Specific Effects of Fibrinogen and the γ A and γ' -Chain Fibrinogen Variants on Angiogenesis and Wound Healing

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In a newly formed wound, the natural fibrin network provides the first temporary matrix for tissue repair. Topical application of fibrin to a new wound may improve wound healing. A matrix of the common natural γ' fibrin variant may further improve wound healing because it is expected to have a different architecture and this will influence angiogenesis, because it possesses increased thrombin and factor XIII binding and decreased platelet binding, when compared with the common γ A fibrin matrix. Our objective was to determine the effect of fibrinogen and its γ A and γ' variants on angiogenesis and wound healing. We used *in vitro* angiogenesis models and an *in vivo* rat full-thickness excisional wound healing model. When comparing γ A and γ' fibrin *in vitro*, more tube-like structures were formed on day 7 in γ A fibrin than in γ' fibrin (13.83 ± 6.12 AU vs. 6.1 ± 1.46 AU). Wounds treated with fibrin demonstrated improved healing *in vivo* with more perfusion ($47\% \pm 3\%$ vs. $26\% \pm 4\%$, $p < 0.01$ in placebo) and higher CD34 density score (2.0 ± 0.4 vs. 2.8 ± 0.1 , $p < 0.01$) on day 21 with fibrin matrices when compared with placebo-treated wounds. Increased perfusion was observed in γ A fibrin-treated wounds on day 21 ($53\% \pm 10\%$ vs. $41\% \pm 7\%$ for γ' fibrin). The other parameters showed slightly improved (not significant) wound healing with γ A fibrin compared with γ' fibrin matrices. In conclusion, the use of fibrin and fibrin variant matrices offers an interesting methodology to stimulate the wound healing process.

Introduction

FIBRINOGEN IS A SOLUBLE plasma protein with a molecular mass of 340,000 Da that comprises two identical disulfide-linked halves, each consisting of three polypeptide chains termed α , β , and γ . At the end of the coagulation cascade fibrinopeptides A and B are spliced off, which results in the transition to fibrin fibers that coalesce into a fibrin matrix.¹ In healthy individuals many fibrinogen variants are present, which are the result of proteolytic degradation of the protein, alternative processing of fibrinogen messenger RNA, or post-translational modifications. A common, naturally occurring variant is γ' fibrinogen, which comprises $10\% \pm 3\%$ of the total circulating fibrinogen in healthy individuals.² γ' Fibrinogen is the result of alternative messenger RNA processing at intron 9 and exon 10, resulting in the replacement of the last 4 amino acids (408–411) at the C-terminus of the γ A-chain by 20 amino acids (408–427).³ γ' Fibrinogen has extra binding sites for thrombin and the factor XIII subunit and lacks the platelet integrin $\alpha_{IIb}\beta_3$ binding site.^{4,5} In the circulation, γ A/ γ A fibrinogen (abbreviated as γ A) and fi-

brinogen molecules containing one or two γ' chain(s) (γ A/ γ' or γ'/γ' , together abbreviated as γ') are present. Several studies reported structural and functional differences between γ A and γ' fibrin matrices, such as slower fibrinopeptide B release, slower fibrin polymerization for γ' fibrinogen, thinner fibers and more branch points with scanning electron microscopy on γ' fibrin matrices, and a stiffer network that lyses slower.^{6–10} We hypothesize that these differences between γ A and γ' fibrin matrices affect wound healing, especially the angiogenesis process.

The conversion of fibrinogen to fibrin is the final step of the coagulation cascade, which is activated following injury. The coagulation cascade results in the formation of a thrombus with as primary function protection against hemorrhage. Fibrin is the principal matrix protein in the thrombus. The second important role of the fibrin matrix is to provide a temporary, biodegradable matrix for endothelial cell invasion in the early phases of the wound healing process.¹¹ Wound healing is a dynamic, interactive, and complex process that involves interactions between the extracellular microenvironment and cells, with, especially at the first crucial stages

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of healing, an important role for endothelial cells. The invasion of endothelial cells results in the formation of new capillaries (angiogenesis), a process that partly depends on the interactions of vascular cells and the extracellular matrix in the wounded area. The fibrin matrix facilitates cell adhesion, migration, and proliferation and thus stimulates angiogenesis.^{12,13} Further, fibrin binds growth factors that influence these processes, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2).¹⁴ For other common variants of fibrinogen, that is, high- and low-molecular-weight fibrinogen, we previously showed that it affects the interaction between endothelial cells and the fibrin matrix because of the different network structure and binding of endothelial cells, resulting in differences in cell adhesion, migration, and proliferation.^{12,13}

In this study, we investigated how unfractionated fibrin and the fibrin γ A and γ' variants influence the fibrin matrix structure, angiogenesis, and wound healing.

Materials and Methods

Purification and characterization of fibrinogen variants

γ A and γ' fibrinogen variants were purified by anion-exchange chromatography¹⁵ from plasma-purified fibrinogen (Enzyme Research Laboratories, South Bend, IN). Purity of the fibrinogen variants was determined with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 4–15% gradient gel (Criterion Tris-HCL precast; Bio-Rad, Hercules, CA) under reducing conditions with a 25 mM Tris, 192 mM glycine, and 0.1% SDS (pH 8.3) running buffer (Bio-Rad) and total protein was stained with colloidal blue (Invitrogen, Carlsbad, CA). Western blotting was performed using polyclonal rabbit anti-fibrinogen (Dako, Glostrup, Denmark) and monoclonal anti-human γ' fibrinogen antibodies (2.G2.H9; Millipore, Billerica, MA) followed by detection with fluorescent-labeled secondary antibodies: goat anti-rabbit 680 (Invitrogen, Paisley, United Kingdom) and goat anti-mouse 800 (Invitrogen) for total fibrinogen and γ' fibrinogen, respectively. The immunoblots were visualized using Osysey imaging system (Li-Cor, Inc., Lincoln, NE). The clottability was determined by measuring the protein content (optical density at 280 nm) in the starting solution and in the supernatant of a squeezed clot after 2-h polymerization of purified fibrinogen (1 mg/mL) at ambient temperature with 1.0 U/mL thrombin (Organon Teknika, Bostel, The Netherlands) and 2.5 mM CaCl_2 .¹⁶ Factor XIII activity was determined photometrically according to manufacturer's instructions (Berichrom Factor XIII; Siemens Healthcare Diagnostics, Deerfield, IL). Plasminogen activity was measured with chromogenic substrate S-2251 following the manufacturer's instructions (Instrumentation Laboratory, Lexington, MA).

Confocal microscopy of fibrin matrices

Purified fibrinogen was labeled with fluorescein isothiocyanate isomer I (FITC; Sigma-Aldrich, Saint Louis, MI) and fibrin matrices (2 mg/mL) were formed in the presence of 0.5 U/mL thrombin and 2.5 mM CaCl_2 (final concentrations) in microchambers. The FXIII concentration in the γ A fibrinogen matrix was supplemented with human plasma coagulation FXIII (Fibrogammin P; CSL Behring, Marburg,

Germany) to compensate for the higher FXIII activity in γ' fibrinogen (final concentration = 0.31 U FXIII/mg fibrinogen), when indicated. Confocal images were visualized on a Zeiss LSM 510 Meta inverted confocal microscope (Carl Zeiss MicroImaging, Jena, Germany) with 63 $\times$ oil immerse objective lens in combination with a 5-W argon laser and 488-nm band-pass laser filter for the excitation. Fibrin fiber diameters ($n=50$) were measured using LSM Image Browser software, version 4.2 (Carl Zeiss Micro Imaging).

In Vitro Endothelial Cell Behavior

Fibrinogen coatings

Polystyrene culture plates (Costar; Corning, Amsterdam, The Netherlands) were coated with γ A and γ' fibrinogen (0.2 μ M in phosphate-buffered saline) as previously described.¹³ Human umbilical vein endothelial cells (HUVECs)^{17,18} were seeded on these fibrinogen coatings to determine their adhesion, migration, and proliferation and compared with a placebo coating of 1% gelatin. Coating of fibrinogen to plastic results in a fibrin-like structure. Pilot studies showed that addition of FXIII did not alter the adhesion/migration/proliferation of endothelial cells.

In vitro adhesion, proliferation, and migration

Adhesion was performed by seeding HUVECs (three independent isolations) on fibrinogen coatings in a density of 2×10^4 cells/cm² in cell culture Medium 199 (M199; Lonza, Verviers, Belgium) supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (p/s) and 1% human serum albumin (HSA; Sanquin, Amsterdam, The Netherlands). After 0.5, 1, 2, and 4 h cells were washed and fixated. Cell counting was performed using Image J 1.42 software (National Institutes of Health, Bethesda, MD).

Proliferation of HUVECs (three independent isolations) was determined using ³H-thymidine incorporation. HUVECs were seeded in a density of 6×10^3 cells/cm² in M199 supplemented with p/s and 1% HSA. Adherent HUVECs were stimulated for 72 h with M199 supplemented with p/s, 10% heat-inactivated newborn calf serum (NBCS; Lonza), and 10 ng/mL VEGF, of which the last 16 h in the presence of 1 μ Ci ³H-thymidine. The beta emission was measured with Ultima Gold scintillation fluid on 1900 TR Liquid Scintillation Analyzer (Packard Bioscience, Waltham, MA).

The migration of HUVECs (six independent isolations) was studied using an adapted version of the scratch assay. To maintain the fibrinogen coating a cell-free area was obtained by inserting a stainless steel bar prior to cell seeding. HUVECs were seeded in a density of 6×10^4 cells/cm² in M199 supplemented with penicillin/streptomycin and 1% HSA. After 4 h of cell adherence, the bar was removed and cells were stimulated with M199 supplemented with penicillin/streptomycin, 10% NBCS, and 10 ng/mL VEGF. After 16 h of migration, the cells were fixed and the migration into the cell-free area was determined by making five pictures per well and performing five measurements of cell-free area per picture with Photoshop 5.0 software (Adobe, San Jose, CA).

In vitro tube formation

The *in vitro* tube formation was determined using three-dimensional fibrin matrices and human microvascular

FIG. 1. Characterization of the γ A and γ' fibrinogen fractions. **(A)** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis with total protein staining and **(B)** western blotting of fibrinogen with polyclonal human fibrinogen antibody (red) and monoclonal human γ' fibrinogen antibody (green). M, protein electrophoresis marker. Color images available online at www.liebertpub.com/tea

endothelial cells (HMVECs; three independent isolations), as described previously by Koolwijk *et al.*¹⁹ γ A fibrinogen was supplemented with human plasma coagulation FXIII (final concentration = 0.31 U FXIII/mg fibrinogen, which made the concentration similar to that in γ' fibrinogen). Fibrin matrices were prepared by addition of thrombin (0.01 U/mL) to 2 mg/mL fibrinogen solution. HMVECs were seeded in confluent density and stimulated with 10 ng/mL tumor necrosis factor- α (TNF α ; Sigma-Aldrich) and 25 ng/mL VEGF or 10 ng/mL FGF-2 (Peprotech, London, United Kingdom). Matrices of γ A fibrin appeared to be opaque; this was circumvented by using visualization with fluorescent labeling of the cells. The tubular structure formation of HMVECs into the fibrin matrices was analyzed after staining the F-actin filaments with rhodamine-phalloidine (Molecular Probes, Eugene, OR) and nuclei with Hoechst (Molecular Probes). The three-dimensional nature of the tubes was confirmed by z-stack analysis. The concentration of fibrin-degradation products in conditioned media of the tube formation assay was determined with the enzyme-linked immunosorbent assay FDP14-DD13.²⁰

In vivo wound healing

Full-thickness excisional wound healing model. The wound healing model was performed as previously described by Tong

*et al.*²¹ Briefly, two full-thickness wounds (15-mm-diameter circles) were excised in male 9-week-old WAG/RijHsd rats (Harlan, Zeist, The Netherlands). Fibrinogen (100 μ L of 2 mg/mL), mixed with 1 U/mL thrombin, was topically applied. On control wounds an equal amount of Tris-HCl buffer was applied.

Fifty-six 9-week-old WAG/RijHsd rats were purchased from Harlan, weighing 150–200 g. They were housed two animals per cage before surgery. The rats were exposed to a 12-h light/dark cycle and fed with a standard laboratory diet (Hope Farms, Woerden, The Netherlands) with food and water available ad libitum. The rats were allowed to acclimatize to their environment for 1 week before the procedure. The experimental protocol was approved by the Animal Experiments Committee under the national experiments on animals act and adhered to the rules laid down in this national law that serves the implementation of “Guidelines on the protection of experimental animals” by the Council of Europe (1986), Directive 86/609/EC. There was no subject loss.

Twelve rats were studied for 7 days and 44 rats were followed for 21 days. Wound-closure rates were quantified with standardized digital photographs of the wounds on days 0, 3, 7, 10, 14, and 21 using Image J 1.42. Skin perfusion was determined using Laser Doppler perfusion measurements of the wounded and normal skins on days 14 and 21 with local heat provocation (Perimed AB, Stockholm, Sweden). The skin area under the thermostatic probe was kept on 33°C until a stable baseline was established for 5 min. Subsequently, the skin was heated to 44°C and maintained to this temperature through the 10-min heating period. The micro-circulatory response to this local heating was monitored and expressed as the percentage of change from the baseline as we previously described.²¹ The breaking strength of the wounded skin was measured on day 21 as described previously.²¹ Paraffin sections of the wounds were immunohistochemically stained for CD34 on endothelial cells (goat anti-rat CD34 IgG; R&D System, Minneapolis, MN),²² and CD68 on macrophages (AbD Serotec, Düsseldorf, Germany). The positive-signal density of CD34 was scored semiquantitatively as 1 (=absent), 2 (=low), 3 (=medium), 4 (=strong), and 5 (=very strong) by four observers, all blinded to the treatment.^{23,24} After each observer's score had been ranked separately, the mean of the scores was calculated for each individual sample.

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Normal data distribution was confirmed by box-plot analysis. Differences between groups were analyzed by

FIG. 2. Confocal microscopy of γ A and γ' fibrin matrices. Fibrin matrices prepared from γ A fibrinogen without additional FXIII (γ A), γ A supplemented with FXIII (γ A + FXIII), and γ' fibrin (γ'). The scale bars indicate 5 μ m. Color images available online at www.liebertpub.com/tea

univariate ANOVA, and when the overall testing was significant, groups were compared using *post hoc* testing with least significant difference correction. Statistical analyses were performed with Statistical Package for the Social Sciences for windows (SPSS), Version 20 (Chicago, IL). Results were considered statistically significant with two-sided *p*-values < 0.05.

Results

Characterization of γ A and γ' fibrinogen

The γ' -chain of ~ 50 kDa was present in the purified fraction of γ' fibrinogen and absent in the γ A fibrinogen fraction on SDS-PAGE (Fig. 1A). Western blotting with fibrinogen- and γ' fibrinogen-specific antibodies confirmed this result (Fig. 1B). In the γ' fibrinogen fraction, an extra band of ~ 77 kDa was visible. Mass spectrometry confirmed that this was the FXIIIb subunit (data not shown). Purified γ A and γ' fibrinogen were >95% clottable. In the γ' fibrinogen fraction, the FXIII activity was significantly higher than measured in the γ A fraction (0.34 U FXIII/mg fibrinogen and 0.01 U/mg, respectively). The plasminogen activity in the γ A and γ' fibrinogen fractions was similar (0.012 and 0.011 U/mg, respectively).

Confocal microscopy on the fibrin matrices showed that the structure of γ' was different from that of γ A, with much larger pores and a structure with shorter and less straight fibers. Further, the fibers were thicker in γ A fibrin without FXIII ($0.37 \pm 0.08 \mu\text{m}$) than in γ' fibrin ($0.29 \pm 0.04 \mu\text{m}$). However, the thickness of the γ A fibers ($0.29 \pm 0.04 \mu\text{m}$) was similar to that of γ' fibrin after supplementation of FXIII (Fig. 2).

In vitro endothelial cell behavior

On coatings of γ A and γ' fibrinogen similar HUVEC adherence and proliferation was observed (Fig. 3A, B). Also, the migration of HUVECs into a cell-free area was similar on coatings of γ A and γ' fibrinogen (Figs. 3C and 4). No difference in HUVEC adhesion, proliferation, and migration was visualized when thin coatings γ A and γ' fibrinogen were compared. In addition, no differences on fibrinogen and 1% gelatin coatings were observed in adhesion, proliferation, and migration of cultured HUVECs (Fig. 3).

Light microscopy of *in vitro* tube formation of HMVECs showed a low amount of tubular structures in γ' fibrin; due to opaqueness, no cells or structures were visible in γ A fibrin (Fig. 5A, B). Fluorescent microscopy showed massive tube formation in γ A fibrin matrices, with many sprouting cells (Fig. 5C). In the γ' fibrin matrix, the endothelial cells formed stable tube-like structures with a normal morphology (Fig. 5D). The three-dimensional nature of these tubular structures was confirmed by z-stack analysis. Quantification of the *in vitro* tube formation revealed $2.2 \times$ more ingrowth of endothelial cells into γ A fibrin (Fig. 5E). In line with this observation, the concentration of fibrin degradation products in conditioned medium of γ A fibrin was higher than in conditioned medium of γ' fibrin (Fig. 5F, *p* < 0.05).

In vivo effects of γ A and γ' fibrin on skin wound healing

On day 7, during the early phase of wound healing in these young adult rats, topical application of fibrin resulted in a significantly increased neovascularization (visualized

FIG. 3. *In vitro* functional characteristics of human umbilical vein endothelial cells (HUVECs) on various fibrinogen coatings. (A) Adhesion of HUVECs to gelatin (solid line), γ A fibrinogen (dashed line), γ' fibrinogen (dotted line), and 1% bovine serum albumin (dashed-dotted line) coatings (*n* = 3). (B) The proliferation of HUVECs on gelatin, γ A, and γ' fibrinogen coatings, measured by ^3H -thymidine incorporation (*n* = 3). The values were corrected for cell adhesion and expressed as percentage of HUVEC proliferation on gelatin coatings. (C) The migration of HUVECs on gelatin, γ A, and γ' fibrinogen coatings; after 16 h of migration, the area of migration was determined (*n* = 6).

by the increased endothelial cell marker CD34 density) in the dorsal skin wounds when compared with placebo (mean score $\pm$ SEM: 4.1 ± 0.1 vs. 2.7 ± 0.5) (Table 1 and Fig. 6). In fibrin-treated wounds, we also saw a small, but not significant, effect on the wound-closure rate and Laser Doppler

FIG. 4. Representative images of endothelial cell migration, based on the *in vitro* assay. The length of the bar is 0.5 mm.

perfusion (Table 1). The unfractionated γ A and γ' fibrin had similar effects on the wound healing parameters.

On day 21, all wounds had closed and no differences were seen for the treatments (Table 1 and Fig. 7). In the histological analysis of the wounds on day 21, a relatively dense network of new blood vessels was seen over the total wound area. The neovascularization (visualized by endothelial cell marker CD34 density) was significantly lower than on day 7 in all wounds, consistent with a later phase of the wound healing process (Fig. 8). Neovascularization was higher in fibrin-treated wounds when compared with the placebo-treated wounds (score = 3.0 ± 0.4 vs. 2.3 ± 0.2) (Table 1). Also, in the wounds treated with fibrin, a higher vasodilator response in the skin was measured, especially at day 21, when compared with the placebo-treated wounds ($47.0\% \pm 3.4\%$ for fibrin, vs. $26.2\% \pm 3.6\%$ for placebo, $p=0.01$). Further, a more active

inflammatory process in wounds treated with fibrinogen was observed (2.8 ± 0.1 vs. 2.0 ± 0.4) (Fig. 9).

When we compared the fibrinogen γ A and γ' variants, we observed several differences that were consistent with improved wound healing in the γ A variant, but these were not significant (Table 1 and Fig. 6). These differences included a higher vasodilator response in the skin for the γ A-treated wounds in the Laser Doppler perfusion measurements when compared with γ' fibrin-treated wounds ($53.0\% \pm 10.0\%$ vs. $41.4\% \pm 6.5\%$, $p<0.01$). Our data also suggest a slightly, but not significantly, increased breaking strength in the γ A fibrin-treated group, when compared with the γ' fibrin-treated group (0.15 ± 0.03 for γ A vs. 0.11 ± 0.02 for γ'). We saw slightly more macrophages (CD68 staining) on day 21 in γ' fibrin than in γ A fibrin-treated wound, but this difference was not statistically significant.

FIG. 5. *In vitro* tube formation by human microvascular endothelial cells (HMVECs). HMVECs were seeded on three-dimensional γ A (A, C) and γ' fibrin (B, D) matrices and tubular structure formation was stimulated. Representative pictures of the *in vitro* tube formation assay, using light microscopy with visible tube-like structures are depicted with black arrows (A, B) and fluorescent staining where white arrows depict tube-like structures (C, D). The fluorescent staining represents the F-actin filaments of endothelial cells; the bright structures represent the tubular structures in the fibrin matrix. In γ A fibrin many structures are present, only structures in the left-lower corner are depicted. An enlargement of figure (C, E) is given. Z-stacks confirmed the three-dimensional nature of the tube-like structures. (E) Quantification of the amount of tube formation in the various fibrin matrices ($n=3$). (F) Fibrin degradation products that were present in conditioned medium of the *in vitro* tube formation assay, as determined by enzyme-linked immunosorbent assay ($n=3$). $*p<0.05$. Color images available online at www.liebertpub.com/tea

TABLE 1. *IN VIVO* WOUND HEALING IN THE RAT MODEL

	Control	Unfractionated fibrinogen	γ A fibrinogen	γ' fibrinogen
Wound closure rate (%)				
Day 3	0.3 $\pm$ 2.6	-0.1 $\pm$ 4.1	0.9 $\pm$ 2.6	-2.1 $\pm$ 3.7
Day 7	43.7 $\pm$ 3.4	37.7 $\pm$ 4.9	48.5 $\pm$ 3.5	45.9 $\pm$ 4.6
Day 10	82.0 $\pm$ 1.8	81.6 $\pm$ 2.1	85.0 $\pm$ 2.8	81.2 $\pm$ 2.8
Day 14	97.2 $\pm$ 0.5	95.4 $\pm$ 0.7	96.2 $\pm$ 0.7	97.1 $\pm$ 0.7
Day 21	100 $\pm$ <0.1	100 $\pm$ <0.1	99.8 $\pm$ 0.2	100 $\pm$ <0.1
Immunohistology				
CD34 density scoring				
Day 7	2.7 $\pm$ 0.5	4.1 $\pm$ 0.1 ^a	4.0 $\pm$ 0.3 ^a	4.3 $\pm$ 0.1 ^a
Day 21	2.3 $\pm$ 0.2	3.0 $\pm$ 0.2 ^b	3.0 $\pm$ 0.4 ^b	3.1 $\pm$ 0.2 ^b
CD68 density scoring				
Day 21	2.4 $\pm$ 0.2	2.3 $\pm$ 0.2	1.8 $\pm$ 0.3	2.5 $\pm$ 0.4
Inflammation				
Day 21	2.0 $\pm$ 0.4	2.8 $\pm$ 0.4	2.8 $\pm$ 0.3	2.6 $\pm$ 0.2
Laser Doppler perfusion (%)				
Day 14	27.3 $\pm$ 6.7	34.5 $\pm$ 6.0	20.4 $\pm$ 5.5	28.6 $\pm$ 6.4
Day 21	26.2 $\pm$ 3.6	37.4 $\pm$ 4.2	53.0 $\pm$ 10.0 ^a	41.3 $\pm$ 6.5
Breaking strength ratio				
Day 21	0.11 $\pm$ 0.02	0.10 $\pm$ 0.01	0.15 $\pm$ 0.03	0.11 $\pm$ 0.02

Data are given as mean $\pm$ SEM. Laser Doppler perfusion is expressed relative to baseline measurements at 33°C (%increase). The breaking strength is expressed as a ratio relative to the breaking strength of normal skin.

^a p < 0.01 fibrinogen (variant) versus control-treated wounds.

^b p < 0.05 fibrinogen (variant) versus control-treated wounds.

Discussion

In this study we showed improved revascularization and perfusion of rat full-thickness wounds when covered with fibrin. Also, the vessel maturation seems to proceed more rapidly in fibrin-treated wounds, as reflected by the significantly decreased revascularization score in fibrin-treated wounds at day 21. We further observed an increased perfusion for γ A fibrin, as compared with γ' fibrin, likely a direct reflection of the increased vascularization of the wound area. This finding also may indicate that γ A fibrin

promotes the *in vivo* wound healing slightly better than γ' fibrin. In our *in vitro* experiments, we also showed that the γ A fibrin matrix shows the highest vascular potential by studying tube-like structure formation of endothelial cells in a three-dimensional fibrin matrix.

We observed that the structure of γ' fibrin matrices comprises of larger pores and shorter fibers, when compared with the γ A fibrin matrices. Matrices of γ A fibrin had thinner fibrin fibers than matrices of γ' fibrin when we used the fibrinogen variants directly after purification, as is also reported by other groups.^{6,7} As we measured very low FXIII levels in γ A fibrinogen, while γ' fibrinogen contains a considerable amount of FXIII, we hypothesized that this difference in fiber thickness may be the result of the lower FXIII concentration in γ A fibrinogen. Indeed, after we supplemented γ A fibrinogen with FXIII, to obtain similar FXIII concentrations, this resulted in γ A fibrin matrices with a similar fiber thickness as γ' fibrin matrices. This observation is in line with Collet *et al.*, who also observed no significant differences in fiber thickness when FXIII was supplemented to γ A fibrin.⁹

The *in vitro* endothelial cell adhesion, proliferation, and migration showed no significant differences between γ A and γ' fibrinogen coatings. We had expected differences, since previously we showed an altered migration and proliferation on high molecular weight and low molecular weight fibrinogen coatings.¹³ These data were consistent with the *in vitro* endothelial cell tube formation in three-dimensional fibrin matrices.^{12,25} The results of our study indicate a minor influence of the C-terminus of the γ chain on endothelial cell interactions. These results are in accordance with a previous report.²⁶

The *in vitro* tube formation showed more tube-like structures and higher fibrinolysis of HMVECs in γ A fibrin than in γ' fibrin. γ' Fibrin facilitated controlled tubular structure

FIG. 6. Revascularization of full-thickness rat wounds treated with fibrinogen or placebo on days 7 and 21. The symbol □ gives data of placebo-treated wounds ($n=6$) and ▨ fibrin-treated wounds ($n=17$). * p < 0.05.

FIG. 7. Representative images of *in vivo* wound closure of the wounds. Color images available online at www.liebertpub.com/tea

formation, whereas γ A fibrin showed a massive fibrinolysis and facilitation of HMVEC sprouting. The increased fibrinolysis rate was confirmed with higher levels of fibrin degradation products in the conditioned media of γ A fibrin matrices. Lysis of the fibrinous matrix is a prerequisite for invasion of endothelial cells, and thus for angiogenesis. The *in vitro* tube formation assay is mainly dependent on plasmin-dependent fibrin degradation.²⁷ Differences in fiber thickness do not explain the effects on angiogenesis and fibrinolysis, since fiber thickness was similar in our γ A and γ' fibrin matrices. However, we did observe that γ A fibrin matrices were more opaque than γ' fibrin matrices. Also, the higher number of branch points and free fiber ends in γ A fibrin matrices, as previously reported by Gersh *et al.*,⁸ may play a role in the observed angiogenic and fibrinolytic effects.

Our *in vivo* experiments, using a full-thickness rat wound healing model, showed improved vascularization during wound healing when the wound was covered with fibrin. Several measures of wound healing, such as the trend to-

ward a decreased number of infiltrating fibroblasts and the trend toward an increased number of inflammatory cells, pointed toward an improved healing. Other measures, like breaking strength, were similar in placebo- and fibrin-treated wounds. An explanation for the apparent discrepancy in results of the parameters may be found in the timing of the experiment. We choose to sacrifice the rats after 7 and 21 days, which was the moment that all wounds had closed. At this stage of wound maturation, the breaking strength was still only 10% of normal skin. On the other hand, when vascularization is considered, blood vessel condensation is more advanced in fibrin-treated wound at this stage. This may indicate improved wound repair. Another factor that may flatten the differences between placebo and fibrin (variants) is that the natural wound healing process is highly competent in normal rats. Therefore, a major improvement cannot be expected with fibrin matrices. Choosing a model in which wound healing is disturbed, such as the diabetic db/db mouse,²⁸ or the streptozotocin diabetic rat, may better

FIG. 8. Representative images of the CD34 immunohistochemistry staining of control wounds and wounds treated with unfractionated, γ A fibrin, and γ' fibrin at day 21. Color images available online at www.liebertpub.com/tea

FIG. 9. Inflammation score in full-thickness rat wounds on day 21 (blank $n=6$, unfractionated $n=5$, γ A fibrin $n=6$, and γ' fibrin $n=6$). * $p < 0.05$.

elucidate differences in improving wound healing of the different fibrinogen variants.

In conclusion, the use of fibrin and fibrin variant matrices improves multiple parameters of wound healing and offers an interesting methodology to stimulate the wound healing process, with promising stimulatory effects for purified γ A fibrin.

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Disclosure Statement

No competing financial interests exist.

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