Megakaryocyte and Hepatocyte Origins of Human Fibrinogen Biosynthesis
Exhibit Hepatocyte-Specific Expression of γ Chain-Variant Polypeptides

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The γ chain of human fibrinogen is heterogeneous in length at the C-terminus due to differential RNA processing of the γ chain-gene primary transcript. We have produced two specific monoclonal antibodies (MoAbs) against the γ chain epitopes generated by this alternative processing event: anti-γ57.5,55.5 (L2B), which reacts with γ57.5 and γ55 chains, and anti-γ50,55.5 (H9B7), which reacts preferentially with γ50 chains. Using these MoAbs, we have studied the expression of γ-chain polypeptides by immunofluorescence microscopy in the tissues of fibrinogen biosynthesis and have determined that γ57.5 polypeptide is expressed in hepatocytes but is absent or present in significantly reduced amounts in megakaryocytes. Therefore, the γ50 chain is found in plasma, platelet, and megakaryocyte fibrinogens, but the γ57.5 chain is found only in plasma fibrinogen. The C-terminal amino acid sequence of γ55 includes the L2B epitope 57.5-55.5. Using MoAb L2B we have determined that γ55, which is a post-translationally modified γ57.5 chain, is found only in plasma fibrinogen and is absent or present in markedly reduced amounts in platelet or megakaryocyte fibrinogen. In addition, the conformation of the L2B epitope is preserved in γ55, as determined by Western blot analysis. The hepatocyte-specific expression of the γ57.5-chain polypeptide and the post-translational modification to γ55 results in a compartmentalization of γ-chain polypeptide expression. This is suggestive of different mechanisms regulating human fibrinogen γ-chain gene expression in hepatocytes vs megakaryocytes that may operate in a tissue-specific manner at the level of 3' RNA processing events.

IN BOTH THE HUMAN and the rat, fibrinogen is composed of three nonidentical polypeptide chains, each of which exist in pairs, Aα, Bβ, and γ. The γ chains of human fibrinogen are heterogeneous in charge and mol wt because of differences in the amino acid sequence of the C-terminus. Rat and human γ chain genes each exist in single copy and are composed of ten exons and nine introns. Differential RNA processing of the γ chain primary transcript generates two distinct γ chain messenger RNAs (mRNAs) in each species. The cloning of two γ chain cDNAs from liver mRNA demonstrated that they encode the γ50 and γ57.5 polypeptide chains in the human. (The smallest γ chain has been termed γ4, γA, and γ50; the largest γ chain: γA, γB, and γ57.5, and the intermediate size γ chain: γ55.) In this report we will use the terminology γ50, γ55, and γ57.5 to distinguish human and γA and γB to distinguish rat γ-chain variants.) and the γA and γB polypeptide chains in the rat. The human γ55 polypeptide chain is generated by a proposed post-translational modification of the γ57.5 chain. The overall amino acid homology between the human and rat γ chains is >83%; however, the differential RNA processing mechanisms differ. In the human, differential polyadenylation site selection as well as alternative splicing produce the γ50 and γ57.5 mRNAs, whereas in the rat, alternative splicing alone produces the γA and γB mRNAs. The alternatively spliced γ-chain gene product, fibrinogen γ57.5, including the post-translationally modified fibrinogen γ55.5, is a relatively abundant plasma protein approximating one tenth of total fibrinogen, or 30 mg/dL, yet its function has not been determined.

The C-terminal sequences of the γ chain where this alternative splicing event occurs are involved in platelet aggregation. Two features distinguish γ57.5 fibrinogen from γ50 fibrinogen. First, only γ50 chains are found in fibrinogen stored in platelet α granules, whereas fibrinogens containing all three forms of γ chain circulate in plasma. Rat platelet (γA only) and rat plasma fibrinogens (γA and γB) display a similar compartmentalization of γ chain type. Second, γ57.5 fibrinogen is functionally less effective than γ50 fibrinogen in supporting adenosine diphosphate (ADP)-induced platelet aggregation when the Aα C-terminus is partially degraded, although platelet γ50 and plasma γ50 fibrinogens are similar in their ability to support platelet aggregation and in their affinity for the activated platelet fibrinogen receptor.

We report here the production of specific monoclonal antibodies (MoAbs) against C-terminal sequences of the γ55 and γ57.5 chain (L2B), the C-terminus of the γ50 chain (H9B7), and a site in common with the γ50, γ55, and γ57.5 chains of human fibrinogen (J88B). Using these MoAbs we determined that the human-fibrinogen γ57.5 polypeptide (and perhaps γ55 if post-translational modifications occur intracellularly) is expressed in hepatocytes but not in megakaryocytes and that platelet fibrinogen does not contain the post-translationally modified γ55 chain.

MATERIALS AND METHODS

Cells and cell culture conditions. Human marrow and whole blood was obtained from consenting adults in accordance with the Committee on Human Subjects Approval. Marrow concentrate...
Fig 1. C-terminal nucleotide and amino acid sequences of fibrinogen γ50 and γ57.5 chains. Upper bar shows exon (open) and intron (stippled) organization at the 3' end of the γ chain gene. Nucleotide (nt) numbers above the bar indicate the exon/intron junctions and the locations of polyadenylation signals, which are underlined. The nt sequences of γ50 and γ57.5 mRNAs are shown below with the numbers above the sequences indicating amino acid position. The γ50 mRNA is generated by splicing of ten exons (exons I-X) and polyadenylation at the downstream polyadenylation signal at nt 8499. The γ57.5 mRNA is generated when intron I is not spliced out, and the upstream polyadenylation signal at nt 8061 is used. The post-translational modification of γ57.5 to γ55 occurs after Pro-423. The boxed amino acids indicate those selected for synthesis of the γ57.5 vaccine peptide (MoAb L2B) and the overlined amino acids indicate those of the γ50 vaccine peptide (MoAb H8B7; sequence taken from Chung and Davis\textsuperscript{39}). Note: MoAb H8B7 binds to γ chain sequences upstream of residue 400 and therefore binds to all three γ chain polypeptides.
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from eight individuals was prepared by centrifugation at 2,500 rpm in a clinical centrifuge for ten minutes at room temperature (RT), and platelets were prepared as described. Platelets were smeared onto glass coverslips, marrow concentrate was smeared onto glass slides, and cells were fixed in formalin for immunofluorescent staining. Washed platelets used for Western blot analysis were immediately lysed by boiling in gel-electrophoresis loading buffer. HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown under culture conditions as described. HepG2 cells for immunofluorescence microscopy were grown on glass coverslips and fixed in formalin.

Immunofluorescence microscopy. Goat antiserum (IgG+ IgM) fluorescein isothiocyanate (FITC–)–conjugated (Boehringer Mannheim Biochemicals, Indianapolis) was used as the secondary antibody for immunofluorescence microscopy as described.

Synthetic peptides. The Hopp and Woods hydrophilicity analysis was performed with an averaging length of six amino acids, which is the number of residues recommended for prediction of protein antigenic determinants. The hydrophilicity analysis of the C-terminal sequence of the γ57.5 chain indicated that the unique sequences are predominantly hydrophilic and, therefore, potentially antigenic (not shown). The amino acid sequence and exon/intron organization of the C-terminus of the human fibrinogen γ chain gene is shown in Fig 1, from which the numbering of the amino acids of the following γ-chain–specific synthetic peptides were assigned: γ57.5γ397.4γ1, Y50KQAGDV, and γ50γ391.1 γYKQAGDV, which were synthesized by Applied Biosystems (Foster City, CA); γ50γ391.1 γQGQHGLGGAKQAGDV was purchased from Bachem Inc (Torrance, CA); and γ50γ391.1 γdodecapeptide: HHLGGAKQQGDV was purchased from Peninsula Laboratories, Inc (Belmont, CA).

Immunization, fusion, and cloning. The peptide γ57.5γ391.1 or γ50γ391.1 was coupled to a carrier, BSA (Sigma, St Louis), using 0.5% glutaraldehyde as described. Sensitized spleen cells from BALB/c mice (Jackson Labs, Bar Harbor, ME) were fused to mouse myeloma cell line X63-Ag8.653 according to manufacturer's instructions. Forty milligrams of IgG1 was purified from 5 mL of ascites fluid and was dialyzed against phosphate-buffered saline (PBS). Rabbit antihuman fibrinogen antiserum was purchased from Dako Corporation (Santa Barbara, CA).

The anti–γ-chain MoAbs selected from the peptide-conjugate fusions were L2B (anti-γ57.5γ391.1 and H9B7 (anti-γ50γ391.1). L2B recognizes γ57.5γ391.1 sequences as well. H9B7 was produced as a control MoAb for comparison of expression of γ50 to γ55/57.5 chains. It was desired to have a C-terminal control MoAb to address the possibility of buried epitope(s) in the γ chain C-terminal sequences. An additional anti–γ–chain MoAb produced against fibrin degradation products, J88B, recognized a site in common with γ50, γ55, and γ57.5 chains. L2B and J88B are IgG1 isotype MoAbs, H9B7 is an IgM isotype, and all three possess κ light chains. The isotype of MoAbs was determined with an isotyping kit purchased from Bio-Rad.

Western blot analysis. Purified fibrinogens γ50, γ55 and γ57.5; purified reduced and S-carboxymethylated γ50 and γ57.5 chains; pooled plasma fibrinogen; or washed platelet lysate were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7% gels. Western blot analysis was performed with modifications. Undiluted MoAb culture supernatant or a 1:2,000 dilution of rabbit antihuman fibrinogen was used in the primary antibody step. Rabbit antiserum Ig-polyvalent or goat antirabbit IgG conjugated to alkaline phosphatase (SIGMA) secondary antibody was followed by 5-bromo-4-chloro-3-indolyl phosphate with 1% nitroblue tetrazolium (SIGMA) substrate for color development.

RESULTS

Characterization of anti-γ57.5/γ55 chain MoAb L2B and anti-γ50 chain MoAb H9B7. To determine the γ-chain specificity, the MoAbs were analyzed by differential ELISA against a panel of γ-chain synthetic peptides and Western blot against reduced and denatured fibrinogens. MoAb L2B reacted only with fibrinogen γ57.5, γ57.5γ408.413, and the peptide γ50/γ57.5γ408.413 in which residues γ400–γ402 are the same in both γ50 and γ57.5 and residues γ408.413 are unique to the γ57.5 chain (Fig 1). MoAb H9B7 reacted with fibrinogens γ50 and γ57.5 (which contain equivalent amounts of γ50 and γ57.5 chains) as well as γ50-containing synthetic peptides. The differential ELISA data suggested that H9B7 reacts preferentially with γ50 chains in that the reactivity of this MoAb was strongest with synthetic peptides containing the γ50 C-terminal residues γ50γ408.413. J88B did not react by ELISA with any of the γ50 or γ57.5 C-terminal synthetic peptides but reacted equally well with all fibrinogens. To confirm the specificity and to determine if the L2B epitope is preserved in γ55 chains, the γ chains of purified fibrinogens γ50, γ55, and γ57.5 were separated by SDS-PAGE (Fig 2, left panel) and analyzed by Western blot, which showed that MoAb L2B reacted equally with γ55 and γ57.5 chains but not with γ50 chains (Fig 2, right panel). Western blot analysis using H9B7 and J88B indicated that each MoAb recognized both γ50, γ55 (not shown), and γ57.5 chains.
Fig 3. Western blot analysis using MoAbs H9B7 (middle) and J88B (right) of chromatographically purified human plasma fibrinogens or reduced and S-carboxymethylated γ chains (left). Lane 1, γ50 chain; lane 2, γ57.5 chain; lane 3, fibrinogen γ50; lane 4, fibrinogen γ57.5. The migration positions of the polypeptide chains of fibrinogen (Aα, Bβ, γ50, and γ57.5) are indicated. Note that the migration of purified γ chains is shifted to higher mol wt after reduction and S-carboxymethylation.

(Fig 3), although H9B7 appeared to bind more avidly to the γ50 chain (Fig 3, middle panel).

Post-translationally processed γ55 chains are not found in platelet fibrinogen. Previous reports were unable to determine if γ55 chains were found in platelet fibrinogen due to the limits of detection of the techniques employed.14,15 To characterize the reactivity of MoAb L2B further, Western blot analysis of plasma fibrinogen compared with platelet fibrinogen was performed (Fig 4). Platelets were collected, washed, and then lysed in SDS gel-loading buffer. Duplicate protein blots were probed with either MoAb L2B or polyclonal antihuman fibrinogen antiserum. A third set of samples was stained with Coomassie brilliant blue. The plasma fibrinogen γ55/57.5 chains reacted with MoAb L2B; however, no protein bands in the platelet lysate reacted with MoAb L2B, indicating the absence or much-reduced amounts of the γ55 chain in platelets. Plasma fibrinogen γ55/57.5 chains were loaded in at least ten-fold excess above the limits of their detection in the Western blot using MoAb L2B (not shown).

Fig 4. Western blot analysis of plasma and platelet lysate using MoAb L2B. In each panel, lanes 1 and 2, approximately 1 x 10^5 and 4 x 10^5 platelets washed and lysed, respectively; lane 3, 20 μg fibrinogen γ57.5; and lane 4, 20 μg pooled plasma fibrinogen. Western blot analysis using L2B, middle panel, and polyclonal antihuman fibrinogen (right) of duplicate blots of proteins resolved by SDS-PAGE as shown in Coomassie brilliant blue-stained gel (left). The gels were overloaded with protein for purposes of Western blotting to detect, within the limits of the procedure, possible trace quantities of γ55/57.5 chains in platelet fibrinogen. The smear of fibrinogen (right) results from the excess protein detected with the monospecific polyclonal antifibrinogen, which cannot be detected in the corresponding Coomassie-stained gel (left). The distinct Aα, Bβ, and γ chain bands of fibrinogen are observed in the platelet lysate using polyclonal antiserum for blotting (lane 1, right). When the enzyme-substrate reaction was developed for shorter periods of time, the expected migration pattern of fibrinogen Aα, Bβ, and γ chain polypeptides were of the same intensity in all three fibrinogen preparations (lanes 2 to 4), indicating equivalent amounts of immunoreactive fibrinogen (not shown).
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Immunofluorescence microscopy reveals hepatocyte-specific expression of \( \gamma \)57.5 chains. Fibrinogen stored in platelet \( \alpha \) granules is synthesized in the megakaryocyte; therefore megakaryocytes from human marrow as well as platelets from human blood were examined for the presence of the \( \gamma \)57.5 chain of fibrinogen by indirect immunofluorescence microscopy. No positive immunofluorescent staining was observed for platelets or megakaryocytes (Fig 5b and 5e, respectively) using MoAb L2B. When L2B was preincubated with an excess of synthetic peptide antigen (\( \gamma \)57.50.46), the amount of background fluorescence (Fig 5c) was similar to that observed for the negative control (Fig 5f).

Marrow from eight individuals was examined, and negative staining with L2B was observed for all megakaryocytes. A positive staining pattern was observed in the cytoplasmic region of megakaryocytes (Fig 5d) using the anti-\( \gamma \)50.00-411 MoAb H9B7, and the characteristic granular pattern for fibrinogen stored in \( \alpha \) granules was observed in platelets (not shown). In addition, using the MoAb J88B, which is an IgG1 MoAb that recognizes a site in common with all \( \gamma \) chains, the characteristic granular staining pattern was observed for platelets (Fig 5a), and positive immunofluorescence was observed in the cytoplasmic region of megakaryocytes (not shown). These observations extend those of Francis et al.\textsuperscript{14} Mosesson et al.\textsuperscript{15} and Kunicki et al.\textsuperscript{16} to show that the \( \gamma \)57.5 or post-translationally processed \( \gamma \)55 chains are absent or present in significantly reduced quantities in platelets and moreover, that the megakaryocyte progenitor of platelet fibrinogen shows the same compartmentalization of \( \gamma \) chain expression: they contain only \( \gamma \)50 chains and not \( \gamma \)57.5 (or \( \gamma \)55) chains.

To demonstrate the \( \gamma \) chain specificity of MoAb staining and the lack of L2B staining as shown for megakaryocytes (Fig 5), the HepG2 cell line was examined by immunofluorescence microscopy (Fig 6). Positive immunofluorescent staining was observed with \( \gamma \)50-H9B7 and \( \gamma \)57.5-L2B in the perinuclear region of the HepG2 cell, which is indicative of a secreted protein (Fig 6a and 6d, respectively). This same positive staining was observed when L2B was preincubated with an excess of a negative control synthetic peptide, the dodecapeptide \( \gamma \)5011 of the \( \gamma \)50 chain (Fig 6f). However, when MoAbs H9B7 and L2B were preincubated with an excess of their respective synthetic peptides against which they were produced, the immunofluorescent staining of HepG2 cells was specifically inhibited to the background level of fluorescence (Fig 6b and 6e compared with 6c).

**Discussion**

Plasma fibrinogen is synthesized in hepatocytes,\textsuperscript{32} while platelet fibrinogen is synthesized in megakaryocytes,\textsuperscript{33} the progenitor cells of platelets. It has been clearly shown by the
Fig 6. Immunofluorescence microscopy of HepG2 cells using anti-γ-chain variants MoAbs. Positive immunofluorescent staining of HepG2 cells is shown using γSO-H9B7 (a) and γ57.5-L2B (d), which, when the γ-chain-specific MoAbs were preincubated with their respective immunogen synthetic peptides H9B7 (b) and L2B (e), is inhibited to background fluorescence equal to the goat antimouse (IgG + IgM)-FITC-conjugated secondary antiserum (c). Positive staining is observed when L2B is preincubated with an excess of a negative control synthetic peptide, the dodecapeptide γ50,85-101 of the γ60 chain C-terminus (f). The bar in (e) represents 20 μm in (a to f).

cDNA cloning of two distinct liver-specific γ-chain mRNAs from both human and rat that differential RNA processing is involved in the hepatocyte expression of the γ chain gene.10,11 The only information available concerning the megakaryocyte is the report of Uzan et al.34 in which an analysis of rat megakaryocyte γA and γB mRNAs using rat γ chain cDNAs as probes indicated the presence of γB mRNA in megakaryocytes. However, two problems limit interpretation of the findings. First, its abundance was more than 50% reduced compared with rat liver γB mRNA when compared with the relative abundance of γA mRNA in both tissues.34 Second, the findings with two γ chain probes were apparently discrepant. Uzan et al used two different γ chain probes to measure γ-chain–specific mRNAs in rat liver and megakaryocytes. Using a γB cDNA probe comprised of part γA sequences as well as the intron sequences specific only to γB mRNA, they demonstrated γA and γB mRNAs in both liver and megakaryocyte RNA. However, for some unexplained reason the γA cDNA probe failed to detect γB mRNA in megakaryocyte RNA even though the γB mRNA contains all of the nucleotide sequence of the γA mRNA plus additional sequence contributed by the last intron.11 Uzan et al concluded that since all rat fibrinogen mRNAs (Aα, Bβ, and γAγB) are expressed in the megakaryocyte, the mechanisms for the production and the processing of fibrinogen mRNAs in hepatocytes and megakaryocytes are likely the same.34

Since γB and γ57.5 are not found in platelets14,16 and no information is available regarding megakaryocyte synthesis of γ-chain–variant polypeptides, we chose to compare the expression of γ57.5 and γ55 polypeptides in two pools of fibrinogen biosynthesis, liver and marrow, using γ-chain–specific MoAbs we describe in this report. Using MoAb L2B, which specifically recognizes γ55 and γ57.5 chains, we showed that expression of γ57.5/55 polypeptides of human fibrinogen is compartmentalized and hepatocyte specific: they are not expressed in megakaryocytes. We were concerned about a negative result in platelets and megakaryocytes using anti-γ57.5 MoAb L2B. We questioned the possibility that fixation and/or the native conformation of the γ57.5 polypeptide C-terminus might not allow its accessibility to the antibody. We reasoned that the C-terminus of the γ50 chain constituted the most appropriate epitope to approximate the conditions we were examining at the γ57.5 C-terminus; therefore we developed the anti-γ50 MoAb H9B7 for the purpose of controlling the immunofluorescent staining. MoAb H9B7 detected γ50 fibrinogen by immunofluorescence in platelets and megakaryocytes. When anti-γ50-H9B7 and anti-γ55/57.5 MoAb L2B were used to stain HepG2 cells, the γ55/57.5 variant was easily detected; therefore if γ55/57.5 were present in platelets and/or megakaryocytes in a similar or slightly reduced ratio, we should have detected it. The method of immunolocalization of polypeptides is a powerful tool, as was demonstrated in
identifying hepatocytes as one of the tissues of factor VIII (factor VIII:C) expression, a coagulation factor found in much-reduced abundance in plasma compared with fibrinogen. Our results indicated that γ55/57.5 polypeptides are absent from platelets and megakaryocytes or present only in much-reduced amounts, extending the observations of Francis et al., Mosesson et al., and Kunicki et al.

The commonest means of tissue-specific gene regulation is transcriptional control involving alternative RNA processing. This results in differential processing of the mRNA in the tissues in which the corresponding polypeptide is translated. Different promoters may be used for RNA polymerase II-directed transcription of eukaryotic genes resulting in multiple mRNAs from a single gene. Alternatively, as in the case of the fibrinogen γ chain gene, the same promoter may be used and differences in 3’ end processing of the primary transcript occur. However, in other systems that involve 3’ end regulation of tissue-specific mRNA expression, low levels of mRNA corresponding to the nonexpressed polypeptide can still be found. Since it is documented in both humans and rats that platelets do not contain the longer γ chain polypeptides, nor do megakaryocytes, as demonstrated in this report, it is conceivable that the platelet progenitor cell is not capable of synthesizing the γB or γ57.5 polypeptide (or detectable levels) because the mRNA is expressed in much-reduced abundance. Recent evidence has indicated that the rat γ chain promoter is not under the same tissue-specific transcriptional control demonstrated for the fibrinogen Αα and Ββ promoters. Therefore an alternative explanation for differential γ chain polypeptide expression is that the megakaryocyte and hepatocyte use different mechanisms to regulate γ chain gene expression, perhaps in a tissue-specific manner at the level of 3’ end RNA processing events.

ACKNOWLEDGMENT
We thank D. Frank for discussions of myeloma-cell fusion techniques; M. Bourgois, S. Lawrence, and M. Vanek for technical assistance; J. Miller for preparation of bone marrow smears; and Carol B. Weed for secretarial assistance. We thank A. Hong from Applied Biosystems for suggestions about synthesis and conjugation of the synthetic peptides.

REFERENCES
25. Kearney JF, Radruch A, Liesegang B, Rajewski K: A new mouse myeloma cell line that has lost immunoglobulin expression...


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