A comparison of endothelial cell characteristics on PER.C6®-derived recombinant and plasma-derived human fibrinogen

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Preliminary data
Abstract

Background: Generic advantages of recombinant proteins are their improved quality with respect to purity and homogeneity as well as product consistency and safety. Recombinant fibrinogen and plasma-derived fibrinogen show similar biochemical properties and fibrin structures. Recombinant fibrinogen an interesting potential scaffold for tissue engineering or direct therapeutic application for wound healing. Thus far, the cellular interactions of endothelial cells on recombinant fibrinogen, known to be pivotal in the described processes, have not yet been investigated.

Objectives: In this study the functional endothelial cell characteristics on recombinant and plasma-derived HMW-fibrinogen were evaluated.

Methods and Results: A production platform yielding high levels of human recombinant fibrinogen in chemically defined medium was set up in PER.C6® cells. Characterization of recombinant fibrinogen showed undegraded fibrinogen, free of commonly co-purified plasma components, with correct polymerization and cross-linking characteristics. Importantly, the adhesion and proliferation of endothelial cells on recombinant and plasma-derived HMW-fibrinogen were similar. Also in vitro tube formation developed to the same extent on recombinant and plasma-derived HMW-fibrin, although it was observed that for certain conditions the cell-mediated fibrinolysis was more extensive on recombinant HMW-fibrin.

Conclusions: In the present study, we described a production platform yielding high levels of recombinant HMW-fibrinogen and showed that the endothelial cell characteristics on recombinant HMW-fibrinogen are very similar to plasma-derived HMW-fibrinogen. These findings provide new information on endothelial cell characteristics on recombinant fibrinogen, which can be directive for the development of tissue engineering applications.
Introduction

Fibrinogen is a soluble plasma protein composed of two sets of three polypeptide chains (A\(\alpha\)\(_2\), B\(\beta\)\(_2\) and \(\gamma\)\(_2\)) that are linked together by 29 disulfide bonds. The fibrinogen chains are encoded by separate genes in the fibrinogen gene cluster that comprises approximately 50 kb and is located on chromosome 4q23-32\(^1,2\). Fibrinogen is primarily synthesized in the liver, but lung epithelial cells can also produce small proportions\(^3\). Plasma fibrinogen is a heterogeneous mixture of fibrinogen variants, which can alter the hemostatic responses and cellular characteristics\(^4-6\). The most abundant naturally occurring fibrinogen variants in the circulation of healthy individuals, are the result of either alternative mRNA processing or proteolytic degradation. Two examples of alternative mRNA processing are A\(\alpha\)-Extended fibrinogen (\(\alpha\)E, 420 kDa, 1 to 3% of total fibrinogen) and \(\gamma\)A/\(\gamma\)′- and \(\gamma\)′/\(\gamma\)′-fibrinogen (5 to 8% of total fibrinogen)\(^7,8\). In \(\alpha\)E-fibrin the structure is more dense and composed of highly branched, thin fibers\(^7\). The fibrinogen variants \(\gamma\)A/\(\gamma\)′ and \(\gamma\)′/\(\gamma\)′ showed altered interactions with thrombin, platelets and FXIIIa, next to its more dense fibrin structure\(^5\). In addition, proteolytic degradation of fibrinogen in the circulation results in multiple molecular weight variants. High molecular weight fibrinogen (HMW, 340 kDa, ~70% of total fibrinogen) becomes partially degraded in the circulation, which results in low molecular weight fibrinogen (LMW, 305 kDa, ~24% of total fibrinogen) that lacks the C-terminus of one of its A\(\alpha\)-chains, and low molecular weight fibrinogen (LMW′, 270 kDa, 6% of total fibrinogen) that is partially degraded at both A\(\alpha\)-chains\(^6\). In HMW-fibrin the structure is more open and composed of thick fibers when compared to LMW-fibrin\(^6\). HMW-fibrin facilitates an increased and accelerated ingrowth, proliferation and 2D-migration of endothelial cells, when compared to LMW-fibrin\(^6,10\).

These naturally occurring fibrinogen variants might in the future be used for tissue engineering applications. However, the isolation of individual fibrinogen variants from donor plasma is difficult due to the high degree of similarity between the variants and relatively low plasma concentrations for some fibrinogen variants.

Studies conducted on angiogenesis and tissue engineering almost exclusively use plasma-derived fibrinogen preparations, here the intrinsic heterogeneity of these preparations can influence the results. Moreover, plasma-derived fibrinogen often includes co-purified plasma components; examples are contaminations with factor XIII (FXIII), albumin, fibronectin, growth factors, enzymes and inhibitors\(^11,12\). Heterogeneity and co-purified plasma components can be avoided by using recombinant preparations. Generic advantages of recombinant fibrinogen are their improved quality with respect to purity and homogeneity as well as product consistency and safety. The recombinant fibrinogen production has been previously described employing baby hamster kidney (BHK)\(^13\) cells, African green monkey kidney cells (COS-1)\(^14\) and Chinese hamster ovary (CHO-K1) cells\(^15\). Recombinant fibrinogen that was produced in CHO cells, showed remarkably similar biochemical properties and fibrin structures to plasma-derived fibrinogen and can serve as a functional \textit{in vitro} model for plasma fibrinogen\(^16\). Recombinant fibrinogen is predominantly produced in its native form with six intact polypeptide chains; HMW-fibrinogen. In addition, other naturally occurring variants can be produced, as was previously shown for the variants; fibrinogen \(\gamma\)A/\(\gamma\)′ and \(\gamma\)′/\(\gamma\)′\(^17,18\), and the dysfibrinogenemia variants fibrinogen\(\text{Vlissingen/Frankfurt IV}\)\(^19\) and fibrinogen\(\text{Matsumoto VII}\)\(^20\). Besides these naturally occurring variants, engineered variants can be produced, for instance with altered fibrinopeptides\(^21,22\).
To investigate the effects of recombinant HMW-fibrinogen (Aα₂Bβ₂γ₂) on functional endothelial cell characteristics, a recombinant fibrinogen production platform was established in human PER.C6® cells. These cells grow in suspension and showed high protein productions with low levels of degradation\textsuperscript{23,24}. In contrast, inhibition of proteolysis during culture with aprotinin (trasylol) was obligatory for the recombinant fibrinogen production in CHO cells, as well as during subsequent fibrinogen purification\textsuperscript{15}. Protease inhibition was not required during tissue culture for recombinant fibrinogen production by PER.C6® cells, nor during subsequent downstream purification.

Scaffolds based on recombinant fibrinogen hold a promising potential in applications for tissue engineering, especially when specific variants can be used to alter the vascularization of scaffolds. Until now, the data on cellular interactions with recombinant fibrin(ogen) are limited to the adhesion of human fibroblasts and platelets\textsuperscript{25,26}. PER.C6®-derived recombinant fibrinogen was used to gain more insight in the endothelial cell characteristics on recombinant fibrinogen. Next to the exploration of tissue engineering possibilities, also a more fundamental question is answered, namely HMW-fibrinogens role in facilitating endothelial tube formation. With recombinant fibrinogen one can control the concentration of relevant plasma factors that normally co-purify with plasma-derived fibrinogen. Functional endothelial cell characteristics were subsequently studied on recombinant HMW-fibrinogen and compared to plasma-derived unfractionated- and HMW-fibrinogen.

**Materials and methods**

*Construction of the expression vectors*

cDNAs encoding the fibrinogen Aα-, Bβ- and γ-chains were synthetically engineered (GeneArt, Regensburg, Germany) according to consensus sequences as archived in the NCBI nucleotide databank (Aα-chain NM_021871; Bβ-chain NM_005141; γ-chain NM_000509) and cloned into pcDNA3.1(+) expression vectors (Invitrogen, Breda, The Netherlands), the gene expression was driven by the cytomegalovirus (CMV) promotor. Correct insertion and coding sequences were confirmed by DNA-sequence analysis (ServiceXS, Leiden, The Netherlands).

*Cell culture, transfection and recombinant fibrinogen isolation*

PER.C6® cells were cultured in suspension according to standard procedures, as previously described\textsuperscript{27}. The cells were co-transfected with 3 constructs, each coding for one of the individual fibrinogen chains, using electroporation\textsuperscript{27}. One day post transfection, the cells were seeded in densities of 1000, 3000 & 10.000 cells per well in medium containing 125 µg/mL G418 (Invitrogen, Breda, The Netherlands) as a selection agent. G418-resistant, clonal growing cell-lines were selected and individually expanded. During this expansion period, the culture supernatants were screened for intact recombinant fibrinogen using G8-Y18 fibrinogen ELISA (see below) and western blotting under reducing conditions using Y18-HRP. Y18 binds the intact N-terminus of the Aα-chain and thereby detects any C-terminal degradation of the Aα-chains. The selected cell-lines were adapted to grow under shear conditions in shaker flasks. Successfully adapted cell-lines with population doubling times below 40h were again screened for expression
of intact recombinant fibrinogen. The stability of growth and fibrinogen production was tested during 50 population doublings. After this selection procedure, the remaining clones were subjected to subcloning by limiting dilution. Briefly, cells were plated at 0.3, 1.0 and 3.0 cells per well in medium without G418. Subsequently, a stringent selection procedure was employed as described before.

The best performing monoclonal cell-lines were used to develop a production platform in chemically defined medium in stirred bioreactors, employing a fed batch strategy. Recombinant fibrinogen was purified from clarified tissue culture harvest using both cationic and anionic column chromatography. Subsequent concentration and formulation was performed employing diafiltration.

Recombinant fibrinogen detection during cell-line generation
Recombinant fibrinogen concentrations in conditioned media were determined using an enzyme-linked immunosorbent assay (ELISA). The monoclonal antibodies G8 and Y18 (TNO, Leiden, The Netherlands) were used to detect intact fibrinogen Aα-chains, with plasma-derived fibrinogen (Fib3, Enzyme Research Laboratories, Swansea, UK) as reference. Monoclonal antibody G8, which recognizes the fibrinogen Aα-chain C-terminus, was used as catching antibody. The monoclonal antibody Y18 conjugated with horseradish peroxidase (HRP), binding the fibrinogen Aα-chain N-terminus, was used as tagging antibody. The coloring reaction was performed using 3,3′,5,5′-tetramethylenyldiamine (TMB Ultra, Thermofisher Scientific, Rockford, IL, USA) and stopped with 1 M H₂SO₄. The optical density at 450 nm was measured using a microplate reader (Labsystems Multiskan RC, Helsinki, Finland).

Proteins in clarified conditioned media were size separated under reducing conditions employing SDS-PAGE (NuPAGE Novex 10% Bis-Tris Gel, Invitrogen), followed by identification of the Aα-chain by western blotting using the monoclonal antibody Y18 conjugated with HRP. Protein bands were detected using a chemiluminescence substrate (ECL, Thermofisher Scientific) and digital imaging was performed on ChemiDoc-It (UVP, Upland, CA, USA).

Recombinant fibrinogen quality
After purification from the tissue culture harvest with both cationic and anionic column chromatography and subsequent concentration, recombinant fibrinogen was structurally and functionally characterized. Purified recombinant fibrinogen was size separated, both under non-reducing and reducing conditions, using SDS-PAGE (NuPAGE Novex 10% Bis-Tris Gel, Invitrogen), followed by total protein staining with Coomassie brilliant blue.

Individual chains of recombinant fibrinogen were also identified by western blotting using a polyclonal rabbit anti-human-fibrinogen antibody (Zebra biosciences, Enschede, The Netherlands) and monoclonal Y18 antibody, and visualized using ECL substrate, as described above.

The thrombin induced fibrin polymerization was evaluated monitoring the optical density increase at 350 nm in time. The polymerization of 1 mg/mL fibrinogen was initiated with 1 U/mL α-thrombin (Enzyme Research Laboratories) in the presence of 2 mM Ca²⁺. Plasma-derived fibrinogen (Fib3, Enzyme Research Laboratories) was used as a control.
The FXIIIa cross-linking susceptibility was evaluated including FXIIIa (HFXIII, Enzyme Research Laboratories) in the thrombin induced polymerization reaction and subsequent solubilization of the fibrin polymer in 8 M Urea. Covalent cross-linking of the α- and γ-chains was evaluated using SDS-PAGE and total protein staining with Coomassie brilliant blue.

**Functional endothelial cell characteristics on recombinant fibrinogen**

Human umbilical vein endothelial cells (HUVEC) from six independent donors and human microvascular endothelial cells (HMVEC) from three independent donors were isolated, cultured and characterized as previously described\(^2^8,2^9\). Fibrinogen coatings were prepared on polystyrene tissue culture plates using 100 µl/cm\(^2\) with 0.2 µM fibrinogen in PBS for 1h at 37°C, followed by consecutive washing with PBS\(^1^0\). HUVEC were seeded on fibrinogen coatings at 2x10\(^4\) cells/cm\(^2\) in Medium 199 (M199) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 1% human serum albumin (HSA, Sanquin, Amsterdam, The Netherlands). After 4h, non-adhering cells were washed away and adhered cells were counted using image analysis (Image J, NIH, Bethesda, USA).

The proliferation rate was evaluated using \(^3\)H-thymidine incorporation, adapted from van Hinsbergh et al.\(^3^0\). HUVEC (6x10\(^3\) cells/cm\(^2\)) were seeded on fibrinogen coatings in M199 supplemented with p/s and 1% HSA. After 4h, the cells were stimulated to proliferate for 72h by addition of 10 ng/mL vascular endothelial growth factor (VEGF) in M199 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin (p/s) and 10% heat-inactivated newborn calf serum (NBCSi). 16h before termination 1 µCi \(^3\)H-thymidine was added per cm\(^2\) HUVEC. The beta-emission, resulting from \(^3\)H-thymidine incorporated into the cellular DNA, was quantified in TCA-treated and sodium hydroxide dissolved cell preparations by liquid scintillation counting using Ultima Gold as a scintillation fluid on Scintillation Analyzer (Packard Bioscience, Massachusetts, USA). The data of six independent HUVEC donors was obtained in triplicate wells.

*In vitro* angiogenesis was evaluated using 3-dimensional fibrin matrices and HMVEC, as previously described\(^3^1\). Prior to fibrin polymerization, all fibrinogen preparations were dialyzed to M199 supplemented with p/s. Fibrin matrices were prepared by addition of α-thrombin (0.1 IU/mL, Organon Technika, Boxtel, The Netherlands) to 2 mg/mL fibrinogen and 2.5 U/mL Factor XIII (Fibrinogamma P, CSL Behring, Marburg, Germany) in M199 medium. 4h after the initiation of polymerization, thrombin was inactivated by a 2h incubation with M199 supplemented with 10% heat-inactivated human serum (HSi) and 10% NBCSi. HMVEC were seeded confluently on the fibrin matrices. After 24h, and subsequently at 48h intervals, the HMVEC were stimulated with the combination of 25 ng/mL VEGF (Invitrogen), 10 ng/mL fibroblast growth factor-2 (FGF-2, Preprotech, London, UK) and 10 ng/mL tumor necrosis factor-α (TNFα, Sigma Aldrich) in M199 supplemented with 10% HSi and 10% NBCSi. After 5 days the *in vitro* tube formation was ended and structures were fixated. The tube-like structures in fibrin were analyzed by phase-contrast microscopy and their length was quantified using Optimas image analysis software (Media Cybernetics, Bethesda, USA). The data of three independent HMVEC donors was obtained in triplicate wells. Fibrin degradation products in conditioned culture media (first 48h culture period) were determined using FDP14-DD13 (TNO, Leiden, The Netherlands) ELISA, as previously described\(^3^2\).
Statistical analysis
Statistical analysis was performed using paired Student t-test or One-way ANOVA with Bonferroni post-hoc test. Numbers and significant P-values are indicated in the text or figures, P< 0.05 was considered significant. Results are shown as mean ± SEM.

Results

Vector construction and expression
The cDNAs for the individual fibrinogen chains were cloned in separate pcDNA3.1(+) expression vectors. The Aα-chain cDNA codes for a 629 amino-acids polypeptide (isoform 2) and resulted in the production of a mature Aα-chain of 610 amino-acids. The Bβ-chain cDNA codes for a 491 amino-acids polypeptide and resulted in the production of a mature Bβ-chain of 461 amino-acids. The γ-chain cDNA codes for a 437 amino-acids polypeptide (gamma-A) and resulted in the production of a mature γ-chain of 411 amino-acid. DNA sequence analysis confirmed that the vectors contained the consensus cDNA sequences, as archived in the NCBI nucleotide databank (Aα-chain NM_021871; Bβ-chain NM_005141; γ-chain NM_000509). After transfection of PER.C6® cells, 300 clones were initially selected and expanded based on growth characteristics only. After 3 consecutive screening rounds, based on growth, production level and product quality, 30 cell-lines were selected for adaptation to shear conditions in shaker flasks. After stability testing during 50 population doublings, 4 cell-lines were subjected to sub-cloning by limiting dilution without selective pressure. Finally, 4 sub-clones were identified, following the earlier described selection strategy. The best sub-clone displayed a population doubling time below 30h and expressed undegraded intact recombinant fibrinogen at 0.6 g/L for more than 50 generations in the absence of selective pressure.

Recombinant fibrinogen characterization
Western blotting of total fibrinogen showed intact fibrinogen and individual Aα-, Bβ- and γ-chains for recombinant fibrinogen at the expected molecular masses after coomassie staining (Figure 1A,B). Western blot analysis with polyclonal anti-human fibrinogen antibody also showed the individual fibrinogen chains (Figure 1C). In order to demonstrate the undegraded nature of recombinant fibrinogen, western blotting was also performed using the Y18-HRP antibody. Full length Aα-chains were detected with an approximate mass of 66.5 kDa which typifies the intact Aα-chain. No measurable amounts of truncated Aα-chains were detected in recombinant fibrinogen (Figure 1D). Similar results were obtained after thrombin-induced fibrin polymerization and subsequent turbidity increase as determined by optical density measurements for recombinant and plasma-derived fibrinogen (Figure 2A). This shows the comparable polymerization and turbidity of recombinant and plasma-derived fibrin matrices. Moreover, the FXIIIa cross-linking was evaluated on SDS-PAGE after solubilization of the fibrin matrices that were generated in the absence and presence of activated FXIII. The results show FXIIIa induced α-oligomers and γ-dimers (Figure 2B). The γ-γ cross-linking is demonstrated by the conversion of the γ-monomer band into γ-dimers with a molecular mass of ~90 kDa. For fibrin generated from plasma-derived fibrinogen the presence of α-oligomers and γ-dimers are apparent without the
addition of FXIII, indicating the presence of FXIII in this plasma-derived fibrinogen preparation. The residual FXIII in plasma-derived fibrinogen will be activated during fibrin polymerization via the presence of thrombin and calcium.

Figure 1. Characterization of recombinant fibrinogen. Plasma-derived (pFbg) and recombinant fibrinogen (rec-Fbg) were separated on SDS-Page gels under non-reducing (A) and reducing conditions (B) and all proteins were stained with coomassie brilliant blue. Western blotting on samples separated under reducing conditions with polyclonal rabbit anti-human-fibrinogen antibody staining for total fibrinogen (C) and monoclonal Y18 antibody staining for Aα-chain species (D; detecting possible degradation).
Endothelial cell adhesion and proliferation

To compare the endothelial cell adhesion and proliferation, HUVEC were cultured on plasma-derived unfractionated- and HMW-fibrinogen and recombinant HMW-fibrinogen coatings. No significant differences were found in the number of endothelial cells that adhered in 4h to plasma-derived unfractionated-, plasma-derived HMW- and recombinant HMW-fibrinogen coatings (Figure 3A, n=6).

Both plasma-derived HMW- and recombinant HMW-fibrinogen coatings supported a significantly increased growth factor induced HUVEC proliferation (30%), when compared to gelatin (Figure 3B, P<0.05, n=6). The proliferation of HUVEC on plasma-derived HMW- and recombinant HMW-fibrinogen coatings was not different from the HUVEC proliferation on plasma-derived unfractionated-fibrinogen coatings. No differences were found in the adhesion and

Figure 2. Polymerization characteristics of recombinant fibrinogen. (A) The thrombin induced polymerization of plasma-derived (squares) and recombinant fibrinogen (circles) at 1 mg/mL with 1 U/mL thrombin was assessed monitoring the optical density (OD) at 350 nm. (B) SDS-PAGE of solubilized fibrin (0.75 mg/mL) after thrombin induced polymerization either in the absence or presence of thrombin (5 U/mL) and/or FXIIIa (0.05 U/mL). Total protein was stained with coomassie brilliant blue. Thrombin induced removal of fibrinopeptides A and B, resulting in desA and desB fibrinogen. Cross-linking with FXIIIa resulted in α-oligomers and γ-dimers. In plasma-derived fibrin, γ-dimers and α-oligomers are also generated after addition of only thrombin. Albumin was present in the dilution buffer of thrombin.
Endothelial cell characteristics on recombinant fibrinogen

Figure 3. HUVEC adhesion, proliferation and morphology, on recombinant fibrinogen coatings. The number of adhering cells (A, n=6) during 4h and the proliferation (B, n=6) during 72h, assessed by ³H-thymidine incorporation on gelatin, plasma-derived unfractionated-fibrinogen, plasma-derived HMW-fibrinogen and recombinant HMW-fibrinogen coatings were determined. (C) Representative pictures of HUVEC morphology on plasma-derived and recombinant HMW-fibrinogen coatings. Bars indicated in the figures represent 200 µm. All quantitative data are expressed as mean ± SEM of six independent HMVEC donors in triplicate wells.

proliferation of HUVEC on plasma-derived HMW- and recombinant HMW-fibrinogen. In addition, no differences in cell morphology were observed upon culture of HUVEC on plasma-derived and recombinant HMW-fibrinogen coatings (Figure 3C).

In vitro tube formation
Prior to the experiments, the fibrin polymerization and cell adherence to 3-dimensional recombinant fibrin matrices was determined. Fibrin matrices generated from recombinant HMW-fibrinogen and subsequent seeding of HMVEC showed contraction after 24h in the absence of 2.5 U/mL FXIII, while plasma-derived fibrin matrices maintained their morphology (data not shown). Cross-linking fibrin matrices with FXIIIa has been shown to increase the stability of the fibrin network. As FXIII is already present in plasma-derived fibrinogen, equal amounts of FXIII for all conditions were employed in the in vitro tube formation assay, to avoid matrix contraction.
Representative images of both the morphology and amount of HMVEC tube-like structures in plasma-derived and recombinant fibrin matrices are shown (Figure 4A). Wide and more open tube-like structures of HMVEC with significant amounts of sprouting cells were observed in recombinant HMW-fibrin matrices. The tube-like structures that were observed in recombinant HMW-fibrin were relatively unstable with a more fibrinolytic phenotype (according to earlier personal observations). The tube formation, induced by simultaneous stimulation with VEGF, FGF-2 and TNFα was increased on fibrin matrices prepared from plasma-derived HMW-fibrin (344 ± 136%) and recombinant HMW-fibrin (350 ± 131%), when compared to its counterparts on plasma-derived unfractionated-fibrin (normalized at 100%, Figure 4B, n=3). Similar results were obtained after stimulation of HMVEC tube formation with the combination of VEGF and TNFα or FGF-2 and TNFα (data not shown). The endothelial cell mediated fibrinolysis of recombinant HMW-fibrin, as determined by the quantification of fibrin degradation products in culture supernatant from 0-48h, which was higher than for plasma-derived unfractionated- and plasma-derived HMW-fibrin (Figure 4C, n=2). The degradation of plasma-derived fibrin during this time interval was 0.01%, whereas the degradation of recombinant fibrin was 0.5% of the total amount of fibrin.

Figure 4. In vitro tube formation of HMVEC in recombinant fibrin. (A) Representative pictures of HMVEC from a single donor after in vitro tube formation in plasma-derived unfractionated-fibrin, plasma-derived HMW-fibrin and recombinant HMW-fibrin. Bars indicated in the figures represent 200 µm. Quantitative data are expressed as mean ± SEM of three independent HMVEC donors in triplicate wells. (B) Quantification of the in vitro tube formation, the results are expressed as percentage of tube formation of HMVEC on plasma-derived unfractionated-fibrin matrices (n=3, 100% is 54.8 ± 13 mm/cm²). (C) Fibrin degradation products measured with FDP14-DD13 ELISA in conditioned HMVEC media from the in vitro tube formation assay (0-48h, n=2).
Endothelial cell characteristics on recombinant fibrinogen

The tube-like structures in recombinant HMW-fibrin matrices were stabilized by increasing the concentration of either fibrin or Factor XIIIa. The tube-like structure formation and fibrinolysis of recombinant HMW-fibrin were compared to plasma-derived HMW-fibrin matrices formed at concentrations of 1.5, 2.0 and 3.0 mg/mL fibrinogen. The elevated fibrinolysis and widened tube-like structures, as observed by microscopic imaging, were clearly stabilized when 3.0 mg/mL recombinant HMW-fibrin was used, here more stable tube-like structures were observed (Figure 5A). The stabilization of fibrinolysis was also quantified, as the levels of fibrin degradation products in the supernatant decreased by 27% when 3.0 mg/mL of recombinant HMW-fibrin was used (Figure 5B,C). In addition, the tube-like structure formation in recombinant HMW-fibrin was stabilized in recombinant HMW-fibrin when more Factor XIII, 12.5 U/mL instead of 2.5 U/mL, was used (data not shown). Although the matrix composition might require additional fine tuning, recombinant HMW-fibrinogen typifies plasma-derived HMW-fibrinogen, with respect to endothelial cell adhesion, proliferation and in vitro tube formation, wherein, the fibrinolytic phenotype could be attenuated by increasing the fibrinogen or Factor XIII concentrations.

Discussion

The present study shows that recombinant HMW-fibrinogen elicits similar endothelial cell responses as plasma-derived HMW-fibrinogen. Fully assembled undegraded recombinant fibrinogen can be produced on large scale by PER.C6® cells and isolated from culture media. Fibrinogen characterization shows comparable polymerization and FXIIIa cross-linking of recombinant and plasma-derived fibrinogen. However, the plasma-derived fibrinogen preparation already contains co-purified FXIII, which induces cross-linking during fibrin polymerization under basal conditions. The endothelial cell adhesion and proliferation are similar on recombinant and plasma-derived HMW-fibrin matrices. In addition, in vitro tube formation in recombinant and plasma-derived fibrin is similar after matrix stabilization. The cell-mediated fibrinolysis is higher on recombinant HMW-fibrin, compared to plasma-derived HMW-fibrin. Increasing the fibrinogen or FXIII concentrations results in an attenuated fibrinolysis, both morphologically and in the amount of fibrin degradation products.

The production of recombinant fibrinogen in CHO cells has previously been well-characterized by Lord et al.15,21,34 and Gorkun et al.16. Due the incorporation of sufficient protease inhibiting measures, functional intact fibrinogen is produced by CHO cells. Until now, evaluation of the functional endothelial cell characteristics was not employed for any type of recombinant fibrinogen. The human PER.C6® cells as described here, secrete undegraded recombinant fibrinogen in a stable manner at levels more than a 100-fold higher than reported for CHO cells, without the requirement for extensive protease inhibition. This new recombinant fibrinogen platform provides sufficient amounts for high turnover 3-dimensional studies on functional endothelial cell characteristics and subsequent high consumptions with in vivo studies.

The endothelial cell responses and angiogenesis are pivotal to wound healing and tissue engineering. A temporary fibrin matrix is formed during wound healing or when blood plasma leaks into tissues. Fibrin not only acts as a barrier preventing blood loss, but also provides new adhesion sites and spatial information that play a role in cell migration and formation of new
capillaries. In the present study, the adhesion, proliferation and tube formation of human endothelial cells occurred at similar rates using both recombinant and plasma-derived HMW-fibrinogen. Increased tube formation was observed in recombinant HMW-fibrin, when compared to plasma-derived unfractionated-fibrin, as was previously demonstrated for plasma-derived HMW-fibrin. The cell-mediated fibrinolysis of recombinant HMW-fibrin was initially elevated, resulting in morphologically different tube-like structures. This difference in tube-like structure morphology was corrected after stabilization of the fibrin matrices with either increased fibrinogen or FXIII concentrations.

Figure 5. The effect of the fibrinogen concentration on in vitro tube formation by HMVEC. (A) Representative pictures of HMVEC from a single donor after in vitro tube formation in fibrin matrices, prepared at fibrinogen concentrations of 1.5, 2.0 and 3.0 mg/mL for both plasma-derived and recombinant HMW-fibrin. Bars indicated in the figures represent 200 µm. All quantitative data are expressed as mean ± SD of HMVEC from a single donor in triplicate wells. (B) Quantification of the in vitro tube formation in the various fibrin matrices (n=1). (C) Fibrin degradation products measured with FDP14-DD13 ELISA in conditioned HMVEC media from the in vitro tube formation assay (n=1).
The formation of tube-like structures by endothelial cells requires proteolysis by cell-bound proteases and cell-matrix interactions\textsuperscript{36}. Proteolysis depends on receptor-bound urokinase-type plasminogen activator (uPA) and plasmin activity and/or the action of membrane-type matrix metalloproteinases\textsuperscript{37-40}. In our \textit{in vitro} tube formation assay, the proteolysis that is accompanied by tube formation, is mainly dependent on uPA/uPAR and plasmin activity\textsuperscript{41}. Plasma-derived fibrinogen preparations contain blood-originated co-purified components, which are absent in recombinant fibrinogen preparations. The plasma components can be responsible for suppression of the ‘fibrinolytic phenotype’ by attenuating the cell-bound proteolysis of plasmin. α2-antiplasmin is one possible candidate, this fibrinolysis inhibitor can be detected cross-linked to fibrinogen by FXIIa\textsuperscript{42-44}. Thrombin activatable fibrinolysis inhibitor (TAFIa) is also able to inhibit fibrinolysis by preventing the positive feedback in plasmin generation\textsuperscript{45}. Although direct binding of TAFI to fibrinogen is not described, TAFI can bind plasminogen and plasmin and thereby fibrin(ogen)\textsuperscript{46}. The presence of the proteolysis inhibitors α2-antiplasmin and TAFI in plasma-derived fibrinogen preparations can be shown using fibrinolysis assays, either by measuring fibrin degradation products and/or the influence on turbidity changes during tissue-type plasminogen activator-mediated proteolysis by plasmin, as was previously described\textsuperscript{47}.

The results presented here, emphasize the exclusive contribution of HMW-fibrin to the angiogenic process. Previously, plasma-derived HMW-fibrin was shown to facilitate an increased angiogenesis, when compared to total unfractionated plasma-derived fibrin. In the present study, plasma component-free recombinant HMW-fibrin shows to harness similar characteristics as plasma-derived HMW-fibrin, therefore, HMW-fibrin manifests as an appropriate matrix to facilitate angiogenesis. The similar functional endothelial cell responses on both recombinant HMW-fibrin and plasma-derived HMW-fibrin underscore the capacity of HMW-fibrin to serve as an optimal matrix for endothelial cell tube-like structure formation.

Numerous therapeutic applications for fibrin are currently in development, amongst which several FDA-approved fibrin sealants\textsuperscript{48,49}. However, all current fibrinogen sealants are sourced from human plasma and contain a heterogeneous mixture of fibrinogen variants and co-purified plasma components. In the present study, we describe a platform for recombinant HMW-fibrinogen production and show that recombinant HMW-fibrinogen typifies plasma-derived HMW-fibrinogen for the endothelial cell characteristics. Producing recombinant fibrinogen variants, using the PER.C6\textsuperscript{®} platform will facilitate a more detailed exploration of these phenomena and could deliver recombinant fibrinogen preparations with unique therapeutic potentials.

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Reference List


