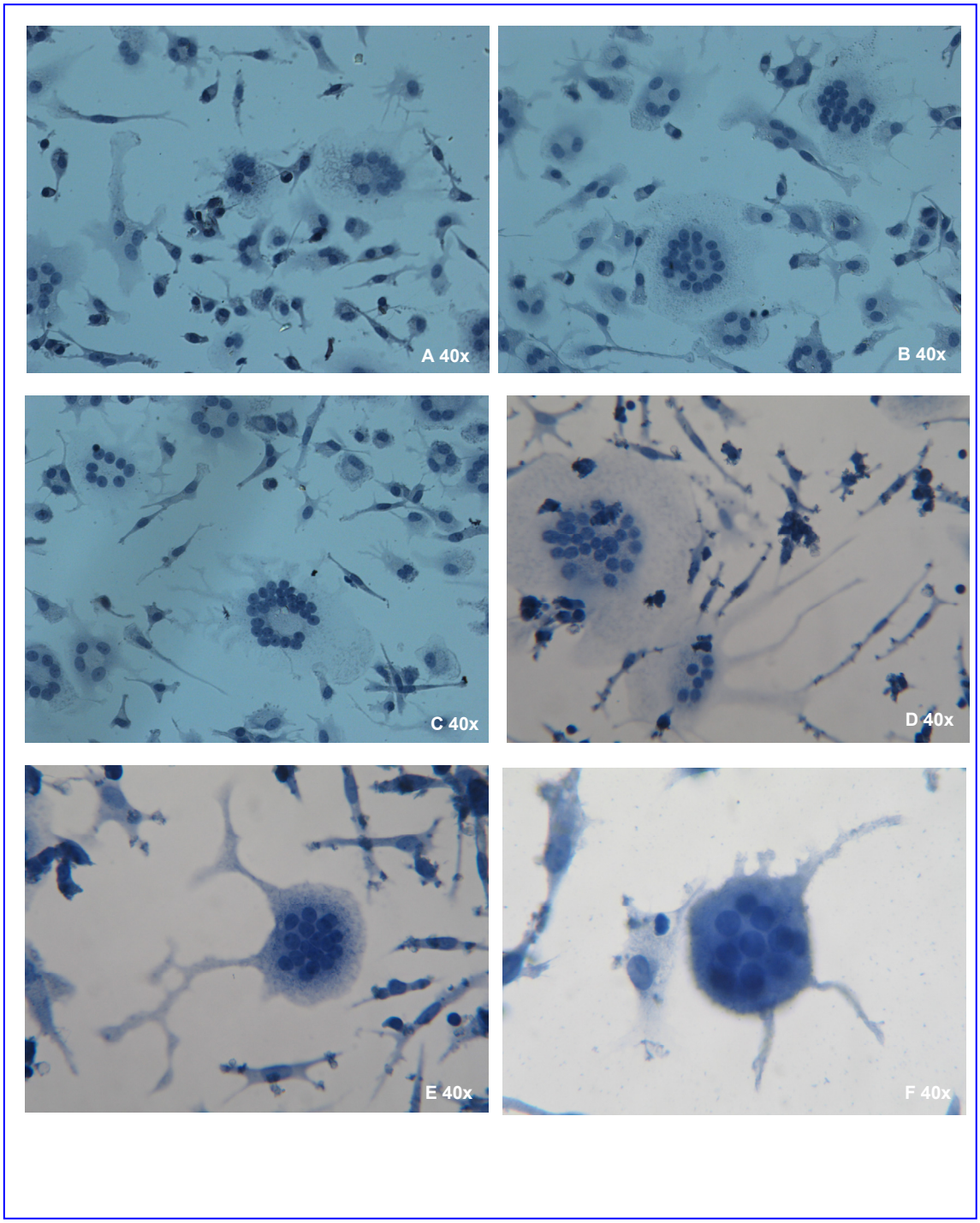


Fig 10. Modified haematoxylin eosin staining. Interference contrast, 40x. A) At the early stage of cells culture, the Mk are represented by low number of nucleus, one or two, and with small proplatelets formation. B)

Cells increase their cytoplasmatic volume and increase in the polyploidy stage (2N, 4N, 8N). The cells present a low number of proplatelets formation. C, D) During maturation megakaryocyte have a high number of nuclei. In this stage of development megakaryocyte begin cytoplasm extension to form the proplatelets. E) In the next step of maturation the cells increase proplatelets formation and undergo further fragmentation and finally; F) nuclei undergo apoptosis.

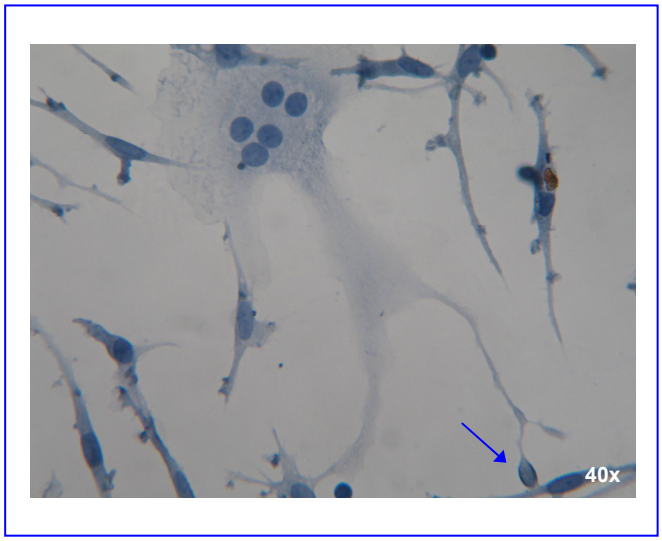


Fig 11. Interference contrast objective 40x. Microtubule bundles at the end of proplatelets, their fragmentation release the new individual platelet.

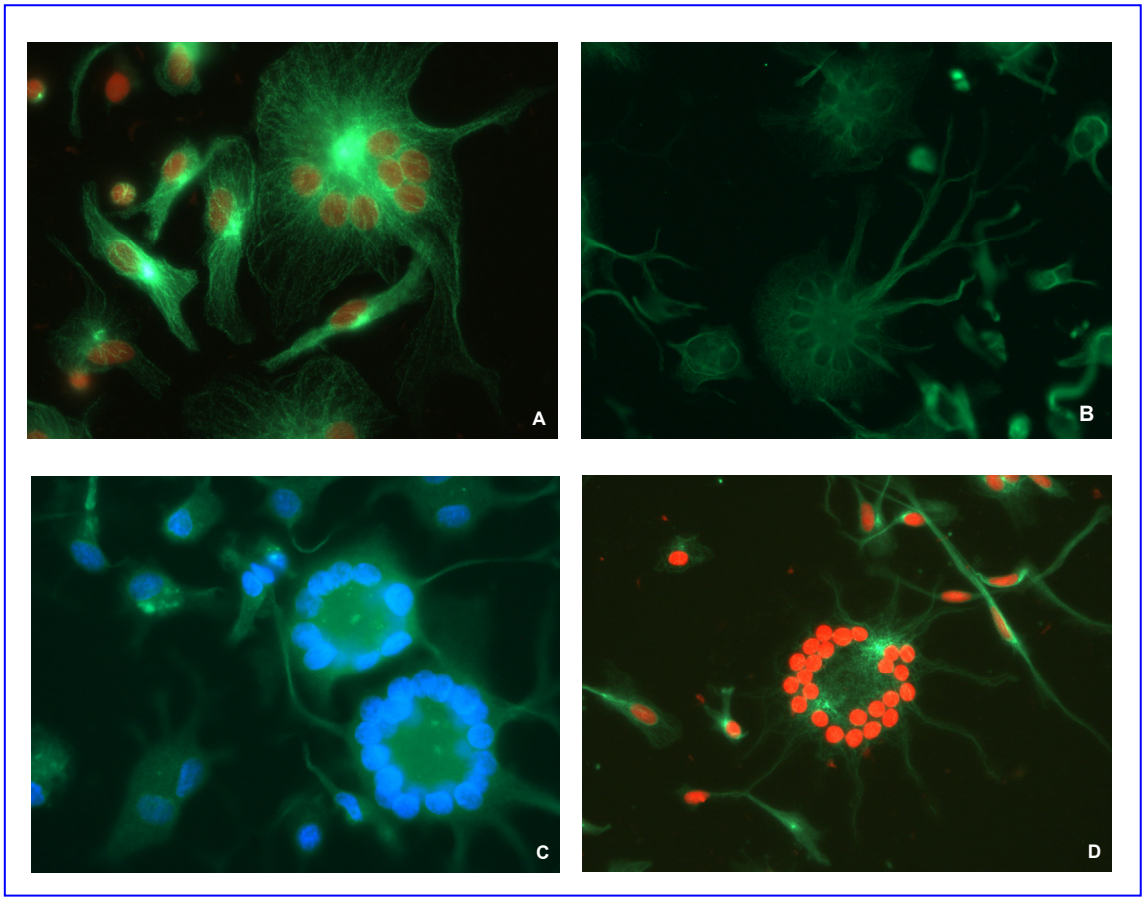


Fig 12. Immunofluorescence images of cytoskeletal proteins involved in the megakaryopoiesis, objective 60X. A) anti β-tubulin B) anti α-tubulin,both proteins are present in the whole cytoplasm and in proplatelets, C) anti γ tubulin is present in the centrosomes, important for the nucleation of megakaryocytes, D) anti β-tubulin, after the release of platelets the nuclei remain without cytoplasm. DNA was counterstained with Höechst (blue) or with propidium iodide (red).

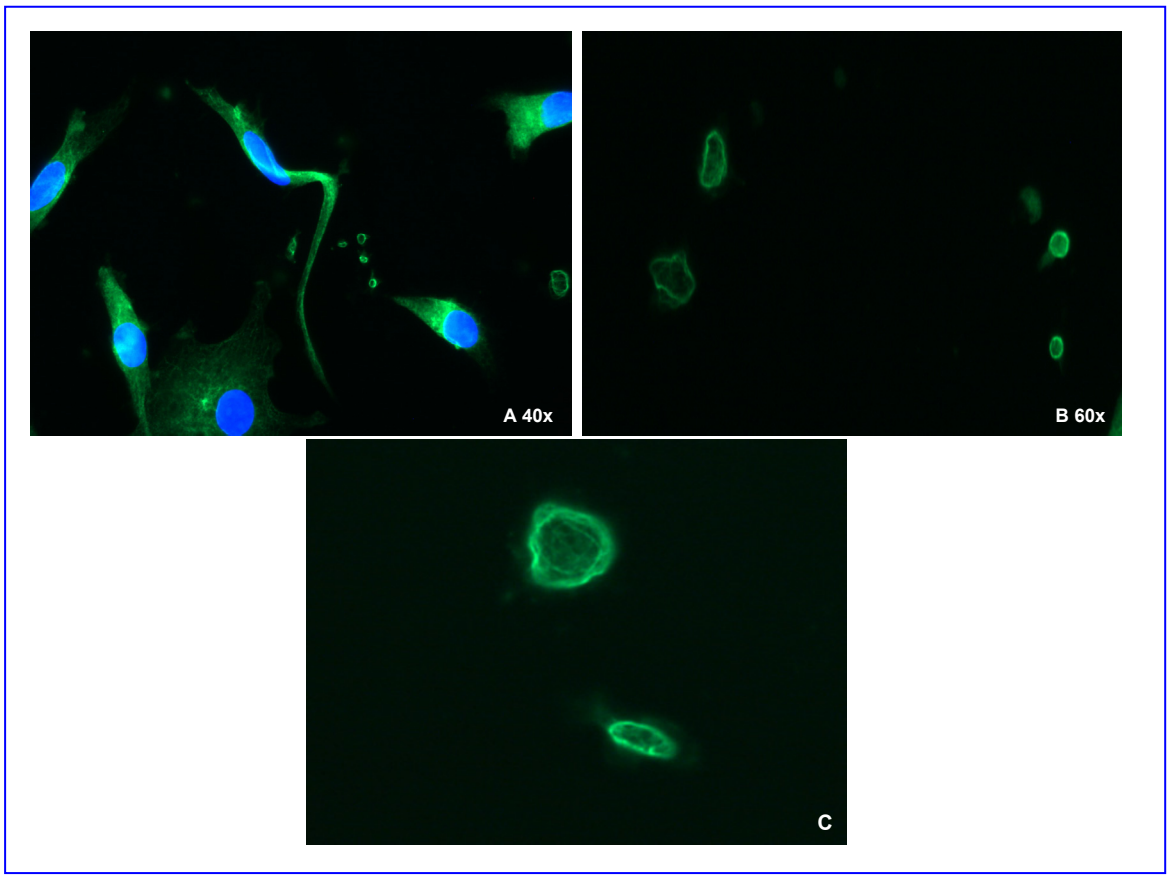
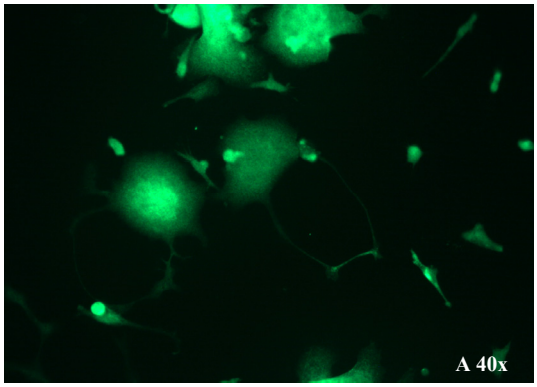
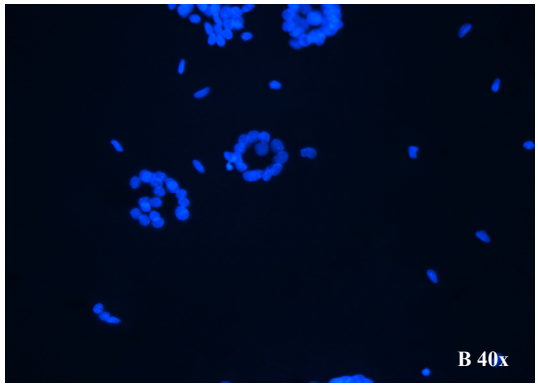


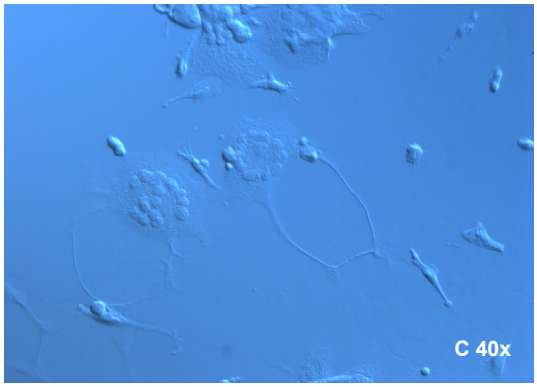
Fig 13. A, B: anti β tubulin immunofluoernce staining of platelets like particles released by megakaryocytes proplatelet. C) Microtubule coil in the new form platelet, (enlarged magnification of panel A).

Protein S expression by cultured megakaryocytes from normal and PS deficiency subjects.

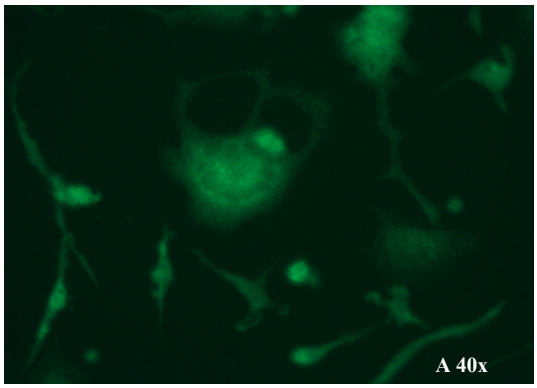
Megakaryocytes derived from normal subjects were cultured in serum free conditions. PS detected in culture MK from day 5 onwards, by indirect immunofluorescence. Mk staining with anti human protein S fluorescein conjugated at different days of culture. PS was localized in the cytoplasm and in forming proplatelets of megakaryocytes and in proplatelets area of fully mature Mk. Mk isolated from PS heterozygous deficiency patients (type I or type II) was analyzed expression of PS. PS was similar localization and the same morphological characteristics of Mk derived from normal subjects (Fig 14). We analyzed the expression of coagulation factor V (FV) and protein C in cultured Mk. Cells was labeled with anti human FV-FITC and anti human PC-HRP. Mk was positive for FV in contrast PC was not present (Fig 15).

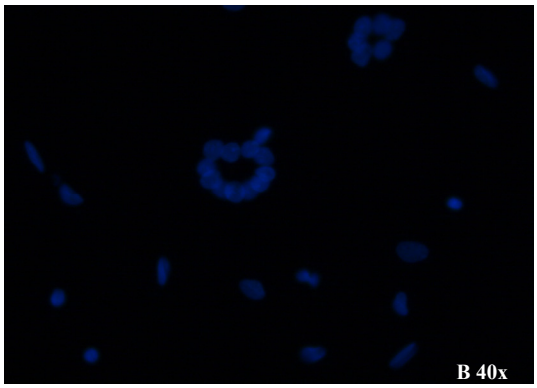


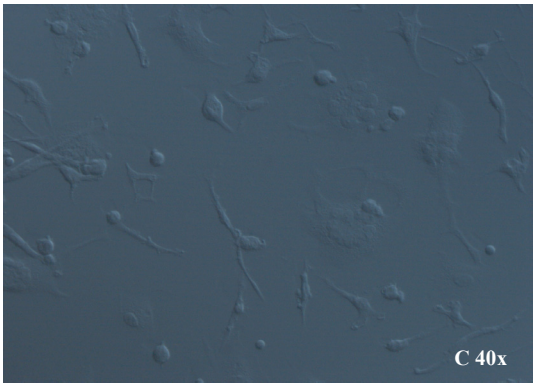




A







B

Fig 14. Megakaryocytes in normal subjects (A) and PS deficient patients(B), immunofluorescent labeling with anti human PS after 10 days of culture. A) Intracellular distributions of PS, PS is present in the cytoplasm and in the proplatelets B) the nucleus was labeled with Höechst. C) Interference contrast. (Obgiective 40x).

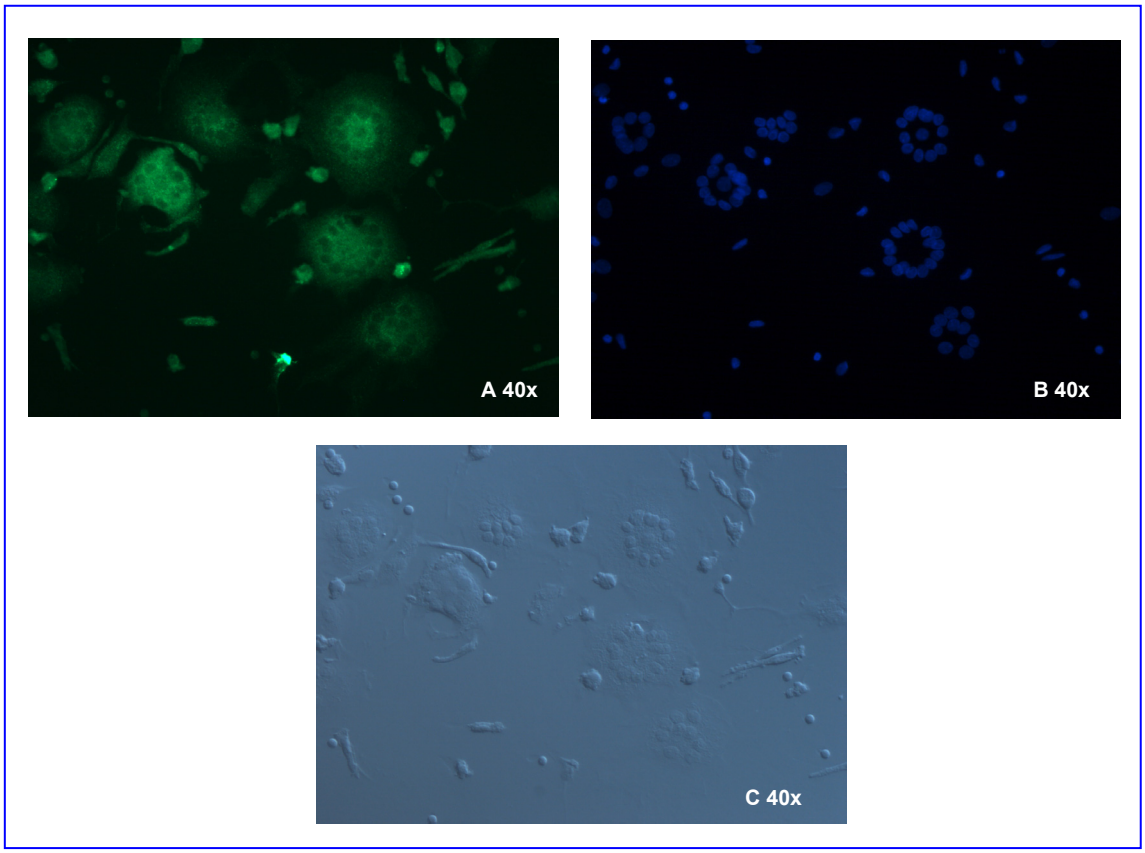


Fig 15**.** Immunofluorescent staining with anti human FV. A) Megakaryocytes result positive for FV staining (at 10 day of culture.) B) DNA staining by Höechst. C) Interference contrast. Objective 40x.

Familial Protein S deficiency; characterizations of plasma and platelets PS.

We unrelated 80 individuals from 15 families with PS deficiency (type I and III). Of these, 27 patients were heterozygous for type I and 15 patients for type III deficient PS and 38 individuals were normal. In all individuals were determined plasma levels of total, free PS antigen and free PS activity and platelets (Plts) PS antigen (Table1).

Table 1.

PS%	Total plasma PS	Plasma free PS antigen	Platelet PS antigen
Normal Individuals	119.27±17.28	110.36±16.6	101±29.95
Type I PS deficiency	62.4±7	37.04±11.97	66±31.7
Type III PS deficiency	85±13	41±13	80±37

Normal cut off: free PS antigen / total PS/Plts antigen: 70-120%

Table 1. PS plasma and platelet levels.

We observed that free, total and platelets PS in type I deficient individuals were lower than type III deficient individuals and both significantly lower than normal individuals. Platelets concentration of platelets PS in type I was lower than in and III deficient PS individuals. The levels of free plasma PS in type I and III PS deficiency was lower than in platelets PS.

No significant difference was detecting between platelets PS and total plasma PS levels in type I and III PS deficiency respectively (Fig 16).

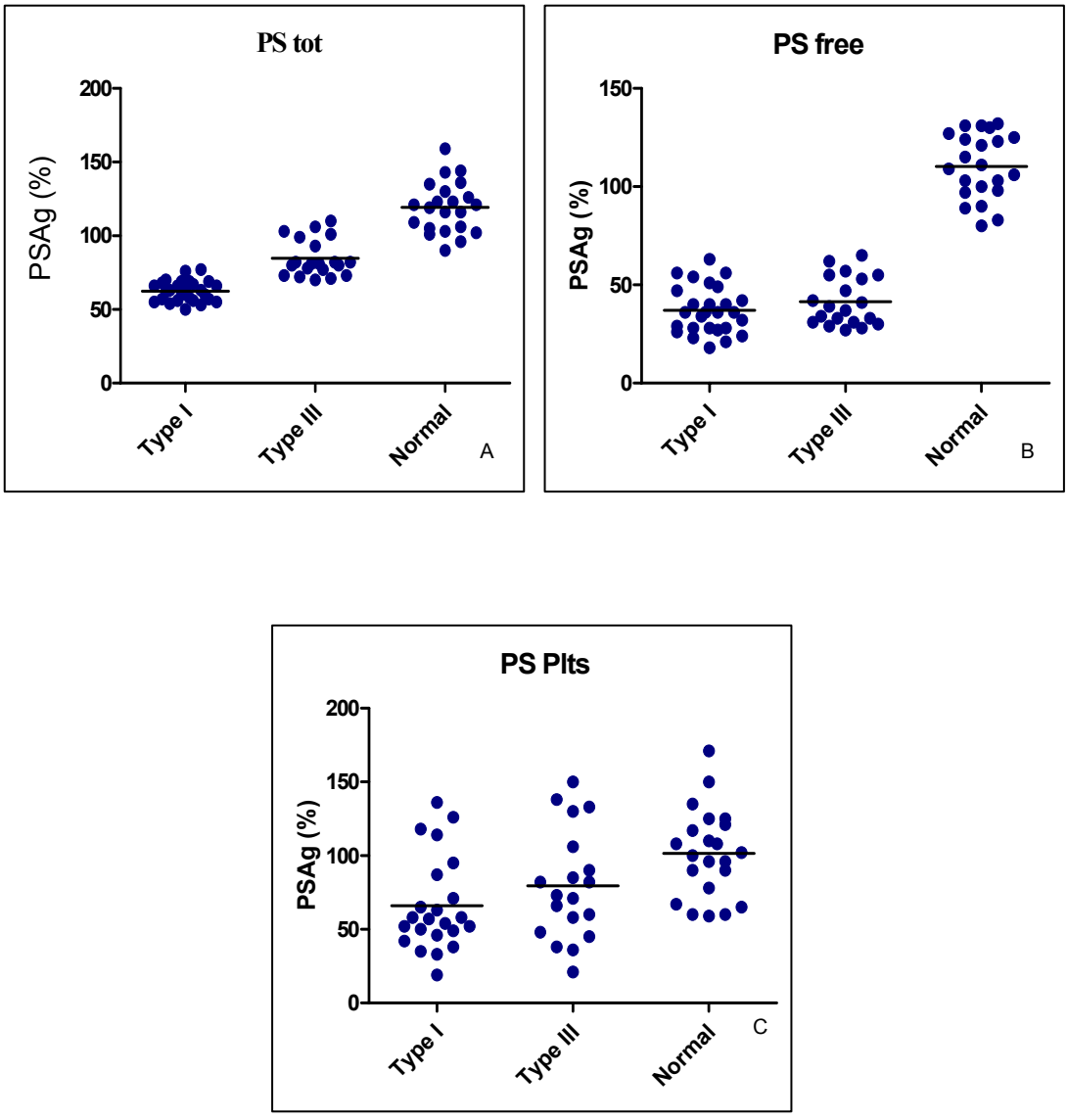


Fig 16. Plots of different distribution of total plasma (A), free plasma (B) and platelets (C) PS in normal, type I and type III PS defects subjects.

Analysis of plasma and platelet PS in normal and PS deficiency individuals.

Plasma free PS for western blotting analysis was purified through barium citrate absorption. Plasma free PS and platelets PS from normal subjects and with PS deficiency were analyzed by 5-15% SDS-PAGE. Wesrern blot demonstrate a single band of approximately 70 kD in normal subjects and in platelets. These results indicating, that PS in plasma has the same relative molecular mobility as in platelets PS. In PS deficiency (type I and Type III) patients blotting analysis give the same molecular mass of normal subjects (Fig 17). In one family two individuals with type III PS deficiency has in western blot two type of band, one of normal PS and the other with lower molecular weigh approximately of 65 kD (Fig 18). This results suggesting that these two patients have a variant PS. One of the possibility explanations for this observation would be the variant PS lacks one glycosylation site and have lower molecular mass, this type of PS variant called PS Heerlen. Because the T to C transition introduces a RsaI restriction site (GTAT to GTAC) in PS Heerlen allele, the mutation could be also followed with the restriction analysis. Enzyme restriction of amplified exon 13 demonstrated the present of Heerlen polymorphism in these subjects (Fig 19).

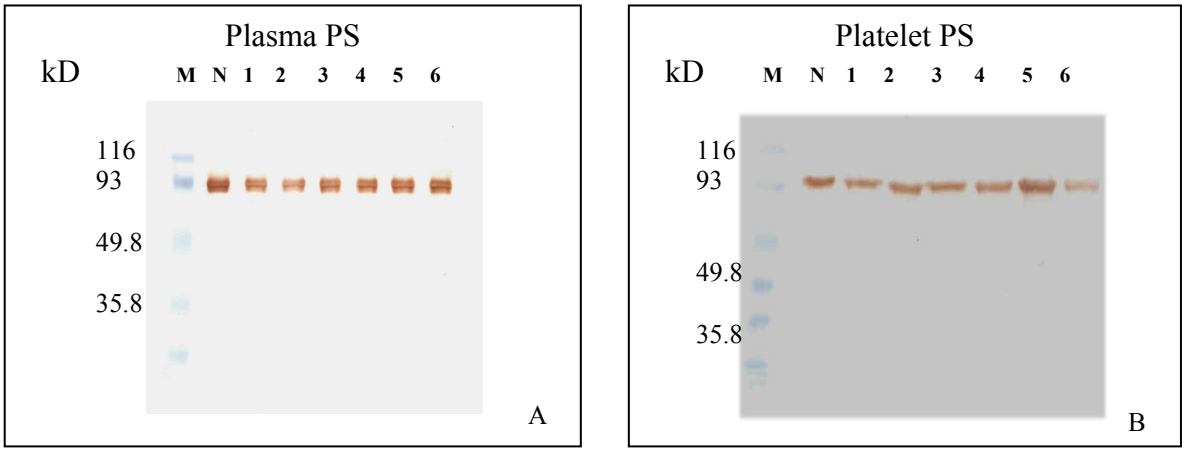


Fig 17. Immunoblotting of plasma and platelet PS. A, B) M: Markers; 1, 2, 3: type I PS deficiency patients; 4,5,6: type III PS deficiency patients. A) N: pooled normal plasma. B) N: pooled normal platelet.

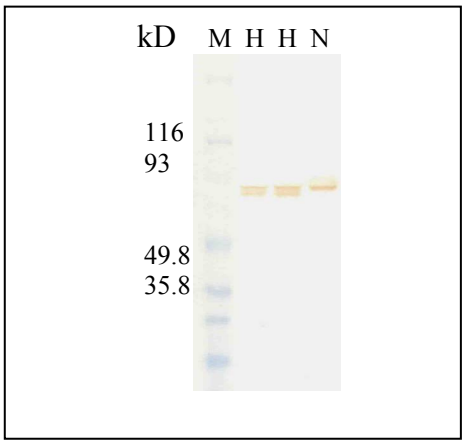


Fig 18. Western blotting of plasma PS, in patient's heterozygotes for type III PS deficiency and with the PS Heerlen allele. M: markers; H: PS Heerlen; N: pooled normal plasma.

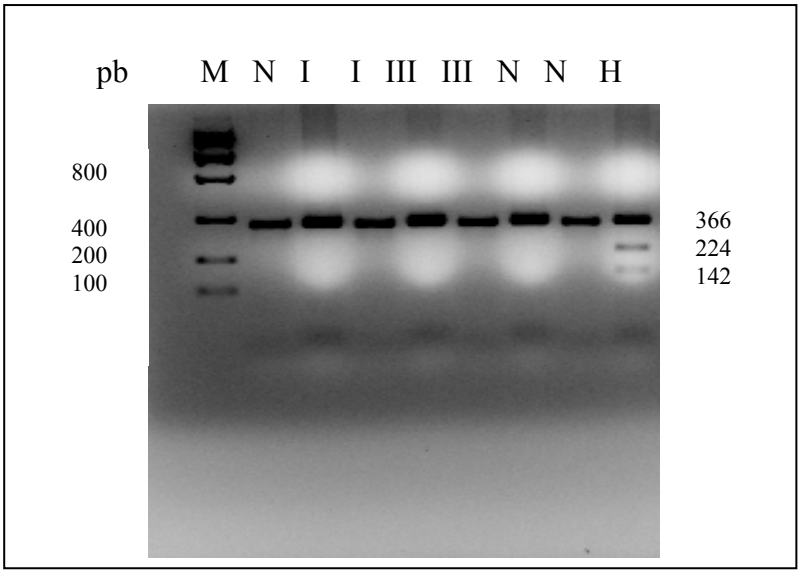


Fig 19. Agarose gel electrophoresis, 2% of Rsa I digest of the amplified exon 13. M: molecular weights; N: normal individual; I: type deficiency PS patients; III: type III deficiency PS patients; H: heterozygote for type III PS deficiency patient, with PS Heerlen variant. Numbers at the right of the gel indicate the calculated molecular weights in base pairs (bp) of the restriction fragments.

Effects of warfarin in plasma and platelets PS concentration

Protein S deficiency was associated with increased risk of thrombosis. The most common therapy in patients after recurrent episodes of thrombosis is with warfarin. Thirty five patients on anticoagulant therapy (warfarin) with 2-3 Prothrombin Time (PT)/International Normalized Ratio (INR) were enrolled after informed consent to study protein S levels. We observed the decreased of free PS levels and the important decreased of platelets PS levels. These results indicates that platelets progenitors cells may be influence in the treatment with warfarin (Table 2). Two patients with PS deficiency in treatment with PS at the moment of anlysis had 41±6 PS platelets levels. After a weak without treatment in warfarin the levels of platelets PS was normal 100±8 in both the patients.

PS%	Total plasma PS	Plasma free PS antigen	Platelet PS antigen
Normal Individuals	119.27±17.28	110.36±16.6	101±29.95
Patients treatment with warfarin	80.5±16	49.6±17	14±7

Table. 2. Normal cut off: free PS antigen / total PS/Plts antigen: 70-120%.

Effect of warfarin. and of vitamin K in megakaryocyte culture.

Because PS is a vitamin K dependent protein, the effects of synthesis of PS by megakaryocytes were examined by immunofluorescent staining. MKs treatment with warfarin (1μg/ml) decrease the levels of synthesis of PS in normal MKs and in patients with PS deficiency (Fig 20, 21). Whereas MKs treatment with high concentration of warfarin (5 μg /ml) decreased levels of PS endogenous synthesis whereas the nucleus of the cells lack the symmetric position, suggesting that warfarin is toxic for the cells. MKs treatment with vitamin K (1 μg /ml) increased the accumulation of PS in megakaryocytes and increased our polyploidization (Fig 22). The addiction of warfarin or vitamin K at the Mks culture in the concentration of 1 μg /ml after 3 days of cultures give the same results, decreased of PS synthesis by MKs when treatment with warfarin whereas increased after vitamin K treatment.

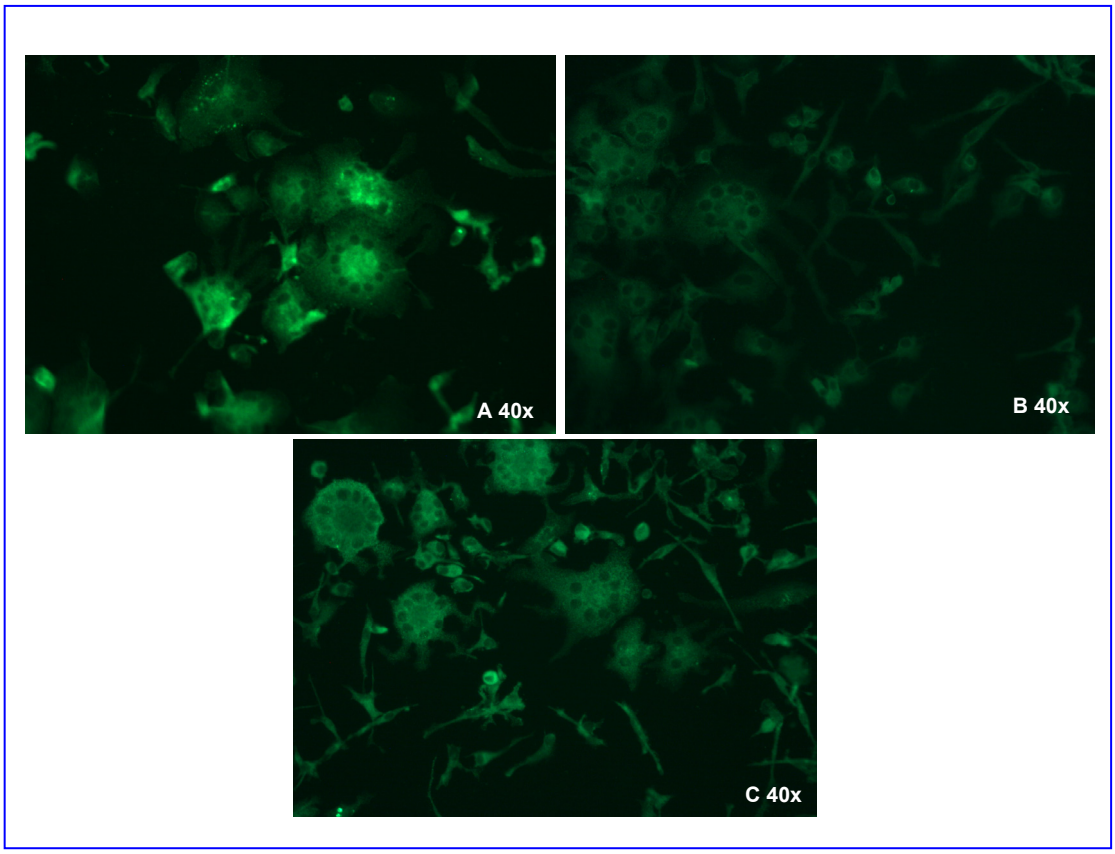


Fig 20. Mk of normal individual, staining with anti human PS antibodies. A) cells without treatment B) Cells treatment with 1 µg /ml warfarin, C) cells treatment with 1 µg /ml of vitamin K.

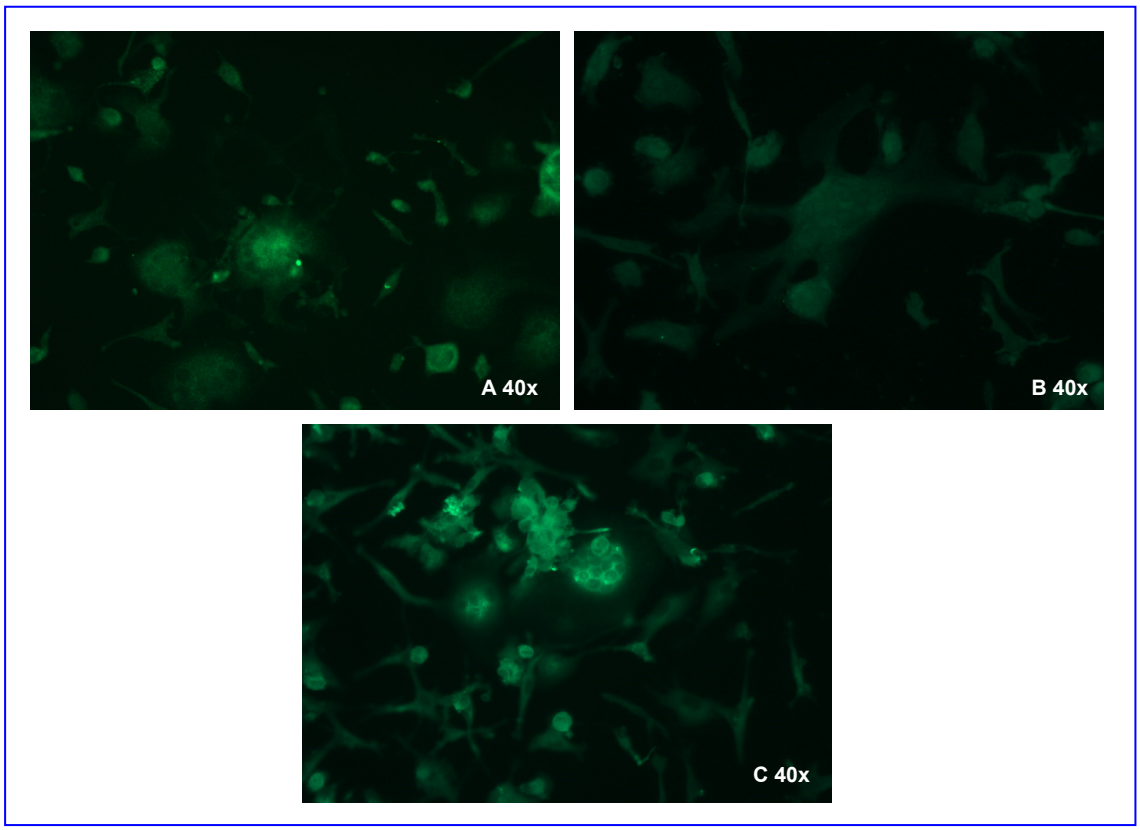


Fig 21. Fig 20. Mk of PS deficiency patient, labeling with anti human PS antibodies. A) Cells without treatment B) cells treatment with 1 µg /ml warfarin, C) cells treatment with 1 µg /ml of vitamin K.

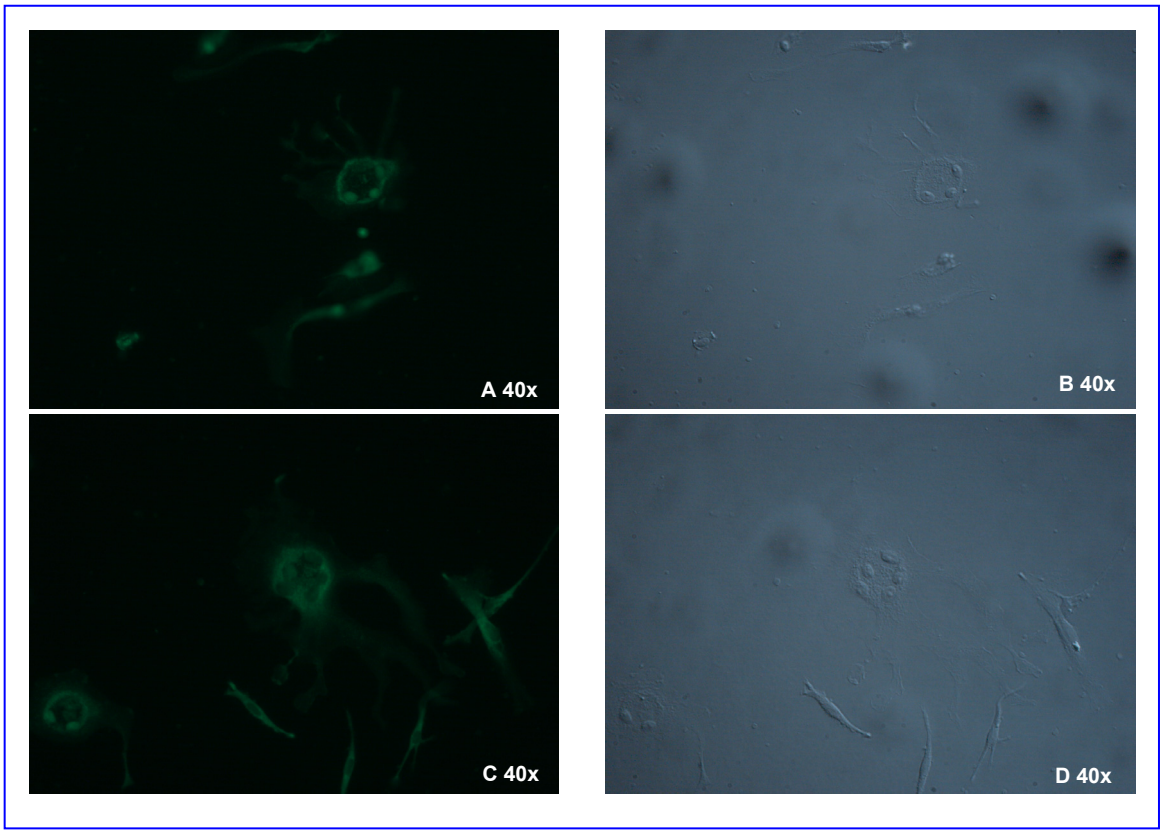


Fig 22. Normal megakaryocytes treatment with warfarin at the concentration of 5 μg/ml. At day 8 cells represented, a lower level of PS synthesis and the nuclei lacks the symmetric position on the cytoplasm. A, C) Cells were labeling with the anti human PS antibodies. B,D) Interference contrast.

DISCUSSION

In vitro model of human megakaryocytes cells

Haematopoietic stem cells were isolated, from whole blood, by histopaque system and were grown in a serum free medium. Cells were differentiating in a megakaryocytes lineage after the addiction in culture of TPO and IL 3. Haematoxylin eosin staining at day 5 of cell culture we observed many immature cells contain one or two nucleus then the cells undergo increased of their DNA contained through multiple rounds of incomplete mitosis termed endomitosis. In this stage cells synthesizes many cytoskeleton proteins such as αβ tubulin and γ tubulin and other organelles. The major important protein for the nucleation of these cells is the γ tubulin. The cells increased in cytoplasmatic volume and on number of nucleus resulting in a cell that content 4N, 8N chromosome. At 10 and 15 days of culture, we observed cells with high ploidy 16 N and 24 N. Therefore, maturation of cytoplasm content, cytoplasm begins to form cytoplasmatic extension. These thick pseudopodia elongate and form thin tubules called proplatelets similar form to the megakaryocyte cell. We analyzed these cells for the expression, at the different stage of cell culture, of a specific megakaryocyte protein the CD41. The 95% of the cells were positive since the early stage of growth for CD41 suggesting that these cells were indeed megakaryocytes. Our experimental conditions generate a fairly enriched megakaryocyte cells (> 95%), at the beginning with different stage of differentiations and at finally with an high polyploid nuclei.

Megakaryocyte cytoskeleton characterization and proplatelet formation

Platelet formation by megakaryocytes is a complex process that appears to be unique in cell biology. Maturation culminates in an exquisite series of events that ends the megakaryocytes life as it converts its entire cytoplasm first into proplatelets and then into platelets. Proplatelets elongate from the megakaryocyte using bundles of microtubules. The first insights into the cytoskeleton mechanics of platelets formation date from the work of Tablin et al, who showed that proplatelets elongation is dependent on microtubules. Our experiments demonstrate the formation of cytoplasmatic extension by multinucleated cells but as such from the mononuclear cells. Then the cytoplasmic spreads elongates and form large pseudopodia and subsequently elongate into thin tubules, called proplatelets. Proplatelets contain multiple swellings linked together by thin cytoplasmic bridges, the proplatelets frequently terminate as bulbous tips. These tubules undergo a dynamic bending and branching process and at finally release new form platelets. At the end of the platelets formation megakaryocyte is transformed into residual naked nuclei. We observed that the major structural proteins involved in these mechanisms of proplatelets formation and platelets release are α and β tubulin. Antitubulin immunofluorescent staining reveals the presence of these proteins in the cytoplasm of the cells since the early stage of develop and in the proplatelets elongation. Proplatelets ends have microtubule bundles arranged into teardrop-shape loops containing microtubules coils. Our experiments support the theory of Italiano J. who proposed the mechanism for platelet production “The flow

model of proplatelet formation", that hypothesis that platelets derived from the proplatelets elongation by megakaryocytes.

Protein S expression in megakaryocytes culture

Megakaryocytes (Mk) derived from normal individuals we analyzed for the expression of different coagulation proteins. Mk were staining for anti human protein S at different days of culture and we observed the positive of these cells for protein S. This results demonstrate, that megakaryocytes, can synthesize coagulation protein S. PS was localized in the cytoplasm and in forming proplatelets of megakaryocytes and in proplatelets area of fully mature Mk. Mk isolated from PS heterozygous deficiency patients, (type I or type II), was labeled for anti human PS antibodies and we observed, the presence of PS. PS was similar localization and the same morphological characteristics of Mk, derived from normal subjects. We analyzed the expression of coagulation factor V (FV) and protein C in cultured Mk. Cells was labeled with anti human FV-FITC and anti human PC-HRP. Mk was positive for FV in contrast PC was not present as expected, because platelet does not contain protein C.

Studies of plasma and platelet PS, in normal and protein S deficiency subjects.

To study plasma and platelet PS we unrelated 80 individuals from 15 families with PS deficiency (type I and III). All the individuals were heterozygous from PS deficiency. In all the subjects were determined total plasma, free plasma and platelets PS levels. The results indicate that type I

and III subjects total and plasma free PS_{Ag} levels were lower than normal individuals. Intra-platelets PS_{Ag} levels in type I and type III were lower than of healthy individuals. Our analysis demonstrates a strict correlation between total and free plasma PS and Plts PS. The reduction of platelet PS mirrors the reduced levels of free and total PS_{Ag} present in carries of the defect even though PS levels in Plts appears unexpectedly higher than the free PS counterpart.

We analyzed with immunobloting assays purified free plasma PS and platelets PS. Wesrern blot demonstrate a single band of approximately 70 kD in normal subjects and in platelets. These results indicating, that PS in plasma has the same relative molecular mobility as in platelets PS. In PS deficiency (type I and Type III) patients blotting analysis give the same molecular mass of normal subjects. In one family two individuals with type III PS deficiency has in Western blot two type of band, one of normal PS and the other with lower molecular weigh approximately of 65 kD. This results suggesting that these two patients have a variant PS. We studies in plasma the presence of a variant PS called PS Heerlen. Through RFLP assays we observed in these two patients the present of Heerlen polymorphism.

Effects of warfarin and vitamin K in patients and in megakaryocyte culture

We study the interaction of anticoagulant drugs on PS_{Ag} levels on 35 patient treatments with warfarin. The levels of total and free plasma PS decreased during treatment with oral anticoagulant, since PS is a vitamin K-dependent protein. Our study demonstrated significant decreased levels of platelet PS respectively plasma free and total PS. We valuated the effect

of anticoagulant drugs (warfarin) and of vitamin K on Mk cells. The Mk were treatment with 1µg/ml of warfarin or 1µg/ml of vitamin K and analyze synthesis of PS. We observed decreased PS synthesis on MKs with warfarin than control MKs; on the contrary, MKs cultured under vitamin K treatment increase PS synthesis. Treatment of culture with high levels of warfarin demonstrated toxic for the cell culture.

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