Thrombosis Research 131 (2013) 89–93

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Differential regulation of fibrinogen γ chain splice isoforms by interleukin-6

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ARTICLE INFO

Article history:
Received 18 May 2012
Received in revised form 14 September 2012
Accepted 20 September 2012
Available online 1 October 2012

Keywords:
Cytokines
Expression
Fibrinogen
Inflammation
Liver

ABSTRACT

Introduction: Fibrinogen is a major structural protein in blood clots, and is also a well-known acute phase reactant. The γ chain gene of fibrinogen has two alternative splice variants, γA and γ' chains. γ' fibrinogen constitutes about 7% of total fibrinogen. Total fibrinogen levels and γ' fibrinogen levels have been associated with cardiovascular disease, but the mechanisms regulating the production of the two isoforms are unknown. Several inflammatory cytokines are known to influence the production of total fibrinogen, but the role of cytokines in the production of γ' fibrinogen has not been examined. However, epidemiologic studies have shown an association between γ' fibrinogen levels and inflammatory markers in humans.
Materials and methods: The expression of γ' fibrinogen and total fibrinogen by HepG2 liver cells was quantitated after treatment with interleukin-1β, transforming growth factor-β, tumor necrosis factor-α, and interleukin-6.
Results: Interleukin-1β, transforming growth factor-β, and tumor necrosis factor-α, known down-regulators of total fibrinogen synthesis, also downregulate γ' fibrinogen synthesis in HepG2 cells. However, interleukin-6 differentially up-regulates the production of total and γ' fibrinogen, leading to a 3.6-fold increase in γA mRNA, but an 8.3-fold increase in γ' mRNA.
Conclusions: These findings indicate that γ' fibrinogen is disproportionately up-regulated by inflammatory responses induced by interleukin-6.

Introduction

γ' fibrinogen is a newly-emerging risk factor for cardiovascular disease, including coronary artery disease [1], myocardial infarction [2–4], and stroke [5]. Recent studies have also shown a significant association between γ' fibrinogen and CRP [4,6], a marker of inflammation that is associated with cardiovascular disease [7]. In particular, patients from the PAVE study who had both periodontal inflammation and a history of coronary artery blockage of 50% or greater had an increased ratio of γ' fibrinogen/total fibrinogen. Whereas γ' fibrinogen typically constitutes about 7% of total fibrinogen in normal individuals [1,8], γ' fibrinogen constituted 18.6% of total fibrinogen in the PAVE cohort. In addition, γ' fibrinogen levels were significantly associated with CRP levels ($P<0.001$) [6]. These results suggest that γ' fibrinogen may display differential regulation from total fibrinogen in the presence of combined cardiovascular and periodontal disease.

The γ' isoform arises from an alternative splicing event within intron 9 where intron 9 is cleaved before it can be spliced out, resulting in the loss of exon 10 and the retention of a portion of intron 9 in the mRNA. Translation of this mRNA isoform reads through the end of exon 9 into intron 9 (also known as exon 9a), leading to a 20 amino acid carboxyl-terminal extension, VRPEHPAETEYDSLYPEDDL, that is not found in the γA chain [9,10]. The γ' chain harbors unique properties, and when incorporated into a mature fibrinogen molecule can lead to clots with altered architecture that are more resistant to breakdown [11].

The regulation of total human fibrinogen expression by cytokines has been investigated in past studies using the HepG2 hepatocellular carcinoma cell line [12]. This model system faithfully recapitulates fibrinogen expression, including γ' fibrinogen [13] and has been

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CRP, C-reactive protein; EBSS, Earle's balanced salt solution; ELISA, enzyme-linked immunosorbant assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; IL-1β, interleukin-1β; IL-6, interleukin-6; MEM, minimal essential medium; PBS, phosphate-buffered saline; RT-PCR, real-time polymerase chain reaction; SE, standard error of the mean; TGF-β, transforming growth factor-β; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF-α, tumor necrosis factor-α.

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http://dx.doi.org/10.1016/j.thromres.2012.09.017

used successfully to study fibrinogen production and regulation *in vitro* [14]. We therefore investigated whether inflammatory cytokines can act directly on HepG2 cells to dysregulate the expression of γ' fibrinogen.

Materials and Methods

Cell Culture

HepG2 cells (ATCC, Manassas, VA), between passage three and seven, were grown in 6-well plates to 70% confluence in HyClone MEM/EBSS (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum/50 units/ml penicillin/50 μg/ml streptomycin. Cells were serum-starved for 24 hours and then treated for 24 hours with the follow recombinant cytokines; human IL-6, IL-1β, TGF-β, or TNF-α (Leinco Technologies, Inc, St. Louis, MO, USA). Conditioned media were harvested from the wells and total fibrinogen and γ' fibrinogen were quantitated by ELISA. γ' and γA mRNAs were isolated from the cells and quantitated by RT-PCR.

Total fibrinogen ELISA

To measure total fibrinogen produced by HepG2 cells, 96 well plates were coated with 1.5 μg/ml AXL203 rabbit anti-human fibrinogen polyclonal antibody (Accurate Chemical, Westbury, NY, USA) and incubated overnight at 4 °C. Wells were washed three times with PBS/0.1% Triton and blocked in PBS containing 1% BSA/0.1% Triton X-100 at 37 °C for 1 hour. Wells were washed with PBS/0.1% Triton, and 50 μl of conditioned medium diluted 1:10 in PBS/1% BSA/5 mM EDTA/0.1% Triton X-100 was added to each well and incubated at 37 °C for one hour. Wells were washed again with PBS/0.1% Triton, and a 1:2500 dilution of sheep anti-human fibrinogen-HRP conjugate (Innovative Research, Novi, MI, USA) was added to the wells and incubated for 1 hour at 37 °C. Wells were washed with PBS/0.1% Triton and incubated with TMB Super Sensitive 1 Component HRP Microwell Substrate (BioFX Laboratories, Owings Mills, MD, USA) for 30 minutes at room temperature. 450 nm liquid stop solution for TMB microwell (BioFX Laboratories) was added to each reaction and absorbance was measured at 450 nm.

γ' fibrinogen ELISA

γ' fibrinogen was measured using an assay that was originally developed to measure γ' fibrinogen in human plasma samples [1]. This assay was modified slightly for measuring γ' fibrinogen levels in cell culture supernatants. Briefly, 96 well plates were coated overnight at 4 °C with the monoclonal antibody 2.G2.H9 which is directed against the unique carboxyl terminus of the γ' chain. Wells were washed three times with PBS/0.1% Triton and blocked in PBS containing 1% BSA/0.1% Triton at 37 °C for 1 hour. Wells were washed with PBS/0.1% Triton, and 50 μl of conditioned medium diluted 1:1 in PBS/1% BSA/5 mM EDTA/0.1% Triton X-100 was added to each well and incubated at 37 °C for one hour. Wells were washed again with PBS/0.1% Triton X-100, and a 1:2500 dilution of sheep anti-human fibrinogen-HRP conjugate was added to the wells and incubated for 1 hour at 37 °C. Wells were washed with PBS/0.1% Triton and incubated with TMB substrate for 30 minutes at room temperature. Stop solution was added to each reaction and absorbance was measured at 450 nm.

RT-PCR

Total RNA was isolated from HepG2 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and 1 μg of polyA mRNA was reverse-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). TaqMan primer/probe pairs (Applied Biosystems Inc., Carlsbad, CA, USA) used in RT-PCR reactions were as follows; GAPDH Inventoried Endogenous Control Assay ABI4333764T, FGG(γ) Inventoried Assay Hs00241038_m1, FGG(γ')-forward 5'-CAATTGGAGAAGGACAGCAACA-3', FGG(γ')-reverse 5'-TC TGAAACTTTGTGGGTCAATAGAAG-3', FGG(γ')-Probe(FAM) 5'-CACCC TGCGGAAACAGAATATGACTCACTT-3'. PCR reactions were performed using TaqMan Universal Master Mix II with UNG (Applied Biosystems, Inc.) and assayed on an ABI 7300 Real-Time PCR System. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the endogenous control.

Western Blots

HepG2 cells were prepared and treated as described above. Following 24 hours of IL-6 treatment, cells were washed with PBS and lysed in RIPA buffer. Total cellular protein concentration was determined using a BCA assay and 20 μg of total protein from the cell lysates were separated using 10% SDS-PAGE. Protein was transferred to a nitrocellulose membrane and the membrane was probed using rabbit anti-human primary antibodies (phospho-p38 Thr180/Tyr182, phospho MEK1/2 Ser217/221, phospho-Jun Ser 63; Cell Signaling Technology). The membrane was stripped and reprobed for total p38, MEK and Jun using rabbit anti-human antibodies (Cell Signaling Technology). Goat anti-rabbit HRP conjugated secondary antibodies were used and membranes were imaged using an ECL Chemiluminescence Kit (Pierce).

Results

IL-1β, TGF-β, and TNF-α Inhibit γ' fibrinogen Production

We tested the direct effects of several inflammatory cytokines on total and γ' fibrinogen expression in HepG2 cells. IL-1β is a member of the proinflammatory IL-1 superfamily produced by macrophages, monocytes and dendritic cells in response to infection. IL-1β has several effects on the coagulation system, including increasing the release of von Willebrand factor [15] and plasminogen activator [16], and inhibiting the anticoagulant protein C pathway [17]. Interestingly, IL-1β is also known to bind to fibrinogen and fibrin, and this binding leads to increased activity of IL-1β [18]. Previous studies have shown that IL-1β has an inhibitory effect on fibrinogen production in HepG2 cells [14], however the effects of IL-1β on γ' fibrinogen have not previously been examined. As shown in Fig. 1, the addition

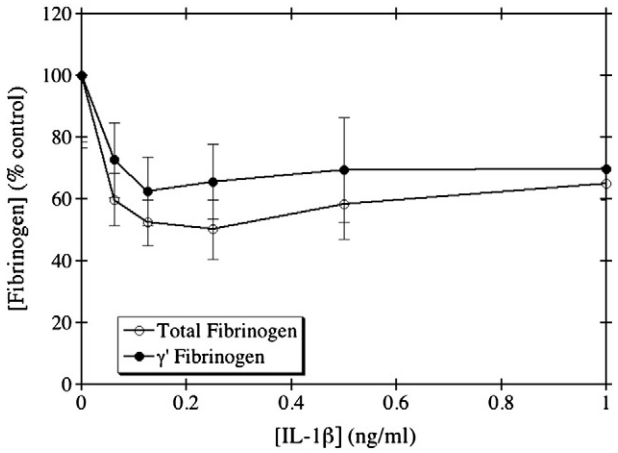

Fig. 1. IL-1β inhibits the production of total and γ' fibrinogen. HepG2 cells were grown to confluence and incubated with medium containing the indicated concentrations of IL-1β for 24 hours. Total and γ' fibrinogen levels were assayed by ELISA and the results normalized to the amount of total protein from whole cell lysates. The average of triplicate experiments is shown ± SE.