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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 (TGF β) as showed by phosphorylation of the SMAD pathway an event blunted by using a TGF β receptor I inhibitor (TGF β RI). FXa- and thrombin-induced upregulation of CTGF/CCN2 was not blocked by TGF β RI. In summary, assembly of the prothrombinase complex occurs on

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DISCLOSURE OF CONFLICT OF INTEREST

The authors state that they have no conflict of interest.

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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.

Keywords

CTGF/CCN2; fibroblasts; TGF β ; thrombin; TSP1; factor X

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 50kDa 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 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 (TGF β) 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]. TGF β is secreted to the extracellular matrix in an inactive form, a large complex that contains the latency associate peptide (LAP) and the latent TGF β binding protein (LTBP). TSP1 activates of TGF β by binding to the LAP and allowing the active TGF β to bind to its receptors [15–20]. TGF β 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 TGF β 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 TGF β [27] that acts as a downstream mediator of TGF β 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, TGF β 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 cell surface and to further evaluate the signaling effects from such reactions within the context of TSP1, TGF β 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). TGF β 1 was purchased from R&D systems, (Minneapolis, MN). TGF β receptor I kinase (TGF β RI) 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 β actin #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 μ g/ml streptomycin. Cells were incubated at 37°C in a humidified control environment with 5% CO₂, 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 2mM CaCl₂. 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 $\times$ 10⁶ cells/mL) were re-suspended in HBSS containing 2 mM CaCl₂ and

preincubated with FVII (5nM) for 10 minutes prior to the addition of a mixture containing FII (1.4 μ M), 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 μ L were removed and placed in a 96-well plate containing 100 μ L 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 prepared 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, not FXa or thrombin were generated under these conditions.

Blocking the activity of the TGF β RI by a specific inhibitor 616451

Monolayer cultures of human fibroblasts were treated with the TGF β RI 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 TGF β RI 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 manufacturer recommendations. Specific primers for CTGF/CCN2 were synthesized by Sigma Genosys (St. Louis, MO) (Forw.: 5' AAGACACATTTGGCCAGAC3' Rev.: 5' TTTTCCTCCAGGTCAGCTTC3'). Reverse transcription was performed using the oligo dT primers and the reverse transcriptase enzyme from Stratagene. The cDNA was amplified, using Sybr green PCR master mix, by the quantitated Polymerase Chain Reaction (PCR) system (7500 real-time PCR system from Applied Biosystems). Beta-actin was utilized as loading control gene.

Western Blot Analysis

Expression of pSMAD2, pSMAD3, SMAD2/3, SMAD4 and β -actin proteins was determined by immuno-blotting. Briefly, extracted proteins from fibroblasts were separated by electrophoresis using SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked, incubated with primary and species-specific secondary antibodies and developed following the same procedures and reagents as previously described [28, 32]. Antibody concentrations of the primary antibodies were as follows, for pSMAD2 and pSMAD3 1:1,000, for SMAD2/3, SMAD4 and β -actin 1:2,000. All of the secondary antibodies were used at a concentration ratio of 1:5,000.

Statistical Analysis

One-way analysis of variances (ANOVA) was used to evaluate the overall differences of multiple measurements of thrombin and FXa generated from fibroblasts. Student *t* test was utilized to analyze the differences of the measurements of thrombin or FXa generated at specific time point of two reactions. Statistical significance was defined as an alpha level less than 0.05.

RESULTS

Tissue factor expressed on the fibroblast cell surface activates FVII

Since fibroblasts in culture express tissue factor on their cell membrane, we performed a series of reactions to assess whether TF expressed on the surface of fibroblasts could activate purified FVII. In the presence of FVII, CaCl_2 , FII, FX and FVa, HFF cells were capable of generating thrombin ($7.99 \pm 0.04 \text{ U/mL} \times 10^{-3}$; reaction II of the panel A, Figure 1) as indicated by the hydrolysis of the chromogenic substrate specific for thrombin as well as FXa ($9.7 \pm 0.8 \text{ nM}$, reaction II of panel B, Figure 1). In contrast, when the same reaction mixture was evaluated in the absence of FVII, thrombin and FXa were not generated as shown by a lack of hydrolysis of their respective chromogenic substrates (Figure 1, reaction I of panels A & B). In addition, the reactions in the absence of FVII are evidence of the absence of protease contamination. Exposure of fibroblasts to FII alone was not sufficient for FX activation and thus for thrombin generation. Overall, these results indicate that the TF expressed on the cell surface of HFF cells in addition to the presence of FVII are absolute requirements for FX activation. In parallel, a set of experiments were conducted to bypass the activation of FVII by adding activated factor X (FXa) into a mixture containing FII, CaCl_2 and FVa. Under these experimental conditions thrombin was efficiently generated ($16.3 \pm 0.81 \text{ U/mL} \times 10^{-3}$), indicating that the amount of thrombin generated is proportional to the amount of FXa available on the HFF cell surface. These results strongly support the assembly of the prothrombinase complex on the surface of human fibroblasts.

TSP1 promotes thrombin generation on the fibroblasts cell surface and modulates the inhibitory effect of the tissue factor pathway inhibitor (TFPI)

We have previously demonstrated the ability of TSP1 to promote thrombin generation on the HL60 cell surface even in the presence of TFPI [7]. Thus, we evaluated the effect of TSP1 in reactions containing FVII, FII, CaCl_2 , FX and FVa. Thrombin generation increased significantly in the presence of TSP1 ($23.3 \pm 0.7 \text{ U/mL} \times 10^{-3}$) when compared to a reaction in the absence of TSP1 ($7.9 \pm 0.04 \text{ U/mL} \times 10^{-3}$) (Figure 2, panel A, $p < 0.05$). Interestingly, no significant differences were noted in the presence of TSP1 for the activity of FX (Figure 2, panel B). We analyzed the effect of TSP1 in the presence and absence of TFPI. Thrombin generation was blunted in the presence of TFPI but the addition of TSP1 neutralized TFPI inhibitory activity with subsequent generation of thrombin equivalent to those levels observed in the absence of TSP1 ($p < 0.05$, data not shown). These data suggest that TSP1 enhances thrombin generation on the fibroblasts cell surface even in the presence of naturally occurring inhibitors.

Assembly of the prothrombinase complex on the surface of fibroblasts is associated with upregulation of CTGF/CCN2 gene expression and is enhanced by TSP1

The mRNA for CTGF/CCN2 is present in fibroblasts and it is induced by purified-FXa and – thrombin [33]. Thus, we evaluated the effect of FXa and thrombin generated from the prothrombinase complex on the fibroblast cell surface on CTGF/CCN2 mRNA expression. Assembly of the prothrombinase complex was associated with a two-fold increment in CTGF/CCN2 mRNA expression when compared to CTGF/CCN2 mRNA baseline levels. The addition of TSP1 to the reaction mixture was associated with a twelve-fold increment in CTGF/CCN2 mRNA expression when compared with baseline values (Figure 3). Interestingly, when TSP1 alone was added, CTGF/CCN2 mRNA expression increased fourfold when compared with baseline values. Thus, assembly of the prothrombinase complex on the HFF cell surface results in upregulation of mRNA for CTGF/CCN2 an effect significantly augmented (eight-fold) by the presence of TSP1 (Figure 3).

TSP1-induced CTGF/CCN2 gene expression is mediated via the TGF β pathway

It has been shown in the literature that activated TGF β is capable of inducing CTGF/CCN2 mRNA expression in fibroblasts [34] and since TSP1 is capable of activating latent TGF β [16], a series of experiments was designed to examine if this mechanism was responsible for TSP1-mediated upregulation of CTGF/CCN2. Our first step was to evaluate the TGF β pathway. A specific TGF β receptor I inhibitor was tested in the presence and absence of purified TSP1 and activated TGF β . CTGF/CCN2 mRNA expression in fibroblasts in the presence of the TGF β RI inhibitor was used as baseline since fibroblasts express CTGF/CCN2 constitutively. Active TGF β increased the basal levels of CTGF/CCN2 mRNA expression three-fold from baseline value and the addition of TGF β RI inhibitor reversed the effect to basal levels. TSP1 alone increased the basal levels of CTGF/CCN2 mRNA expression 2.5-fold and the addition of TGF β RI inhibitor reversed the effect to basal levels. Thus, the TSP1 effect upon CTGF/CCN2 mRNA expression is mediated via the TGF β pathway. Noteworthy to mention is that the addition of TSP1 and TGF β together (2.4-fold increment compared to control) was not additive or synergistic at least at the concentrations tested (Figure 4).

To further evaluate the TGF β pathway, we examine the SMAD pathway, which upon activation of TGF β pathway leads to the phosphorylation of SMAD 2 and 3 proteins. A series of experiments using the TGF β RI inhibitor were performed to evaluate the effect of purified TSP1 and/or exogenously activated TGF β . Phosphorylation of SMAD 2 and SMAD3 was observed on fibroblasts in the presence of activated TGF β , an event that was found blunted by the presence of the TGF β RI inhibitor. TSP1 alone induced phosphorylation of SMAD2 and SMAD3 but not as efficiently as the one seen with active TGF β . Phosphorylation of SMAD2 and SMAD3 in the presence of active TGF β in conjunction with TSP1 was not different from the one observed with active TGF β alone, however phosphorylation was completely blunted by the addition of TGF β RI inhibitor (Figure 5).

DISCUSSION

This study demonstrates the ability of HFF to assemble the prothrombinase complex on their surface due to the expression of tissue factor by these cells. The requirements to form the prothrombinase complex were examined by using purified coagulation factors including FVII, FX, FVa and prothrombin at the physiological concentrations in plasma. The use of specific chromogenic substrates for FXa and thrombin allowed us to follow the generation of these serine proteases independently and sequentially as a function of time. Under our experimental conditions, adding purified FX to fibroblasts in the absence of FVII was not sufficient to activate FX or generate thrombin. Thus FX cannot be directly activated by the fibroblast cell surface. We tested this particular experimental condition since some cells express proteases on its membrane surfaces with the ability to directly activate FX [35].

Since TSP1 enhances thrombin generation via the prothrombinase complex on the surface of HL-60 cells [7], we examined the effect of added TSP1 on fibroblasts to assess FXa and thrombin generation. Only trace amounts of TSP1 are found in circulation physiologically, however in certain pathologic conditions, the level of TSP1 significantly increase in circulation and in inflamed tissue. Thus, we used the plasma concentrations of TSP1 found in rheumatoid arthritis patients [36]. In fibroblasts, addition of TSP1 increased significantly thrombin generation but not FXa generation. It is noteworthy to mention that purified TSP1 was tested for traces of FXa or thrombin contamination, since traces of thrombin can activate FV in the absence of FXa. The purified TSP1 showed no FXa or thrombin contamination.

The role of FXa and thrombin generated on the anionic cell surface of human fibroblasts was explored within the context of TSP1, TGF β and CTGF/CCN2. Assembly of the prothrombinase complex on the fibroblast surface was associated with a two-fold increment in CTGF/CCN2 mRNA expression when compared to baseline values and the addition of TSP1 to such reactions increased the CTGF/CCN2 mRNA expression 12-fold. Pendurthi et. al., demonstrated by microarray and Northern analysis that purified FVIIa induced CTGF/CCN2 expression on human lung fibroblasts in a time- and dose-dependent manner. Our study is in agreement with such observation in that FXa and thrombin generated led to increased CTGF/CCN2 mRNA expression and documents for the first time the ability of the prothrombinase complex to be assembled on the fibroblast surface. In contrast to Pendurthi's study we added inactive FVII. The activation of FVII comes from the TF/FVIIa complex formation. Chambers et al. [33], extended Pendurthi's observations by demonstrating that PAR-1 is primarily responsible for the thrombin effect on CTGF/CCN2 mRNA expression using lung fibroblasts from PAR-1 knockout mice [33, 37]. Studies using human fibroblasts validated that FXa signals mainly via PAR-2 receptor increasing proinflammatory and profibrotic responses *in vitro*. In addition, the Src pathway was linked to FXa-induced cell migration, which suggested that ERK1/2 activation might be responsible for the profibrotic effects of FXa [38]. Experiments in this study focused on the effect observed by the addition of TSP1, which not only favored thrombin generation but also directly induced an increase in CTGF/CCN2 expression via SMAD pathway and activated inert TGF β .

Our observations have clinical relevance; one of the target cells in rheumatoid arthritis are the synovocyte-like fibroblasts and high concentrations of TSP1 and TGF β have been found on synovial tissue and circulating plasma of rheumatoid arthritis patients. Recent studies have documented the ability of synovocyte-like fibroblasts to induce human platelet microparticles [39]. Thus, the ability of human fibroblasts to assemble the prothrombinase complex with subsequent FXa and thrombin generation may lead to signaling the fibroblast to increase proliferation and fibrosis and to activate other cells such as platelets. In addition, the experiments adding TFPI and TSP1 during the assembly of the prothrombinase complex, in which TFPI cannot regulate the serine proteases generated in the presence of TSP1, support the idea of the effect of these local coagulation events in fibroblast proliferation and fibrosis.

In summary, this study documents the assembly of the prothrombinase complex on the surface of human fibroblasts an event associated with upregulation of CTGF/CCN2. TSP1 favors thrombin generation and directly drives the upregulation of CTGF/CCN2. Disrupting the ability of TSP1 to participate within the prothrombinase complex or to interact with TGF β may be of benefit in regulating the expression of CTGF/CCN2 in inflammatory and/or fibrotic diseases.

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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	TGF β receptor I
TFPI	tissue factor pathway inhibitor
LAP	latency associate peptide
LTBP	latent TGF β 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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Figure 1.

Human foreskin fibroblasts assembled the prothrombinase complex on their cell surface. Washed fibroblasts were incubated in suspension on HBSS with 2mM CaCl for 5, 10, 15, 20, 25 and 30 minutes. Two sets of experiments were conducted simultaneously. In reaction I, fibroblasts (1×10^6 cells per time point) were incubated with FII, FVa, FX, in the absence of FVII (black dotted line). In reaction II, fibroblasts were pre-incubated with FVII and then FII, FVa, and FX were added (white dotted line). Thrombin generation (panel A) and Factor X activity (panel B) were assessed by chromogenic assays. Representation of three different experiments is plotted on the graphs.

Figure 2.

TSP1 enhanced thrombin generation on the cell surface of fibroblasts. Washed fibroblasts were incubated in suspension on HBSS with 2mM CaCl for 5, 10, 15, 20, 25 and 30 minutes. Two sets of experiments were conducted simultaneously. In the first reaction (black dotted line, labeled: coag.), fibroblasts (1×10^6 cells per time point) were incubated with FVII, FII, FVa, and FX. In the second reaction (white dotted line, labeled: coag. +TSP1), fibroblasts were incubated with FVII, FII, FVa, FX, and purified TSP1. Thrombin generation (panel A) and Factor X activity (panel B) were assessed by chromogenic assays. Representation of three different experiments is plotted on the graphs.

Figure 3.

CTGF/CCN2 gene expression is augmented by TSP1. Fibroblasts were incubated with coagulation factors and with the addition of purified TSP1. RNA was extracted and CTGF/CCN2 gene expression was assessed by qPCR.

Figure 4.

CTGF/CCN2 gene expression. Fibroblasts cultures were treated with active TGFβ and/or purified TSP1 and/or with a TGFβRI inhibitor. RNA extracted was reverse transcribed and CTGF/CCN2 expression was assessed by qPCR. Open bars represent fibroblasts exposed to the TGFβ receptor inhibitor, *p<0.05.

Figure 5.

Phosphorylation of SMAD2 and SMAD3 of the TGFβ pathway. Fibroblasts cultures were treated with active TGFβ and/or purified TSP1 and/or with a TGFβRI inhibitor. Extracted protein was separated by electrophoresis and transferred onto a PVDF membrane. Blots were exposed to specific antibodies against pSMAD2, pSMAD3, total SMAD 2/3, SMAD4 and β-actin.