The origin of platelet fibrinogen is controversial. It may arise either exogenously by endocytosis of plasma fibrinogen, or endogenously by synthesis. We explored the second possibility because we previously demonstrated that the first mechanism does occur. Fibrinogen synthesis by human megakaryocytes (MK) was investigated by in situ hybridization and the polymerase chain reaction (PCR) applied to mRNA. MK differentiating from narrow CFU-MK were cultured in suspension. In situ hybridization using the 32P α and β fibrinogen chain antisense riboprobes was totally negative in MK in comparison with negative controls (λ phage and α and β fibrinogen chain sense riboprobes). In contrast, synthesis of fibrinogen was detected by this technique in a hepatoma cell line (HepG 2). Furthermore, mRNA for α and β chains of fibrinogen was not detected by the PCR performed on mRNA from cultured MK enriched to 90% purity, by the immunomagnetic bead technique, even after Southern blotting of the amplified products. In addition, fibrinogen mRNA was undetected in narrow MK and in platelets by the same technique, whereas a specific megakaryocyte gene transcript (GPβc) was easily detected. These observations demonstrate that the only mechanism responsible for the presence of fibrinogen in platelets is endocytosis of fibrinogen from plasma.

We attempted to determine whether human MK synthesize Fg by two approaches: (a) in situ hybridization of cultured human MK, and (b) polymerase chain reaction (PCR) of RNA from purified fractions of MK and from platelets. Using these two sensitive techniques, we could not detect Fg mRNA in MK and platelets, demonstrating that platelet Fg has its origin exclusively in the plasma.

Fibrinogen is not synthesized by human megakaryocytes

By Fawzia Louache, Najet Debili, Elisabeth Cramer, Janine Breton-Gorius, and William Vainchenker

Platelets contain numerous proteins stored in their α-granules that are secreted after platelet activation. Until recently, all these proteins believed to be synthesized by megakaryocytes (MK). Clearly, however, some plasma proteins also present in platelet α-granules such as albumin and immunoglobulins are taken up by MK from the plasma and incorporated into α-granules. Fibrinogen (Fg), a plasma protein involved in platelet aggregation and blood clot formation, is a major α-granule protein. This protein consists of three chains, α, β, and γ, coded by separate genes. Plasma Fg is synthesized by hepatocytes. Several reports have indicated that purified preparations of MK from guinea pigs, rats, and humans synthesize Fg. Therefore, platelet Fg has been suggested to have an origin different from that of its plasma counterpart. This hypothesis was further supported by the fact that among the three γ chains arising by alternative splicing of the same precursor RNA and by posttranslational modification, only the γ5 chain is present in significant amounts in platelets. However, Handagama et al. recently provided evidence showing that plasma Fg is taken up by platelets and MK and is subsequently stored in α-granules. First, intravenous (IV) administration of Fg in guinea pigs resulted in its rapid uptake by MK and storage in α-granules. The same result was obtained in a patient with congenital afibrinogenemia. Furthermore, 24 hours after such an infusion, most platelets contained Fg. Therefore, not only MK but also platelets were assumed to take up Fg. Second, after injection of rats with anercod, which degrades plasma Fg, only a small amount of Fg was detected in platelets and MK. Third, in vitro, Fg is detected at the end of MK maturation later than endogenously synthesized α-granule proteins such as von Willebrand factor (vWF). It is present in MK α-granules only when Fg is added to the culture medium. Finally, MK from a patient with congenital afibrinogenemia accumulated Fg in their α-granules after addition of normal plasma to the culture medium, demonstrating endocytosis of exogenous Fg.

Therefore, these different studies clearly show that some of the Fg contained in α-granules of MK obtained either directly from the marrow or from culture is endocytosed from the plasma. When previous results were taken into account, however, platelet Fg apparently had two origins, ie, exogenous (plasma) and endogenous (synthesis).
In PCR, 90% of MK. Human platelets were isolated from healthy adult calcium-free phosphate-buffered saline (PBS) containing 13.6 mmol/L Na citrate, 11.1 mmol/L glucose, 1 mmol/L adenosine; 2 mmol/L theophylline, 2.3 $10^{10}$ mol/L prostaglandin E1, (pH7 and 300 mosm/L) (MK medium), cytocentrifuged on glass slides for 1 minute at 700 rpm and fixed in 4% paraformaldehyde in phosphate buffer pH 7.4 for 3 minutes. Slides were then stored in 70% ethanol until use.

Probes and hybridization. cDNA of 920 and 670 base pairs (bp), respectively, were isolated from 60E1 and 58B clones (a gift from Dr G. Marguerie, Grenoble, France) and were subcloned into the pGEM 4 vector (Promega Biotech, Madison, WI). RNA probes were produced from the sense and anti-sense strands of the fragments using $^{32}$P-UTP (Amersham, Les Ulis, France). Full-length transcripts were hydrolyzed to a mean size of 150 bp and purified. This procedure generated probes with a specific activity of about 1 $10^{9}$ cpm per microgram of RNA. As a positive control and controls for non-specific binding identically labeled and sized anti-sense murine $\beta$-actin (a gift from Dr F. Dautry, Villejuif, France) and $\lambda$-phage were hybridized in the same experiments.

The technique of in situ hybridization was largely derived from the work of Harper et al.29 Probe (10 $^6$ cpm) contained in 20 $\mu$L methionine and was hybridized to cells at 52°C for 16 hours. Slides were washed sequentially in three baths at 52°C (a) 2xSSC, (b) 2xSSC/50% formamide, (c) 1xSSC) Slides were then dehydrated in ethanol until use.

**PCR**

Total cellular RNA were extracted by the guanidinium isothiocyanate method.32 PCR was run essentially as described previously.22 100 ng of total RNA was reverse-transcribed by 10 U avian myeloblastic virus reverse transcriptase (Promega Biotech) for 40 minutes at 42°C using anti-sense oligonucleotides as primers (Table 1) in a final volume of 50 $\mu$L. One half of the resulting cDNA was amplified in a final reaction volume of 100 $\mu$L containing 10 mmol/L Tris-Cl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 0.01% gelatin, 2 mmol/L of each deoxynucleotide triphosphate, 70 mmol/L of each primer (Table 1), and 1 U TAQ polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification was performed on a Perkin-Elmer Cetus PCR 1000 DNA thermal cycler for 35 cycles. Each cycle consisted of heating at 94°C for 1 minute, cooling to 55°C for 1.5 minutes, and heating to 72°C for 1 minute. 10-$\mu$L aliquots of each resulting reaction mixture were applied to 2% agarose gel, electrophoresed, and visualized by Southern blot hybridization with an oligonucleotide probe specific for the amplified fragments labeled with $^{32}$P (Amersham).

**RESULTS**

To assess whether Fg is synthesized by MK, we performed in situ hybridization on MK obtained from in vitro culture of marrow CFU-MK. We used $^{35}$S-labeled anti-sense RNA synthesized from cloned $\alpha$ and $\beta$ Fg chain cDNA. MK were studied at day 12 of culture because previous studies have shown that their content of Fg increases with terminal differentiation.32 MK were identified by their morphology after May-Grunwald-Giemsa staining. As shown in Fig 1A and B, no hybridization was observed with either the $\alpha$ or $\beta$ anti-sense probes in more than 500 MK analyzed. The number of silver grains per MK was less than five. A similar low background was observed both with the two sense probes and the $\lambda$-phage probe (Fig 1D). No significant hybridization was observed even when preparations were autoradiographed for more than 2 weeks.

Two positive controls were used. First, MK preparations were hybridized with the anti-sense murine $\beta$-actin RNA probe (Fig 1C) and a strong signal was observed in all MK. Second, cultured human hepatoma cells (HepG2) were used to test the probes against the $\alpha$ and $\beta$ Fg chains. As shown in Fig 2A and C, mRNA for $Fg$ and $\beta$ chains were detected in HepG2 cells. This hybridization was very heterogenous, and about 30% of the cells exhibited a strong signal (> 40 silver grains). After IL-6 induction of HepG2 for 3 days, the signal markedly increased and nearly all cells strongly hybridized with the two probes. In contrast, no significant hybridization (less than five silver grains) was observed with either the two sense probes (Fig 2B and D) or the $\lambda$-phage probe (data not shown).

**PCR**

The lack of synthesis of Fg by the platelet/MK lineage was further confirmed by the PCR after reverse transcription of mRNA. MK from culture were purified by the immunomagnetic bead technique. This technique yielded MK with high purity, which was difficult to quantitate.

**Table 1. Sequences of Oligonucleotide Primers and Probes for PCR**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Sense 5' to 3'</th>
<th>Anti-sense 5' to 3'</th>
<th>Probe</th>
<th>RNA Fragment Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fg $\alpha$ chain</td>
<td>CCAGAGTGGAAGGCGATTA</td>
<td>TCCAGTTCCAGAGCTCCC</td>
<td>GAGGAGCTGCACTGTG</td>
<td>291</td>
</tr>
<tr>
<td>Fg $\beta$ chain</td>
<td>CAGAGTCTCAAGGCTGCAATGAGGAG</td>
<td>CAGGCTCAGGCTGGAGGACTGCAACCTG</td>
<td>CCTGCCCCACCCCTATC</td>
<td>216</td>
</tr>
<tr>
<td>GPIb$\alpha$</td>
<td>GACGGAGCTGCGAGGTGACG</td>
<td>TTTGAGACCTGAGATG</td>
<td>GCTCTAGCTGTCTTCTC</td>
<td>138</td>
</tr>
</tbody>
</table>

For Fg$\beta$ and GPIb$\alpha$, primer sequences are separated by an intron in their respective gene.*32; therefore, the length of the DNA amplification products is different from that of RNA.

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ABSENCE OF FIBRINOGEN mRNA IN MEGAKARYOCYTES

Fig 1. Absence of Fg mRNA in cultured human MK studied by in situ hybridization. MK were cultured for 12 days in suspension from bone marrow and studied by in situ hybridization: 35S anti-sense Fg α strand (A), 35S anti-sense Fg β strand (B), 35S anti-sense β-actin strand (positive control) (C), and 35S λ-phage probe (negative control) (D). No specific hybridization was observed on cultured MK with the two Fg probes.

however, because of cells clustered around beads. After purification, cells were labeled by a polyclonal antibody against vWF and more than 90% of the cells were clearly stained. RNA was extracted from these preparations, from purified platelets, and from a fraction of MK obtained directly from marrow and enriched by Percoll density gradient; in one case, marrow MK were further purified to 90% purity by the immunomagnetic bead technique.

No amplification products for the α and β Fg mRNA chain were observed either in MK or platelets (Fig 3A and B). In contrast, HEPG2 cells yielded the 291- and 216-bp amplification products to Fg α and β chain mRNA, respectively. Furthermore, Fg mRNA was not detected by this technique in the preparation with 90% marrow MK (data not shown).

As another control, amplification of the GPIbα mRNA (a gene specific of the MK lineage) was performed. This mRNA was detected in preparations from MK and platelets but not from the HepG2 cell line (Fig 3C).

To determine the sensitivity of the technique, HepG2 RNA was serially diluted and RNA was amplified for the Fg β chain. The threshold of sensitivity corresponded to 10 pg RNA, which, in our experiments, is the equivalent to the RNA present in three HepG2 cells (Fig 4).

DISCUSSION

Platelet α-granules contain numerous proteins. Some are specific or restricted to the platelet-MK line, such as platelet factor 4 or vWF, and are synthesized by MK. Other proteins, present in large amount in the plasma, such as albumin and immunoglobulins, are endocytosed and incorporated into α-granules. In contrast, the origin of platelet Fg remains controversial: on the one hand, several reports have provided evidence for its synthesis by MK; on the other hand, several experiments have demonstrated that plasma Fg can be endocytosed into MK. Therefore, platelet Fg appeared to have two origins, one exogenous and the other endogenous. In this study, however, we were unable to detect synthesis of the α and β chains of Fg using in situ hybridization in MK and the PCR applied to mRNA from MK and platelets.

No α or β Fg chain mRNA could be detected by in situ hybridization in cultured human MK. This technique is particularly sensitive because it clearly permits detection of Fg synthesis by uninduced HEPG2 cells (hepatoma cell line). Previous studies have shown that uninduced HEPG2 cells synthesize little Fg. Induction of HEPG2 by IL-6 resulted in a 10-fold increase in Fg synthesis. A similar
Detection of Fg mRNA in the Hop02 cell line by in situ hybridization: 35S anti-sense Fg α strand (A), 35S sense Fg α strand (negative control) (B), 35S anti-sense Fg β strand (C), and 35S sense Fg β strand (negative control) (D). A specific hybridization was observed in about 30% of uninduced Hop02 cells.

phenomenon was also observed by in situ hybridization because the number of positive cells increased from 30% to 100% after IL-6 induction. In addition, the number of silver grains per cell increased. In contrast, in MK, no specific hybridization in comparison to the sense RNA probe was observed, even after a long exposure of 2 weeks.

To confirm this result, cultured MK were purified by the immunomagnetic bead technique, which results in a purity of more than 90%. mRNA for the α and β chain transcripts was studied by PCR. No sequence corresponding to the mRNA was detected after amplification, even after Southern blotting of the amplification products with a specific oligonucleotide. In contrast, using this technique, Fg mRNA was detected in the HEPG2 cell line; conversely, mRNA of GPIb α was easily detected in the MK preparation. We also studied MK purified directly from marrow and platelets and were also unable to detect Fg transcripts by PCR. This technique of amplification is very sensitive, much more so than Northern blotting, which was previously used to detect synthesis of Fg by MK.7 Its threshold of sensitivity is difficult to establish precisely, but we could detect α- or β-chain transcripts in an amount of RNA equivalent to that extracted from three HEPG2 cells. Because mRNA from 5 x 10⁴ MK was used in each amplification reaction, one can calculate that MK express a quantity of Fg mRNA of less than 0.006% of that of a single HepG2 cell. This threshold of detection corresponds to less than one Fg transcript per MK. This absence of synthesis by cultured MK is in agreement with results of another study that did not detect Fg synthesis by MK.26 Furthermore, in previous studies, we were unable to visualize the presence of Fg, in contrast to vWF, in the Golgi apparatus by an immunoultrastuctural technique.12

Three hypotheses may explain the discrepancies with previous results. First, MK may synthesize Fg only after induction by a cytokine. In the present study, however, Fg was not synthesized by aplastic plasma-stimulated MK. The culture supernatant of these cultures contain large amounts of IL-6, the main inducer of Fg synthesis in hepatocytes,27 which is endogenously produced in culture (unpublished observations). Second, there may be differences in synthesis of Fg among species. In another recent study, however, the researchers were unable to detect Fg mRNA from murine and guinea pig MK by PCR.28 Third, a marrow cell type other than MK might be able to synthesize Fg, and these cells might have contaminated the fractions enriched in MK used in previous studies. Handagama et al17 reported that some marrow macrophages, osteoclasts, and endothelial cells were labeled by an anti-Fg antibody. Therefore, this
ABSENCE OF FIBRINOGEN mRNA IN MEGAKARYOCYTES

last hypothesis appears to be the one that most likely explains the discrepancies among investigators.

The mechanisms responsible for Fg endocytosis remain unknown; however, it may be mediated by the GPIIb/IIIa complex, which is the Fg receptor on platelets. This complex is present on both the plasma and α-granule membranes, and there is a recycling pool. Patients with severe Glanzmann’s thrombasthenia type I have a deficiency of both this complex and platelet Fg. Also in favor of this hypothesis is the observation that among the γ chains of Fg only the γ6 has a high affinity for the GPIIb/IIIa complex. This may lead to preferential endocytosis by MK of one species of plasma Fg and may explain the absence in platelet of the abnormal Fg in the dysfibrinogenemia Paris I, which abnormality involves only the γ' chain.

Our study shows that in contrast to platelet factor 4 or vWF, Fg is not synthesized by human MK or platelets and is only endocytosed. This mechanism of uptake and storage of proteins by cells may be more general than was previously believed. A similar mechanism may be found for other proteins and other types of cells.

NOTE ADDED IN PROOF

After the revision of this manuscript, a work was published showing that albumin, IgG, and fibrinogen are not synthesized by rat and mouse megakaryocytes (J Clin Invest 86:1364, 1990).

ACKNOWLEDGMENT

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