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Regular Article

Assembly of the prothrombinase complex on the surface of human foreskin fibroblasts: Implications for connective tissue growth factor

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ABSTRACT

Activated factor X (FXa) and thrombin can up-regulate gene expression of connective tissue growth factor (CTGF/CCN2) on fibroblasts. Since tissue factor (TF) is expressed on these cells, we hypothesized that they may assemble the prothrombinase complex leading to CTGF/CCN2 upregulation. In addition, the effect of thrombospondin-1 (TSP1) on this reaction was evaluated. Human foreskin fibroblasts were incubated with purified factor VII (FVII), factor X (FX), factor V (FV), prothrombin and calcium in the presence and absence of TSP1. Generation of FXa and of thrombin were assessed using chromogenic substrates. SMAD pathway phosphorylation was detected via Western-blot analysis. Pre-incubation of fibroblasts with FVII led to its auto-activation by cell-surface expressed TF, which in turn in the presence of FX, FVa, prothrombin and calcium led to FXa (9.7 ± 0.8 nM) and thrombin (7.9 ± 0.04 U/mL$\times$10–3) generation. Addition of TSP1 significantly enhanced thrombin (23.3 ± 0.7 U/mL$\times$10–3) but not FXa (8.5 ± 0.6 nM) generation. FXa and thrombin generation leads to upregulation of CTGF/CCN2. TSP1 alone upregulated CTGF/CCN2, an effect mediated via activation of transforming growth factor beta (TGFβ) as shown by phosphorylation of the SMAD pathway, an event blunted by using a TGFβ receptor I inhibitor (TGFβRI). FXa- and thrombin-induced upregulation of CTGF/CCN2 was not blocked by TGFβRI. In summary, assembly of the prothrombinase complex occurs on fibroblast's surface leading to serine proteases generation, an event enhanced by TSP1 and associated with CTGF/CCN2 upregulation. These mechanisms may play an important role in human diseases associated with fibrosis.

Introduction

Coagulation is a complex process that involves multiple molecules and cell types and can be even more intricate during inflammation [1–4]. In normal conditions, serine proteases can be generated on the surface of cell membranes by the ability of such cells to express tissue factor (TF) which is a 50 kDa membrane-associated protein directly involved in the activation of the extrinsic pathway of blood coagulation [5]. TF binds to and activates factor VII (FVIIa) forming the TF/FVIIa catalytic complex that leads to the activation of factor X (FXa). Subsequently, the generation of FXa results in the assembly of the prothrombinase complex and in the conversion of prothrombin (II) to thrombin [5]. The prothrombinase complex is comprised of FXa, FVa and Ca^{++} ions that in turn convert prothrombin to thrombin. These series of reactions are closely controlled, in part by the presence of the naturally occurring inhibitor, tissue factor pathway inhibitor (TFPI) [6].

Previously, we demonstrated the assembly of the prothrombinase complex on a monocytic cell line (HL60) due to the presence of TF, leading to thrombin generation. The enzymatic reaction in this cell line was favored by thrombospondin-1 (TSP1) [7] an adhesive, secreted molecule found in platelets, neutrophils, endothelial cells and fibroblasts [8,9]. The effect of TSP1 during the prothrombinase complex formation in HL60 cells is attributed to the ability of TSP1 to form a

Abbreviations: FXa, activated factor X; CTGF/CCN2, connective tissue growth factor; TF, tissue factor; TSP1, thrombospondin-1; HFF, human foreskin fibroblasts; FVII, factor VII; FX, factor X; FV, factor V; II, prothrombin; TGFβ, transforming growth factor beta; TGFβRI, TGFβ receptor I; TFPI, tissue factor pathway inhibitor; LAP, latency associate peptide; LTBP, latent TGFβ binding protein; cDNA, complementary DNA; qPCR, real-time polymerase chain reaction; DMEM, Dulbecco's modified eagle medium; HBSS, Hank's balance salt solution; SDS-PAGE, sodium dodecyl sulfate polyacrylamide; PVDF, polyvinylidene fluoride; ANOVA, analysis of variances; PAR, protease- activated receptor.

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complex with coagulation FV/FVa and to bind and neutralize TFPI [7,10]. The formation of the prothrombinase complex and the subsequent activation of multiple serine proteases may lead to activation of molecules and pathways not directly associated with coagulation, especially during inflammation.

The multifunctional cytokine, transforming growth factor beta (TGFβ) is involved in physiological events such as cell proliferation, differentiation and apoptosis [11,12] as well as in pathological conditions such as in inflammation, angiogenesis, cancer and fibrosis [13,14]. TGFβ is secreted to the extracellular matrix in an inactive form, a large complex that contains latency associate peptide (LAP) and latent TGFβ binding protein (LTBP). TSP1 activates TGFβ by binding to LAP and allowing active TGFβ to bind to its receptors [15–20]. TGFβ binds to two serine/threonine kinase receptors namely type I and type II receptors. The type I receptor is activated by the type II receptor upon ligand binding and transduces signals primarily via the SMAD proteins [21]. Connective tissue growth factor (CTGF/CCN2) is one of the many proteins produced downstream of the SMAD pathway after TGFβ receptor activation [22].

CTGF/CCN2 is secreted by multiple cells such as endothelial cells, osteoblasts, chondrocytes, fibroblasts and synoviocytes [23–25]. CTGF/CCN2 plays a significant role in diverse biological functions including regulation of cell growth and differentiation, embryonic development, angiogenesis, endochondral ossification, wound healing and apoptosis [26]. It is also involved in pathological processes such as fibrosis and atherosclerosis. CTGF/CCN2 is a cofactor for TGFβ [27] that acts as a downstream mediator of TGFβ in mesenchymal cells and fibroblasts [28] and modulates the effect of a variety of cytokines as well as the growth and differentiation of vascular endothelial cells [29]. A recent study demonstrated the presence of CTGF/CCN2 in complicated atherosclerotic plaques, an event that was found associated with mononuclear cell chemotaxis *in vitro* [30].

TSP1, TGFβ and CTGF/CCN2 have been proposed to comprise a pro-inflammatory axis in rheumatoid arthritis in which the synovial fibroblasts are targeted by the autoimmune process and become the effectors cells [31]. We have examined a series of reactions, using purified coagulation factors, on fibroblasts to investigate the possibility that these cells can assemble the prothrombinase complex on their surface and to further evaluate the signaling effects from such reactions within the context of TSP1, TGFβ and CTGF/CCN2.

Methods

Reagents

Purified TSP1 and coagulation factors II, Va, VII, X and Xa were purchased from Haematologic Technologies Inc., (Essex Junction, VT), TFPI was acquired from American Diagnostica, Inc, (Stamford, CT) the thrombin-specific chromogenic substrate, S2238, and the FXa-specific chromogenic substrate, S2222, were obtained from DiaPharma Inc., (West Chester, OH). Human purified thrombin was obtained from Dr. J.W. Fenton (Wadsworth Center for Laboratories and Research, New York, NY). TGFβ1 was purchased from R&D systems, (Minneapolis, MN). TGFβ receptor I kinase (TGFβRI) inhibitor #616451 was obtained from Calbiochem (an affiliate of Merck KGaA, Darmstadt, Germany). RNeasy kit was obtained from Qiagen (Qiagen USA, Valencia CA). Accuscript high fidelity first strand cDNA synthesis kit was purchased from Stratagene (an acquired company of Agilent Technologies, Santa Clara, CA). Real-time polymerase chain reaction (qPCR) Sybr green master mix was acquired from Applied Biosystems (Foster City, CA). Primary antibodies against pSMAD2 #3101, pSMAD3 #9520, SMAD2/3 #3102, SMAD4 #9515 and βactin #4967 proteins were purchased from Cell signaling Technology (Danvers, MA). All cell culture reagents such as Dulbecco's modified eagle's medium (DMEM), L-glutamine and fetal bovine serum were obtained from Cellgro, Mediatech Inc. (Manassas, VA). All other chemicals and reagents were purchased from Fisher Scientific Inc., (Hampton, NH).

Culture of human foreskin fibroblasts

The human foreskin fibroblast cell line (HFF), HS-68, was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) and cultured in DMEM supplemented with 1% L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C in a humidified control environment with 5% CO_2, replacing the media every 3 days until the cells reach 90% confluence. Cells used in the studies were within the 3rd and the 6th passages from the original ATCC vial. For chromogenic assays, the cells were pelleted, washed twice with Hanks Balanced Salt Solution (HBSS) counted and then re-suspended in HBSS containing 2 mM $CaCl_2$. Cell viability was determined by the trypan blue dye exclusion method (95-98% of viability). For RNA isolation, cells were washed in cold HBSS and collected in lysis buffer from the RNeasy kit and quickly frozen in dry ice and then stored at −80 °C. All tissue culture media, buffers, reagents and supplies used were endotoxin free.

Generation of thrombin and activated factor X (FXa) assessed by chromogenic substrates

Coagulation factors and TSP1 proteins were tested for traces of FXa and/or thrombin prior to their use in any experimental assay by over exposure of them to the chromogenic substrate for thrombin and FXa. There was no evidence of the proteases studied in any of the reagents tested. The experiments were conducted at 20 °C under a UV-light laminar flow hood. HFF (1×10^6 cells/mL) were re-suspended in HBSS containing 2 mM $CaCl_2$ and preincubated with FVII (5nM) for 10 minutes prior to the addition of a mixture containing FII (1.4 μM), FX (5nM) and FVa (45nM) prepared in the same buffer used to resuspend the fibroblasts. At different time intervals (0, 5, 10, 15, 20, 25 and 30 minutes) aliquots of 100 μL were removed and placed in a 96-well plate containing 100 μL of the chromogenic substrate for thrombin or FXa. The reaction mixture was stopped using 50% acetic acid. Optical density was determined using a micro-titer plate reader (Revelation® Micro-plate Reader MRX, Model #20330 from Dynex Technologies, Inc., Chantilly, VA, USA) at optical density of 405 nm. Thrombin and FXa enzymatic activity were quantified by comparing the activity with standard preparations obtained from human purified thrombin and FXa. The same experimental conditions as described above were used to determine the requirements of each coagulation factor by excluding them from the reaction mixture. In addition, the role of TSP1 (20nM) and TFPI (8nM) was assessed by their inclusion into the reaction mixture. To examine the requirement of TF which is cell membrane bound, additional reactions were tested in which no cells or phospholipid vesicles were added (in substitution of a cell surface) in the absence of FVII. As anticipated, no FXa or thrombin was generated under this conditions.

Blocking the activity of the TGFβRI by a specific inhibitor 616451

Monolayer cultures of human fibroblasts were treated with the TGFβRI inhibitor 616451 at different concentrations and at different time points (data not shown). The cells were washed with HBSS and then incubated for two hours with the TGFβRI inhibitor at 500 nM concentration dissolved in free-serum culture media.

RT-PCR analysis

Total RNA was extracted from HFF cells using RNeasy kit following the manufacture recommendations. Specific primers for CTGF/CCN2 were synthesized by Sigma Genosys (St. Louis, MO) (Forw.: 5'AAGACACATTTGGCCCAGAC3' Rev.: 5'TTTTCCTCCAGGTCAGCTTC3'). Reverse