Activation of platelet transforming growth factor β-1 in the absence of thrombospondin-1

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This work is supported by National Institutes of Health grant HL28749 from the National Heart, Lung and Blood Institute (to J.L.), and by the Biotechnology and Biological Sciences Research Council (BBSRC)-CASE studentship supported by Johnson & Johnson Medical Ltd (to A.L.).

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Thrombospondin-1 (TSP-1) has been shown to bind and activate transforming growth factor-β1 (TGF-β1). This observation raises the possibility that TSP-1 helps to sequester TGF-β1 in platelet α granules and activates TGF-β1 once both proteins are secreted. Herein, we evaluated the level of active and latent TGF-β1 in the plasma and in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type mice on two genetic backgrounds (C57BL/6 and 129Sv). The plasminogen activator inhibitor-1/luciferase bioassay and an immunological assay were used to determine active and latent TGF-β1. No significant differences were observed in the levels of active and latent TGF-β1 in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type mice. Active and latent TGF-β1 were significantly increased in the plasma and platelets of C57BL/6 mice as compared to 129Sv mice. In addition, there was an increase of plasma level of latent TGF-β1 in TSP-1 null mice as compared to wild-type mice on the C57BL/6 background but not on the 129Sv background. No active TGF-β1 was observed in the plasma of either TSP-1 null and wild-type mice. These data indicate that TSP-1 does not function as a chaperon for TGF-β1 during platelet production and does not activate significant quantities of secreted TGF-β1 despite a vast excess in the number of TSP-1 molecules as compared to TGF-β1 molecules. Because platelet releasates from TSP-1 null mice contain active TGF-β1, we suggest that other important mechanisms of physiological activation of TGF-β1 probably exist in platelets.
INTRODUCTION

Transforming growth factor-β (TGF-β1, 2 and 3) are mammalian cytokines with a wide range of biological effects (1). They are involved in the regulation of development, proliferation, angiogenesis, inflammation, extracellular matrix production, integrin expression, protease activity and apoptosis (2). They play a pathologic role in inflammation and fibrosing diseases such as nephrosclerosis (3). Mice null for either, TGF-β1, 2 and 3 do not survive beyond a few days or weeks (4-8). Surviving pups of animals null for TGF-β1 exhibit dysregulated myelopoiesis and a wasting syndrome characterized by an inflammatory response targeting the heart, lung, pancreas, stomach, liver and striated muscle that has been attributed to an autoimmune process (9, 10). Overexpression of TGF-β1 causes lethality in utero or just after birth (11).

TGF-β1 is synthesized by cells in a latent form that must be activated in order to be recognized by cell-surface receptors and to trigger biological responses (2). Small latent TGF-β1 is a dimeric complex of ~100 kDa, composed of two identical chains in which an amino-terminal 278 amino acid latency-associated peptide (LAP) is noncovalently associated with the carboxyl-terminal 112 amino-acid active peptides (12). This latent complex is the product of a single gene. Prior to secretion, LAP is enzymatically cleaved from the active peptide, and the integrity and latency of the secreted complex are presumably maintained via electrostatic interactions (13). Latent TGF-β1 can exist as a large complex in which it is associated with a latent TGF-β1-binding protein (LTBP). LTBP has features in common with extracellular matrix proteins, and targets latent TGF-β1 to the matrix (2). The latency of TGF-β1 is dependent on the presence of LAP; the presence of LTBP is neither necessary nor sufficient for prevention of activation (14).

Physiological mechanisms of activation of TGF-β1 are not well understood (12), although proteolytic processing by plasmin, exposure to reactive oxygen species, and binding to αvβ6 integrin may participate in TGF-β1 activation (2, 15). Interaction of latent TGF-β1 with thrombospondin-1 (TSP-1) results in activation of latent TGF-β1 (16-19). TSP-1 is a trimer of disulfide-linked 180 kDa subunits found at high concentrations in platelet α granules and also produced by a number of other cell types (20). It is an adhesive protein with a number of domains available for binding to cell surface or matrix proteins. TSP-1 deficient mice are viable and exhibit subtle abnormalities in development (21). The adult mice exhibit increased inflammatory cell infiltrates and epithelial cell hyperplasia in the lungs, suggesting that TSP-1 is involved in normal lung homeostasis (21).
TSP-1 purified from human platelets has been shown to contain TGF-β1 (22). TSP-1 also activates TGF-β1 in cell culture assays when added to endothelial cells. The site in TSP-1 responsible for latent TGF-β1 activation has been localized to the type 1 repeats, specifically the K412RFK415 sequence located between the first and second type 1 repeats of TSP-1 (16). More recently, Ribeiro et al., (23) showed that the TSP-1 sequence KRFK binds LAP through interactions that involve a specific sequence at the amino terminus of LAP (L54SKL57). The binding of TSP-1 to LAP appears to induce a conformational change that renders the TGF-β1 active.

Mechanisms controlling conversion of the latent complex to the active state are key regulators of TGF-β1 activity. TSP-1 is the first activator of TGF-β1 shown to function in natural, untreated, nondiseased tissues in vivo (24). The previous observations that purified platelet TSP-1 contains associated TGF-β1 (16-19), raises the possibility that TSP-1 activates platelet TGF-β1 or serves as a carrier for TGF-β1 during α granules formation. Here, we evaluated and compared the level of active and latent TGF-β1 in the plasma and in platelet α granules of TSP-1 null and wild-type mice using the plasminogen activator inhibitor-1/luciferase bioassay (25) and the commercial TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Boston, MA). We observed that (i) there is no active TGF-β1 in the plasma of both TSP-1 null and wild-type mice, (ii) there is an increase in the level of plasma latent TGF-β1 in TSP-1 null C57BL/6 mice compared to wild-type, and (iii) there is no significant difference in the level of active and latent TGF-β1 in platelets from TSP-1 null and wild-type mice. Despite the absence of TSP-1 in TSP-1 null mice, active TGF-β1 is observed in the platelet releasates. These data suggest that TSP-1 is not the only physiological activator of TGF-β1 in platelet α granules, and that TSP-1 does not play a role of carrier for active TGF-β1 during platelet biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Animals**—TSP-1 null animals were generated by homologous recombination in 129Sv-derived ES cells, as previously described (21).

**Preparation and stimulation of mouse platelets**—Mice were anesthetized with 2.5% avertin. Blood was drawn by periorbital insertion of a heparinized capillary tube (~1.5 cm in length). Blood from 5-8 TSP-1 null and wild-type (C57BL/6 or 129Sv) was drawn into tubes containing acid-citrate dextrose and pooled
separately. Platelets were isolated by differential centrifugation and washed in pH 6.5 buffer containing 0.102 M NaCl, 3.9 mM K₂HPO₄, 3.9 mM Na₂HPO₄, 22 mM NaH₂PO₄, and 5.5 mM glucose. The platelets were resuspended in 15 mM Tris-HCl (pH 7.6), 0.14 M NaCl, 5 mM glucose, and 2 mM CaCl₂. The platelet counts were determined and platelet concentration was adjusted to 1.8 x 10⁵ platelets/µl. Platelets were activated with human thrombin (0.5 U/ml; Sigma Chemical Co., St Louis, Mo) for 3 min under constant stirring. Platelet aggregates were centrifuged at 3,000 g and the releasates of thrombin-stimulated platelets were collected and stored at −20°C for further quantification of total and active TGF-β1 by both the TGF-β1 ELISA kit and the plasminogen activator inhibitor-1/luciferase bioassay (25). In other experiments, thrombin-activated platelets are treated with 10 mM EDTA for 10 min before the collection of the releasate of thrombin-stimulated platelets.

Quantification of total and active TGF-β1 by ELISA kit—Total and active TGF-β1 concentration in the plasma and in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type mice were assayed by a sandwich TGF-β1 ELISA kit (Genzyme, Boston, MA) according to the manufacturer’s specifications. Plasma and supernatant of thrombin-treated platelets were thawed and divided into two pools. In the first pool, acid activation was required in order to convert latent to active TGF-β1 and to record detectable levels of total TGF-β1. By contrast, no acid activation is used in the second pool; the TGF-β1 levels in the samples are therefore representative of active TGF-β1 in the plasma or in the supernatant of thrombin-treated platelets. Plasma (10 µl) and supernatant of thrombin-treated platelets (50 µl) were added to sample diluent with or without 1N HCl for 60 min at 4°C followed by neutralization with 1 N NaOH if samples were activated with HCl. Samples were plated on microtiter plates coated with anti-TGF-β1 antibody and incubated at 37°C for 60 min. After vigorous washing, wells were incubated with a second biotin-conjugated anti-TGF-β1 antibody and the peroxidase reaction was initiated. A standard curve was constructed using serial dilutions of human TGF-β1 (Genzyme) as standard. TGF-β1 levels in samples were compared to known standards and read as nanograms per milliliter.

Quantification of total and active TGF-β1 by the plasminogen activator inhibitor-1/luciferase bioassay—Total and active TGF-β1 concentration in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type mice
were assayed using the plasminogen activator inhibitor-1/luciferase (PAI/L) assay, first described by Abe et al., (25). This assay is based on the ability of TGF-β1 to induce plasminogen activator inhibitor-1 (PAI-1) expression in mink lung epithelial cells (MLEC’s) transfected with a construct containing a truncated PAI-1 promoter fused to a firefly luciferase reporter gene. Transfected MLEC’s were a generous gift from Dr. D. B. Rifkin (New York University Medical Center). Cells were maintained in high glucose (4500 mg/l) Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Paisley, UK) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 200 µg/ml geneticin (G418-sulphate) (Calbiochem-Novabiochem Ltd., Nottingham, UK). Cells were cultured in a humid atmosphere at 37°C, 5% CO₂ and passaged maximally 30 times. As described above, the supernatants of thrombin treated platelets were divided into two pools. This time however, the first pool was diluted in serum-free DMEM containing 0.1% pyrogen-poor bovine serum albumin (Pierce & Warriner UK Ltd., Chester) and heat activated for 10 min at 80°C. As before, the second pool remained untreated so as to measure the amount of active TGF-β1 present. MLEC’s were trypsinised and washed and the cell density adjusted to 1.6 x 10⁵ cells/ml before plating 100 µl/well into a 96-well tissue culture plate (Falcon, Becton Dickenson, Oxford, UK). Cells were incubated for 3-4 h to allow for optimal attachment to the plastic. Following aspiration of the growth medium from the attached cells, 100 µl of the sample was added in triplicate. Cells and samples were then incubated for 14-16 h at 37°C, 5% CO₂. Following incubation, all wells were checked microscopically for cell viability before washing twice with 100 µl of phosphate buffered saline. Cells were then lysed using 100 µl/well of 1 x lysis buffer (Promega, Southampton, UK) and incubated with agitation at room temperature for 20 min. Forty five microlitres of the cell lysates were transferred to an opaque Microlite™ 1 Microtiter® read plate (Dynex Technologies Ltd., West Sussex, UK). Lysates were analysed for luciferase activity using an MLX Luminometer (Dynex Technologies Ltd.) following the injection of 110 µl/well of substrate solution [20 mM tricine, 1.07 mM Mg(CO)₃Mg(OH)₂.5H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 750 µM ATP and 800 µM luciferin (Promega, Southampton, UK) ]. The flash of light obtained upon mixing the lysates with the substrate was recorded as relative light units (RLU). The mean values of the triplicates were then converted into concentrations of TGF-β1 in picograms per millilitre using a standard curve obtained with human recombinant TGF-β1 (R&D Systems, Abingdon, UK).
STATISTICAL ANALYSIS
Data are expressed as the mean ± SEM. Statistical evaluation of the data was
performed using the unpaired t-test, considering p values <0.05 as significant.

RESULTS AND DISCUSSION
Since TGF-β1 binds to TSP-1 with high affinity (16-19), we hypothesized that
TSP-1 may function as a carrier protein for TGF-β1. To test this hypothesis, we
assayed the quantity of TGF-β1 that is secreted from wild-type and TSP-1 null
platelets in response to thrombin. As shown in Figure 1, the level of total TGF-β1
is equivalent in the supernatants from the wild-type and TSP-1 null platelets. We
have confirmed this result by employing two different assays for TGF-β1 and two
different strains of TSP-1 null mouse. In our hands, the bioassay consistently gives
higher values than the TGF-β1 ELISA kit, however, the relative levels of active
TGF-β1 as compared to total TGF-β1 are consistent between the two assays.

TSP-1 reportedly binds to the platelet membrane after secretion when calcium
is present (26). Thus, TGF-β1 that is complexed with TSP-1 may also become
associated with the platelet membrane. If this is the case, then the TGF-β1 levels
that we measured in the supernatant of the thrombin-treated wild-type platelets
may be anomalously low. To determine if this is the case, we treated the platelets
with 10 mM EDTA after thrombin treatment to remove the TSP-1 from the platelet
membrane (Fig. 2). Treatment of the platelets with EDTA resulted in an increase in
the level of TGF-β1 in the supernatants of both the wild-type and the TSP-1 null
platelets (Fig. 2). Thus, while there appears to be a calcium-dependent mechanism
for the association of TGF-β1 to the platelet membrane, it does not require TSP-1.

It is known that LAP contains an arginine-glycine-aspartate (RGD) sequence
which might function to localize latent cytokine to the cell surface by binding
integrins (2). Grainger et al., (27) reported indirect evidence that the RGD
sequence in platelet-derived latent TGF-β1 may be recognized by platelet
integrins. Like several other matrix proteins, human latent TFG-β binding protein
(LTBP) also contains an RGD sequence, however, there are no reports that this
sequence serves as an integrin ligand (2). In wild-type mice, LAP can remain
associated with the TSP-1/TGF-β1 complex without inhibiting the activity of TSP-
1-associated TGF-β1 (23).

Surprisingly, we have found that the absence of TSP-1 has no effect on the
level of active TGF-β1 in the supernatant of thrombin-treated platelets (Fig. 1 &
2). In the lung, pancreas and liver, TSP-1 null mice exhibit pathologies that are
similar to, although generally not as severe as, TGF-β1 null mice (21, 24). The abnormalities include epithelial cell hyperplasia and focal inflammation. Treatment of the TSP-1 null mice with the TGF-β1 activating peptide KRFK normalizes these abnormalities in the lung and pancreas (24). Furthermore, treatment of wild-type mice with a peptide (LSKL) that inhibits the ability of TSP-1 to activate TGF-β1 induces lung and pancreas pathologies similar to those seen in the TSP-1 null mice (24). These data indicate that constitutive activation of TGF-β1 by TSP-1 contributes to epithelial homeostasis in some organs. It remains possible that TSP-1 is an important activator of TGF-β1 in platelets from wild-type mice and that an alternative pathway is upregulated in the absence of TSP-1. Since active TGF-β1 is present in the supernatant of thrombin-treated TSP-1 null platelets, alternative mechanisms for TGF-β1 activation must be present in platelets. These mechanisms may involve the association of TGF-β1 with another protein that is present in the α-granules or shed from the platelet membrane after activation. Since the platelets are removed shortly after thrombin-treatment, it is unlikely that the activation of TGF-β1 is due to the release of a constituent of the lysosomal compartment. It is also unlikely that the amount of thrombin used or the duration of exposure to thrombin would be sufficient to activate significant levels of TGF-β1. In fact, human thrombin (0.1, 0.5 and 1 U/ml) added to both serum and platelet releasate samples for 30 min did not increase the amount of active TGF-β1, as compared to non-thrombin-treated samples, demonstrating that thrombin was not responsible for the active TGF-β1 detected in our assays (data not shown). An alternative interpretation of the data is that TSP-1 is not a significant activator of TGF-β1 in platelets. In wild-type platelets, the majority of TGF-β1 is inactive despite a vast excess in the number of TSP-1 molecules as compared to TGF-β1 molecules (12, 28). Since the type 1 repeats appear to be important for protein-protein interactions in general, it is possible that TGF-β1 binding to TSP-1 is inhibited by the presence of another protein. It is also possible that post-translational modifications of TSP-1 that occur in megakaryocytes inhibit TGF-β1 binding. The data clearly show that coexpression of TSP-1 and TGF-β1 does not necessarily mean that TGF-β1 will be activated via a TSP-1-dependent mechanism. Because TSP-1 and TGF-β1 are stored and secreted together from platelet α-granules, and because active TGF-β1 has a short half-life, an independent spatial and temporal mechanism for regulating TGF-β1 activation may be necessary. This mechanism may not be active in epithelial cells because TGF-β1 and TSP-1 are constitutively secreted and a basal level of activated TGF-β1 is maintained.
We also assayed the levels of TGF-β1 in the plasma of wild-type and TSP-1 null mice to determine if there is a systemic increase in TGF-β1 levels that might compensate for the lack of a TSP-1-dependent activation mechanisms that exist in epithelial tissues (21, 24). A statistically significant (P<0.01) increase in the total TGF-β1 was observed in the plasma of the TSP-1 null mice as compared to wild-type mice on the C57BL/6 background (Table I). By contrast, no difference was observed in the plasma levels of TGF-β1 in the TSP-1 null mice and the wild-type mice on the 129Sv background. No active TGF-β1 was found in the plasma of either TSP-1 null and wild-type mice. Free TGF-β1 can interact with and be inactivated by a number of soluble or matrix molecules, including α2-macroglobulin, decorin, betaglycan, and fucoidan (29, 30). Furthermore, TGF-β1 in plasma is found almost exclusively bound to α2-macroglobulin and presumably represents activated TGF-β1 that will be cleared by the liver (31). The physiological mechanisms for the increase in the level of latent TGF-β1 in TSP-1 null C57BL/6 mice is currently unknown.

In this study, we have shown that the levels of latent and active TGF-β1 in the supernatant of thrombin-treated platelets are equivalent in the presence or absence of TSP-1. Thus, TSP-1 does not appear to function as a chaperon for TGF-β1 during platelet production. The lack of correlation between TSP-1 expression and the level of active TGF-β1 indicates that (i) the ability of TSP-1 to activate TGF-β1 is inhibited in wild-type mice and (ii) alternative mechanisms for activation of TGF-β1 are present in platelets. Elucidation of the mechanisms underlying each of the observations will have important implications for the regulation of TGF-β1 activation in vivo.

Acknowledgements—The technical assistance of Mark Duquette is greatly appreciated. The authors wish to thank Professor Mark W.J. Ferguson for his helpful comments on this manuscript.
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TABLE I

*Plasma TGF-β-1 in C57BL/6 and 129Sv mice*

TGF-β1 levels in the plasma of C57BL/6 and 129Sv mice were determined by a TGF-β1 ELISA kit. Results are expressed as the means ± S.E.M. of two duplicate determinations of twenty two to twenty three separate experiments for C57BL/6 mice and twenty two to twenty six separate experiments for 129Sv mice.
FIGURE LEGENDS

FIG.1. **Active TGF-β1 is present in the supernatant of thrombin-treated platelets from TSP-1 null mice.** Mice platelets from TSP-1 null and wild-type mice were activated with 0.5 U/ml human thrombin for 3 min, platelet suspensions were centrifuged and platelet supernatants were removed. Total and active TGF-β1 were assayed in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type C57BL/6 (A) and 129Sv (B) mice by a TGF-β1 ELISA kit and the plasminogen activator inhibitor-1/luciferase bioassay. Results are expressed as the means ± S.E.M. of duplicate determinations of 4-6 separate experiments (ELISA) and 3-4 separate experiments (bioassay).

FIG.2. **Treatment of platelets with EDTA increases the level of TGF-β1 in the supernatants of both the wild-type and the TSP-1 null platelets.** Mice platelets from TSP-1 null and wild-type were activated with 0.5 U/ml human thrombin for 3 min and treated with 10 mM EDTA for 10 min. Platelet suspensions were centrifuged and platelet supernatants were removed. Total and active TGF-β1 were assayed in platelet releasates from TSP-1 null and wild-type C57BL/6 (A) and 129Sv (B) mice by a TGF-β1 ELISA kit and the plasminogen activator inhibitor-1/luciferase bioassay. Results are expressed as the means ± S.E.M. of duplicate determinations of 3-5 separate experiments (ELISA) and 3-4 separate experiments (bioassay).
Table I

|            | C57BL/6 mice | 129Sv mice |
|------------|--------------|------------|
|            | TSP-1 +/+    | TSP-1 -/-  | TSP-1 +/+    | TSP-1 -/-  |
| Total TGF-β1 | 13.4 ± 1.7   | 13 ± 2.2   | 11.2 ± 1.3   | 11.2 ± 1.3 |
| Active TGF-β1 | 0           | 0          | 0            | 0          |
|            | 20 ± 3       | 0          | 0            | 0          |

*p < 0.01
FIG. 1

A

TSP-1 +/+  

TSP-1 -/-

ELISA  
Bioassay

Total TGF-β1  
Active TGF-β1

B

TSP-1 +/+  

TSP-1 -/-

ELISA  
Bioassay

Total TGF-β1  
Active TGF-β1
**FIG. 2**

(A) TSP-1+/+ vs. TSP-1-/-

- **ELISA**: Black bars
- **Bioassay**: White bars

**Y-axis**: TGF-β1 ng/ml

**X-axis**: Total TGF-β1, Active TGF-β1, Total TGF-β1, Active TGF-β1

(B) TSP-1+/+ vs. TSP-1-/-

- **ELISA**: Black bars
- **Bioassay**: White bars

**Y-axis**: TGF-β1 ng/ml

**X-axis**: Total TGF-β1, Active TGF-β1, Total TGF-β1, Active TGF-β1
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J. Biol. Chem. published online April 14, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C000132200

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