Short Communication

A Total Fibrinogen Deficiency Is Compatible with the Development of Pulmonary Fibrosis in Mice

Victoria A. Ploplis, Julie Wilberding,
Laura McLennan, Zhong Liang, Ivo Cornelissen,
Melanie E. DeFord, Elliot D. Rosen, and
Francis J. Castellino

*From the W. M. Keck Center for Transgene Research and the
Department of Chemistry and Biochemistry, University of Notre
Dame, Notre Dame, Indiana*

In addition to their well-known roles in hemostasis, fibrinogen (Fg) and fibrin (Fn) have been implicated in a number of other physiological and pathophysiological events. One of these involves the fibroproliferative response after acute lung injury, which is the focus of the current study. Mice with a total Fg deficiency ($FG^{-/-}$) were generated by breeding heterozygous ($FG^{+/-}$) pairs, each of which contained an allele with a targeted deletion of its Fg - γ -chain gene. The resulting $FG^{-/-}$ animals did not possess detectable plasma Fg. $FG^{-/-}$ mice were then used to assess the roles of Fg and Fn in a bleomycin-induced acute lung injury model. Intratracheal administration of bleomycin in wild-type and $FG^{-/-}$ mice resulted in equivalent deposition of interstitial collagen and fibrotic lesions at days 7 and 14 after administration. This indicates that Fg and/or Fn are not essential for the development of bleomycin-induced pulmonary fibrosis. (*Am J Pathol* 2000, 157:703–708)

Fibrinogen (Fg) is a 340,000 kd trinodular dimeric protein consisting of three pairs of nonidentical polypeptide chains, namely, $(A\alpha/B\beta/\gamma)_2$,¹ which is altered during blood coagulation into an insoluble polymeric network of fibers that forms the basis of the blood clot.² Additionally, it is believed that Fg participates in events other than hemostasis. For example, Fg is an acute phase reactant that responds to injury and stress^{3,4} and its depletion in mice has been shown to contribute to host susceptibility to infection.⁵ This protein also seems to be necessary for early events in immune inflammatory responses via stimulation of leukocyte adhesion and migration across endothelial cell layers.⁶ Fibrin (Fn) co-localizes with collagen-rich fibrotic lung lesions and has been identified as a

key molecular effector of the fibroproliferative response after injury.⁷ In addition, Fg and Fn have been implicated in the progression of certain types of tumors,^{8–11} in severity of atherosclerosis,¹² and in the efficacy of wound healing.^{13,14} The ability of Fg and Fn to interact with a variety of cell types through integrin¹⁵ and nonintegrin¹⁶ receptors likely accounts for the roles of these proteins in several different cell-related pathophysiological conditions.

Before secretion of the intact Fg, its three chains are assembled in a stepwise manner in the rough endoplasmic reticulum.¹⁷ If genetic defects in the $A\alpha$ chain,¹⁸ $B\beta$ chain,¹⁹ or γ chain^{20,21} exist, impaired secretion can occur that results in congenital hypofibrinogenemia or afibrinogenemia. Complexes of $A\alpha/B\beta$ and $B\beta/\gamma$ chains associate with γ and $A\alpha$ chains in the luminal space of the endoplasmic reticulum, forming Fg, which is then secreted as the native dimeric protein.²²

A targeted deletion of the $A\alpha$ chain in mice led to animals without detectable plasma Fg levels.²³ In the current investigation, we wished to similarly generate mice with a targeted deletion of the γ -chain gene (FG - γ), reasoning that these mice would also lack plasma Fg because of the requirement for α/γ and β/γ chains to serve as intermediates for the synthesis of the heterodimeric protein. In addition, not only would the availability of Fg-deficient mice allow analysis of the role of Fg in various disease models, but generation of specific γ -chain-deficient mice would allow facile and rapid development and phenotypic analyses of transgenic animals containing mutations of the γ chain, which possibly possess loci for platelet binding,^{24–28} leukocyte adhesion,^{6,15} Fn polymerization,^{21,29–31} and cleavage sites for MMPs.³² Therefore, the γ -chain gene of Fg was deleted from mice and an analysis of the effect of a Fg deficiency

Supported by grants HL13423 (to F. J. C.) and HL63682 (to V. A. P.) from the National Institutes of Health, a National Scientist Development Award (9630009N) from the American Heart Association (to V. A. P.), the Kleiderer-Pezold endowed professorship (to F. J. C.), and a grant from the W. M. Keck Foundation (to F. J. C.).

Accepted for publication May 23, 2000.

Address reprint requests to Dr. Francis J. Castellino, Department of Chemistry and Biochemistry, 229 Nieuwland Science Hall, University of Notre Dame, Notre Dame, IN 46556. E-mail: castellino.1@nd.edu.

on fibrotic lesion development after acute lung injury is the subject of the current report.

Materials and Methods

Targeted Inactivation of the Murine *FG-γ* Chain

FG-γ chain-deficient mice were generated by targeted inactivation of the murine γ chain of Fg by homologous recombination in R1 embryonic stem (ES) cells. The steps involved have been described in earlier reports.^{33–35}

Southern Blot Analysis of ES Cells

Correctly targeted ES cells were identified by Southern blot analysis of *Eco*RI digests of purified ES cell genomic DNA. A 0.85-kb fragment located downstream of the 3' homologous flanking region was used as a probe to identify an 11.5-kb *WT* and 6.5-kb disrupted *FG-γ*-chain allele. A full description of the methods used has been published.³⁵

Polymerase Chain Reaction (PCR) Analysis of Genomic DNA

FG-γ genotyping of mice was conducted by PCR analysis of purified genomic DNA from ear-punch tissue. An aliquot of 1 μ l of genomic DNA was used in a single PCR reaction. The reaction mixture contained the following three primers: a *NEO* gene-specific primer (5'-GGTTCTA-AGTACTGTGGTTTC-3'), a sequence within the 5'-non-coding flank of the *FG-γ* chain (5'-CACAGCGGCTTGTCATTAG-3'), and a sequence internal to the *FG-γ* chain (5'-CTGAAAGACCTGTCTTTGC-3'). The PCR reaction consisted of an initial denaturation step (94°C for 3 minutes) and 30 cycles of the following (94°C for 30 seconds, 58°C for 40 seconds, and 72°C for 50 seconds), with a final annealing step at 72°C for 5 minutes. The PCR products, 420 bp for the *WT* allele and 550 bp for the *FG-γ*-chain disrupted allele, were resolved on a 1% agarose gel.

Thrombin Clot Time of Plasma

The assay was conducted in a standard fibrometer (BBL Microbiology Systems). Whole blood (0.8 to 0.9 ml) was isolated from the vena cava of anesthetized *WT*, *FG*^{+/-}, and *FG*^{-/-} mice and placed into 0.1 ml of a 4% sodium citrate solution. The samples were subjected to centrifugation at 3,000 rpm for 10 minutes in a microcentrifuge and the plasma was transferred to a fresh tube for analysis. A volume of 100 μ l of 50 mmol/L Tris-HCl, pH 7.4, was incubated for 3 to 10 minutes at 37°C in a fibrometer cup, after which 100 μ l of fresh plasma was added and allowed to incubate an additional 1 minute. After 1 minute, 100 μ l of 5 NIH U/ml of human α -thrombin (Enzyme Research Laboratories, South Bend, IN) was

added and the fibrometer timer was started. Samples were analyzed in duplicate.

Western Blot Analysis of Plasma

Western blot analysis was carried out using standard procedures using a rabbit-anti-human Fg antibody which recognizes all three chains (to differing extents) and cross-reacts with mouse Fg (DAKO, Carpinteria, CA). Plasma samples were prepared as described above for thrombin times and 0.1 μ l was loaded onto a 9% sodium dodecyl sulfate gel.

Bleomycin-Induced Pulmonary Fibrosis

WT ($n = 5$) and *FG*^{-/-} ($n = 6$) anesthetized mice, 4 weeks of age, were administered, intratracheally, a single dose (4.5 U/kg) of bleomycin in 25 μ l saline by infusion through the vocal cords using a fiber-optic light source for illuminating the tracheal opening. Control animals of similar genotypes were treated with saline alone. At 7 and 14 days after bleomycin infusion, anesthetized mice were perfused with saline and the left lung excised for determination of the collagen content (14 days), while the right lung was processed for histological analyses (7 and 14 days).

Quantitation of Lung Collagen Content

Total lung collagen content in saline and bleomycin-treated mice was determined by a colorimetric assay measuring hydroxyproline levels of acid hydrolyzed lung tissue.³⁶

Histology and Immunohistochemistry

Tissues were processed, embedded in paraffin, and sectioned at 3 to 4 μ m. Hematoxylin and eosin (H&E) staining of tissue was performed to identify the tissue and to detect alterations in cellular architecture. Masson's trichrome staining was used to identify collagen-rich fibrotic lesions in the lung.³⁷ Fn, in the lung, was identified by immunohistochemistry using a polyclonal goat-anti-mouse fibrin(ogen) antibody (Accurate Chemical, Westbury, NY) and a secondary rabbit-anti-goat IgG (DAKO), followed by goat peroxidase/anti-peroxidase (DAKO). The substrate, 3'-aminoethyl carbazole (AEC, Biomed, CA), was then used to detect peroxidase activity. The slides were counterstained with hematoxylin.

Statistical Analysis

Where appropriate, values were expressed as the mean $\pm$ SEM using a Microsoft Excel program.

a. Plasmids

b. EcoRI, probe 1, ES cells

c. PCR, mouse DNA

d. Western, mouse plasma

Figure 1. The strategy for targeted inactivation of the murine *FG- γ* chain gene by homologous recombination in ES cells. **a:** The top line represents the targeting vector, *pND.457*, in which the entire coding sequence of the *FG- γ* chain has been replaced with a neomycin phosphotransferase (*NEO*) gene to allow for positive selection. A cytosine deaminase (*CDA*) gene has also been inserted downstream of the *FG- γ* chain gene for negative selection of homologous recombination events. The middle line illustrates the wild-type (*WT*) allele, with vertical black extensions originating from the line representing the gene indicating the approximate positions of the 10 *Fg- γ* chain exons. On homologous recombination at 5' and 3' flanking locations (blue), the *NEO* gene replaces an approximate 9.0-kb genomic fragment comprising the sequences encoding the entire mature *Fg- γ* chain. The bottom line represents the resulting targeted chromosomal locus (*TL*). The homologous flanking regions of the targeting vector are shown in blue and include a 5' *NotI/EcoRV* 5.5-kb fragment of the *FG- γ* chain gene and a 3.5-kb *NheI/NcoI* fragment starting at a position 700 bp from the termination codon. **b:** Southern blot of *EcoRI*-digested DNA from ES cells to identify correctly targeted cells with 3'-directed probe 1 (red, see **a**). This probe, a 0.85-kb *NcoI/NsiI* 3' fragment immediately downstream of the *Fg- γ* chain, hybridizes to an 11.5-kb *WT* and 6.5-kb disrupted *FG- γ* chain allele. A properly targeted stem cell showing both alleles is indicated by the *. **c:** Genotyping of mouse DNA. Amplification of the disrupted allele was accomplished with forward primer 2 in the 5'-noncoding flanking region of *FG- γ* and with reverse primer 3, located in the *NEO* gene, leads to a 0.55-kb amplicon, whereas amplification of the wild-type *FG- γ* allele with forward primer 2 in the *FG- γ* 5'-flank and reverse primer 4 within the *FG- γ* gene results in a 0.42-kb amplicon. The approximate locations of these primers are indicated in **a** by magenta arrows. **Lane 1**, DNA markers (top to bottom: 2.0 kb, 1.5 kb, 1.0 kb, 0.75 kb, 0.5 kb, and 0.25 kb); **lane 2**, *FG- γ* ^{+/+} mouse DNA; **lane 3**, *FG- γ* ^{-/-} mouse DNA; **lane 4**, *FG- γ* ^{+/+} mouse DNA. **d:** Western analysis of mouse plasma using a polyclonal antibody that recognizes (to differing extents) all three murine Fg chains. **Lane 1**, purified mouse Fg; **lane 2**, molecular mass markers (top to bottom: 101 kd, 79 kd, and 50 kd); **lane 3**, plasma from a *FG- γ* ^{+/+} mouse; **lane 4**, plasma from a *FG- γ* ^{+/+} mouse; **lanes 5 and 6**, plasma from *FG- γ* ^{-/-} mice. These latter two lanes provide additional support that none of the Fg chains are detected in these plasmas.

Results

Targeted Inactivation of the *FG- γ* Gene

A targeting vector was constructed in which the *NEO* gene replaced all coding regions of the *FG- γ* chain (Figure 1a). This altered gene was inserted in place of the *FG- γ* -chain gene in the mouse genome by homologous recombination. Two independent correctly targeted ES clones (Figure 1b) were aggregated with Swiss morula stage embryos, yielding seven chimeric animals with 80 to 95% coat color chimerism. Chimeric mice transmitted the inactivated *FG- γ* gene to their offspring. Intercrossing of these heterozygous *FG- γ* -chain-deficient mice (*FG*^{+/-}) generated homozygous *FG- γ* -deficient (*FG*^{-/-}) progeny

for phenotypic analysis (Figure 1c). Fg antigen was not detected in *FG- γ* -chain null offspring as determined by Western blot analysis of plasma (Figure 1d) and immunostaining of liver sections (not shown). All animals used in this study were 50% 129SvJ/50% C57Bl/6.

Spontaneous Neonatal Bleeding

A significant (~25%) percentage of *FG*^{-/-} neonates suffered from hemorrhaging events. The bleeding was primarily abdominal but was also observed in the cranial, neck, and nasal areas. Although these events initially seemed dramatic, many *FG*^{-/-} mice were able to resolve the bleeding and recover.

Figure 2. Histology of lung tissue from *WT* and *FG*^{-/-} mice 14 days after intratracheal administration of bleomycin or saline control. H&E stain of lung tissue from *WT* (a) and *FG*^{-/-} (d) mice treated with bleomycin demonstrated interstitial fibrosis (original magnification, $\times 200$). Masson's trichrome stain of a serial section of lung tissue from *WT* (b) and *FG*^{-/-} (e) mice identified collagen deposits (blue) within the fibrotic lesion (original magnification, $\times 200$). Fg immunohistochemical stain of a serial section of lung tissue from *WT* (c) and *FG*^{-/-} (f) mice demonstrated co-localization of Fg within the collagen-rich lesions in *WT* mice and not in *FG*^{-/-} mice (original magnification, $\times 200$). g: H&E stain of lung tissue from a *FG*^{-/-} mice treated with saline demonstrated normal lung architecture (original magnification, $\times 200$). h: Masson's trichrome stain of lung tissue from *FG*^{-/-} mice treated with saline demonstrated normal collagen deposition associated mainly with bronchioles (original magnification, $\times 200$). i: Fg immunohistochemistry of lung tissue from *FG*^{-/-} mice treated with saline demonstrated the expected lack of Fg deposition (original magnification, $\times 200$). Results from the three stains performed on lung tissue from *WT* mice treated with saline were indistinguishable to that observed in *FG*^{-/-} mice.

Thrombin Clot Time of Plasma

Thrombin-induced clot times were significantly delayed in *FG*^{-/-} mice relative to *WT* mice (>17 minutes, $n = 2$, versus 35.5 ± 3.5 seconds, $n = 3$, respectively). When it was observed that *FG*^{-/-} samples did not clot after 17 minutes, timing was stopped. Other samples left untreated never formed a clot. *FG*^{+/-} mice demonstrated thrombin clot times that were similar to *WT* mice (35.9 ± 4.3 seconds, $n = 3$).

Bleomycin-Induced Pulmonary Fibrosis

WT and *FG*^{-/-} mice developed pulmonary fibrosis to a similar extent, as evidenced by the equivalent increase, approximately two-fold, of total lung collagen levels, reflected by the hydroxyproline content 14 days after intratracheal administration of bleomycin relative to saline controls ($2.0 \mu\text{g}/\text{mg}$ dry lung, $n = 6$, versus $3.0 \mu\text{g}/\text{mg}$ dry lung, $n = 4$, for *WT* mice, and $1.3 \mu\text{g}/\text{mg}$ dry

lung, $n = 2$, versus $2.4 \mu\text{g}/\text{mg}$ dry lung, $n = 5$, for *FG*^{-/-} mice). H&E staining of lung tissue from *WT* and *Fg*^{-/-} mice receiving bleomycin identified focal areas of interstitial fibrosis (Figure 2, a and d). Masson's Trichrome staining of lung tissue from these mice indicated that collagen deposition was associated within the fibrotic lesions to a similar extent in *WT* and *FG*^{-/-} mice (Figure 2, b and e). In *WT* mice treated with bleomycin, Fg/Fn co-localized with the collagen-rich lesions (Figure 2c). *FG*^{-/-} saline-treated animals demonstrated normal lung architecture (Figure 2g) as well as significantly diminished collagen levels relative to bleomycin-treated mice (Figure 2h). Masson's trichrome staining of lung tissue from *WT* and *FG*^{-/-} mice, as early as 7 days after bleomycin treatment, indicated collagen-rich fibrotic lesions (Figure 3, a and b). Analysis of H&E-stained lung tissue from 7-day treated mice indicated enhanced levels of neutrophils and macrophages in both *WT* and *FG*^{-/-} relative to their respective saline controls (data not shown).

Figure 3. Histology of lung tissue from *WT* and *FG*^{-/-} mice 7 days after intratracheal administration of bleomycin. Masson's trichrome stain of lung tissue from *WT* (a) and *FG*^{-/-} (b) mice identified collagen deposits (blue) within the fibrotic lesion (original magnification, $\times 200$).

Discussion

Fn formation has been identified as a hallmark feature of several forms of pulmonary fibrosis, and it is believed to be a key regulator in the fibroproliferative response after lung injury. In an animal model of bleomycin-induced pulmonary fibrosis, it was observed that there was an induction of plasminogen activator inhibitor-1 and tissue factor expression in perilesional alveolar cells which supported the persistent development of Fn formation within the injured lung tissue.³⁸ Additionally, in patients with idiopathic pulmonary fibrosis, tissue factor and Fn have been identified in type II pneumocytes, but not in normal lung tissue.³⁹ These studies indicate that an environment supporting Fn formation develops after lung injury and that Fn could potentially play a role in the repair process.

In this investigation, mice deficient for the γ chain of Fg, that consequently possessed a total deficiency of plasma Fg, were generated and the development of bleomycin-induced pulmonary fibrosis assessed. Early inflammatory response, 7 days after bleomycin treatment, seemed to be equivalent between *WT* and *FG*^{-/-} mice. It was also observed that there was no significant difference in the development of collagen-rich pulmonary fi-

brotic lesions between *WT* and *FG*^{-/-} mice 7 and 14 days after administration of bleomycin. This indicates that fibrin(ogen) or fibrinopeptides may not be required for fibroblast proliferation and possibly other related events such as fibroblast migration after bleomycin treatment.⁴⁰ Other matrix proteins may be involved in supporting the fibrotic process after lung injury. For example, studies of lung biopsies from patients with pulmonary fibrosis have indicated that fibroblasts increase their synthesis of collagen and fibronectin, coordinately, within airspaces.⁴¹ Therefore, although Fn is not essential for the progression and persistence of fibrosis, matrix proteins, other than collagen, may play prominent roles in supporting the lung repair process after injury.

A recent study wherein bleomycin-induced pulmonary fibrosis was investigated in mice deficient for plasminogen (Pg), tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator, and urokinase plasminogen activator receptor demonstrated enhanced fibrotic lesion formation in Pg and activator deficient mice.⁴² Collagen levels were especially enhanced in *PG*^{-/-} and *TPA*^{-/-} mice, relative to *WT* mice. In the *PG*^{-/-} mice a persistence of Fn deposition was observed that co-localized with collagen-rich fibrotic lesions, thus implicating an involvement of Fn with the progression of the repair process. The current study with *FG*^{-/-} mice presents a key investigation that expands an analysis of the involvement of the fibrinolytic process in bleomycin-induced pulmonary fibrosis and suggests another role for plasmin in this process, possibly acting as a regulatory protein of collagen turnover through activation of matrix metalloproteinases, specifically collagenases.

Acknowledgments

The authors wish to thank Mayra Sandoval-Cooper for assistance with the histology; Stacey Raje for assistance with the animal colony; and Drs. Harm HogenEsch and Mark Suckow for assistance with evaluation of the histopathology.

References

1. Doolittle RF: Fibrinogen and fibrin. *Ann Rev Biochem* 1984, 53:195-229
2. Nemerson Y, Furie B: Zymogens and cofactors of blood coagulation. *CRC Crit Rev Biochem* 1980, 9:45-85
3. Benson MD: Acute-phase reactants. *Curr Opin Rheumatol* 1989, 1:209-214
4. Rokita H, Neta R, Sipe JD: Increased fibrinogen synthesis in mice during the acute phase response: co-operative interaction of interleukin 1, interleukin 6, and interleukin 1 receptor antagonist. *Cytokine* 1993, 5:454-458
5. McRitchie DI, Girotti MJ, Glynn MF, Goldberg JM, Rotstein OD: Effect of systemic fibrinogen depletion on intraabdominal abscess formation. *J Lab Clin Med* 1991, 118:48-55
6. Languino LR, Duperray A, Joganic KJ, Fornaro M, Thornton GB, Altieri DC: Regulation of leukocyte-endothelium interaction and leukocyte transendothelial migration by intercellular adhesion molecule 1-fibrinogen recognition. *Proc Natl Acad Sci USA* 1995, 92:1505-1509
7. Olman MA, Simmons WL, Pollman DJ, Loftis AY, Bini A, Miller EJ, Fuller GM, Rivera KE: Polymerization of fibrinogen in murine bleomycin-induced lung injury. *Am J Physiol* 1996, 271:L519-L526

8. Costantini V, Zacharski LR: The role of fibrin in tumor metastasis. *Cancer Metastasis Rev* 1992, 11:283-290
9. Nagy JA, Masse EM, Herzberg KT, Meyers MS, Yeo KT, Yeo TK, Sioussat TM, Dvorak HF: Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. *Cancer Res* 1995, 55:360-368
10. Nagy JA, Meyers MS, Masse EM, Herzberg KT, Dvorak HF: Pathogenesis of ascites tumor growth: fibrinogen influx and fibrin accumulation in tissues lining the peritoneal cavity. *Cancer Res* 1995, 55:369-375
11. Hatton MWC, Ross B, Bardossy L, Southward SMR, DeReske M, Richardson M, Blajchman MA: The procoagulant state of the VX-2 tumor in rabbit lung *in vivo*—relative accumulation of fibrinogen, prothrombin, plasminogen, antithrombin and heparin cofactor II within the tumor. *Thromb Haemost* 1999, 82:1694-1702
12. Lassila R, Peltonen S, Lepantalo M, Saarinen O, Kauhanen P, Manninen V: Severity of peripheral atherosclerosis is associated with fibrinogen and degradation of cross-linked fibrin. *Arterioscler Thromb* 1993, 13:1738-1742
13. Brown LF, Lanir N, McDonagh J, Tognazzi T, Dvorak AM, Dvorak HF: Fibroblast migration in fibrin gel matrices. *Am J Pathol* 1993, 142:273-283
14. Kao WWY, Kao CWC, Kaufmann AH, Kombrinck KW, Converse RL, Good WV, Bugge TH, Degen JL: Healing of corneal epithelial defects in plasminogen- and fibrinogen-deficient mice. *Invest Ophthalmol Vis Sci* 1998, 39:502-508
15. Altieri DC, Plescia J, Plow EF: The structural motif glycine 190-valine 202 of the fibrinogen gamma chain interacts with CD11b/CD18 integrin (alpha M beta 2, Mac-1) and promotes leukocyte adhesion. *J Biol Chem* 1993, 268:1847-1853
16. Languino LR, Plescia J, Duperray A, Brian AA, Plow EF, Geltosky JE, Altieri DC: Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway. *Cell* 1993, 73:1423-1434
17. Zhang JZ, Redman CM: Identification of B β chain domains involved in human fibrinogen assembly. *J Biol Chem* 1992, 267:21727-21732
18. Koopman J, Haverkate F, Grimbergen J, Egbring R, Lord ST: Fibrinogen Marburg: a homozygous case of dysfibrinogenemia, lacking amino acids A alpha 461-610 (Lys 461 AAA→stop TAA). *Blood* 1992, 80:1972-1979
19. Duga S, Asselta R, Santagostino E, Zeinali S, Simoncic T, Malcovati M, Mannucci PM, Tenchini ML: Missense mutations in the human beta fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion. *Blood* 2000, 95:1336-1341
20. Terasawa F, Okumura N, Kitano K, Hayashida N, Shimosaka M, Okazaki M, Lord ST: Hypofibrinogenemia associated with a heterozygous missense mutation gamma 153 Cys to arg (Matsumoto IV): *in vitro* expression demonstrates defective secretion of the variant fibrinogen. *Blood* 1999, 94:4122-4131
21. Brennan SO, Fellowes AP, Faed JM, George PM: Hypofibrinogenemia in an individual with 2 coding (gamma 82 A→G and B beta 235 P→L) and 2 noncoding mutations. *Blood* 2000, 95:1709-1713
22. Yu S, Sher B, Kudryk B, Redman CM: Fibrinogen precursors. Order of assembly of fibrinogen chains. *J Biol Chem* 1984, 259:10574-10581
23. Suh TT, Holmback K, Jensen NJ, Daugherty CC, Small K, Simon DI, Potter S, Degen JL: Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. *Genes Dev* 1995, 9:2020-2033
24. Hawiger J, Timmons S, Kloczewiak M, Strong DD, Doolittle RF: Gamma and alpha chains of human fibrinogen possess sites reactive with human platelet receptors. *Proc Natl Acad Sci USA* 1982, 79:2068-2071
25. Peerschke EI, Francis CW, Marder VJ: Fibrinogen binding to human blood platelets: effect of gamma chain carboxy terminal structure and length. *Blood* 1986, 67:385-390
26. Hantgan RR: Localization of the domains of fibrin involved in binding to platelets. *Biochim Biophys Acta* 1988, 968:36-44
27. Haidaris PJ, Peerschke EI, Marder VJ, Francis CW: The C-terminal sequences of the gamma 57.5 chain of human fibrinogen constitute a plasmin sensitive epitope that is exposed in crosslinked fibrin. *Blood* 1989, 74:2437-2444
28. Kirschbaum NE, Mosesson MW, Amrani DL: Characterization of the gamma chain platelet binding site on fibrinogen fragment D. *Blood* 1992, 79:2643-2648
29. Bantia S, Mane SM, Bell WR, Dang CV: Fibrinogen Baltimore I: polymerization defect associated with a gamma 292 Gly→Val (GGC→GTC) mutation. *Blood* 1990, 76:2279-2283
30. Niwa K, Takebe M, Sugo T, Kawata Y, Mimuro J, Asakura S, Sakata Y, Mizushima J, Maeda A, Endo H, Matsuda M: A gamma Gly-268 to Glu substitution is responsible for impaired fibrin assembly in a homozygous dysfibrinogen Kurashiki I. *Blood* 1996, 87:4686-4694
31. Okumura N, Furihata K, Terasawa F, Nakagoshi R, Ueno I, Katsuyama T: Fibrinogen Matsumoto I: a gamma 364 Asp→His (GAT→CAT) substitution associated with defective fibrin polymerization. *Thromb Haemost* 1996, 75:887-891
32. Bini A, Itoh Y, Kudryk BJ, Nagase H: Degradation of cross-linked fibrin by matrix metalloproteinase 3 (stromelysin 1): hydrolysis of the gamma Gly 404-Ala 405 peptide bond. *Biochemistry* 1996, 35:13056-13063
33. Rosen E, Chan JCY, Idusogie E, Clotman F, Vlasuk G, Luther T, Jalbert LR, Albrecht S, Zhong L, Lissens A, Schoonjans L, Moons L, Collen D, Castellino FJ, Carmeliet P: Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. *Nature* 1997, 390:290-294
34. Jalbert LR, Rosen ED, Moons L, Chan JCY, Carmeliet P, Collen D, Castellino FJ: Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. *J Clin Invest* 1998, 102:1481-1488
35. Dewerchin M, Liang Z, Moons L, Carmeliet P, Castellino FJ, Collen D, Rosen ED: Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. *Thromb Haemost* 2000, 83:185-190
36. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, Simon RH: Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest* 1996, 97:232-237
37. Masson P: Trichrome stainings and their preliminary technique. *J Tech Meth* 1929, 12:75-90
38. Olman MA, Mackman N, Gladson CL, Moser KM, Loskutoff DJ: Changes in procoagulant and fibrinolytic gene expression during bleomycin-induced lung injury in the mouse. *J Clin Invest* 1995, 96:1621-1630
39. Imokawa S, Sato A, Hayakawa H, Kotani M, Urano T, Takada A: Tissue factor expression and fibrin deposition in the lungs of patients with idiopathic pulmonary fibrosis and systemic sclerosis. *Am J Respir Crit Care Med* 1997, 156:631-636
40. Gray AJ, Bishop JE, Reeves JT, Mecham RP, Laurent GJ: Partially degraded fibrin(ogen) stimulates fibroblast proliferation *in vitro*. *Am J Respir Cell Mol Biol* 1995, 6:684-690
41. Kuhn C, Boldt J, King TE, Crouch E, Vartio T, McDonald JA: An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *Am Rev Respir Dis* 1989, 140:1693-1703
42. Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA: The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system. *Am J Pathol* 2000, 157:177-187