Plasma transforming growth factor $\beta_1$ and platelet activation: implications for studies in transplant recipients

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Abstract

**Background.** Evidence from animal models supports the hypothesis that dysregulated transforming growth factor $\beta_1$ (TGF$\beta_1$) expression plays a role in chronic allograft rejection, the progression of diabetic nephropathy and fibrotic glomerulopathies. However, more evidence is required to support this hypothesis in man, and the current literature concerning blood TGF$\beta_1$ levels in clinical studies is highly confused. We have investigated: (i) the hypothesis that the widespread practice of activating clinical samples prior to measurement of TGF$\beta_1$ is detecting the platelet-released pool of TGF$\beta_1$, artefactually generated on venepuncture and unrepresentative of the real circulating in vivo TGF$\beta_1$ pool; and (ii) the effect of different immunosuppressive drugs on apparent TGF$\beta_1$ plasma levels.

**Methods.** The effect of two different venepuncture procedures on plasma TGF$\beta_1$ was compared in 10 healthy volunteers, one procedure designed to minimize platelet activation and the other representing standard venepuncture practice in a clinic situation. Blood samples from 52 renal transplant recipients on either cyclosporine or tacrolimus immunosuppression were taken by standard venepuncture to investigate the effect of immunosuppressive drugs on plasma TGF$\beta_1$. Plasma TGF$\beta_1$ and $\beta$ thromboglobulin were measured by ELISA.

**Results.** Among 10 healthy volunteers who underwent two different methods of venepuncture, eight of 10 had undetectable levels of TGF$\beta_1$ (<100 pg/ml) under conditions that minimize platelet activation. In contrast, all 10 paired plasma samples collected by vacutainer had measurable TGF$\beta_1$ (median 7.70 ng/ml, interquartile range 5.87–13.64 ng/ml) following acid/urea activation. The median $\beta$TG level (a measure of platelet degranulation) was 0.71 $\mu$g/ml (interquartile range 0.53–1.19 $\mu$g/ml) in the special collections compared with 3.39 $\mu$g/ml (interquartile range 2.27–4.33 $\mu$g/ml) in the vacutainer samples ($P=0.0029$).

Among 52 allograft recipients there was a significantly higher mean TGF$\beta_1$ level in plasma from patients on cyclosporine therapy compared with patients on tacrolimus (28 090±26 860 pg/ml vs 7173±10 610 pg/ml, respectively; $P<0.002$). Mean plasma $\beta$TG levels were also significantly higher during cyclosporine therapy compared with tacrolimus (8.14±5.54 $\mu$g/ml vs 3.66±3.32 $\mu$g/ml, respectively; $P<0.002$). However, when TGF$\beta_1$ values were corrected for the degree of platelet activation (by factoring with $\beta$TG) there was no significant difference between TGF$\beta_1$ levels on cyclosporine or tacrolimus (4117±2993 pg/µg $\beta$TG vs 2971±658 pg/µg $\beta$TG, respectively; $P=0.294$).

**Conclusions.** To avoid erroneous hypotheses concerning TGF$\beta_1$ and perpetuating confusion in the literature over levels in health and disease, it is imperative that proper internal controls for platelet activation are used. The effects of experimental treatments and drugs on platelet biology must be rigorously controlled when attempting to measure and interpret plasma levels of TGF$\beta_1$ in clinical practice.

**Keywords:** immunosuppression; platelets; renal allografts; TGF$\beta_1$; immunoassay

Introduction

There is little doubt that TGF$\beta_1$ is profibrotic in many disease conditions. In an experimental model, persistent expression of active TGF$\beta_1$ in the vasculature promotes fibrosis in the kidney [1]. Neutralizing the effects of TGF$\beta_1$ in experimental models with natural antagonist [2] protects against the development of tissue fibrosis.
Clinical studies of fibrotic disease have measured circulating blood levels of TGFβ1 in patients and healthy individuals to investigate the link between TGFβ1 expression and the clinical presentation and prognosis of disease. A highly confused literature on blood levels of TGFβ1 has developed with claims that values in normal controls are low [3] or high [4], that certain drugs induce high levels of circulating TGFβ1 [5], and that patients in end-stage renal failure (ESRF), in particular African Americans [6], have high levels of circulating TGFβ1.

Attention has been drawn recently to the difficulties involved in interpretation of data on TGFβ1 blood levels [7]. The complex interaction of factors including the choice of blood sample (serum or plasma), the deliberate sample activation, the technical measurement of TGFβ1 by ELISA, and the presence of latent and active pools of TGFβ1 have a profound influence on the interpretation of TGFβ1 levels in a clinical context. The fundamental problem in estimating a real in vivo circulating level of TGFβ1 is how to avoid the enormous platelet pool of readily releasable TGFβ1. TGFβ1 was originally isolated from platelets [8], where it is stored at high concentration as a latent complex. Most of the confusion in the literature relates to whether or not researchers adopt a strategy to avoid, minimize or control for platelet-released TGFβ1 occurring in the sample ex vivo.

The choice of serum as the medium in which to quantitate TGFβ1 seems particularly inappropriate for two reasons. First, there is a significant correlation between the total platelet count and TGFβ1 levels [9] (measured after activation of the latent molecule, see below), which is common to other platelet-stored proteins e.g. β-thromboglobulin [10]. Secondly, it has been demonstrated that 95% of the TGFβ1 in serum is artefactually derived from platelets on blood clotting [3]. This accounts for serum TGFβ1 levels being reported as 10- to 20-fold [11], 20-fold [3] and 3.84-fold [9] higher than plasma levels.

TGFβ1 levels in plasma (measured after activation of the latent molecule and often referred to as total level) have also been reported by workers in clinical studies of TGFβ1 expression as representing a circulating blood level [3]. The recommendation that plasma TGFβ1 levels should be related to the level of a recognized platelet release protein (β-thromboglobulin or platelet factor 4) has unfortunately not been adopted as standard practice [3]. Workers who recognize the danger of platelet-released TGFβ1 recommend the use of platelet poor plasma [12], but this does not address or control for the variable platelet release of proteins that occurs during the trauma of venepuncture.

This study is therefore designed (i) to illustrate the effect of venepuncture on the non-specific release of TGFβ1 from platelets at the point of blood collection, and (ii) to demonstrate how a failure to correct for platelet activation can lead to erroneous conclusions about the effect of different immunosuppressive drugs on plasma TGFβ1 expression. This study is not designed to recommend the optimum method of venepuncture or quantitation of plasma TGFβ1, but is investigating the effects of two important modulators of platelet activation (trauma at venepuncture and drug-platelet effects) on the subsequent measurement of plasma TGFβ1 after deliberate sample activation and how these impact on our interpretation of TGFβ1 biology in renal transplantation.

Subjects and methods

The effect of venepuncture: the healthy volunteer group

Ten healthy volunteers (mean age ± SEM 34.4 ± 2.3 years; 7 males, 3 females; 9 Caucasians, 1 non-Caucasian) were subjected to two venepunctures from two different sites on the same occasion by both the special procedure and the standard procedure. Informed consent was obtained for venepuncture.

Special venepuncture procedure

This procedure is recommended to minimize platelet activation during venepuncture [12]. Blood samples were collected from healthy volunteers without the use of a tourniquet by introducing a wide bore (16 gauge needle) into the antecubital vein with minimal trauma and allowing blood to flow slowly without suction from syringe or vacutainer into a pre-chilled EDTA bottle. Samples were separated immediately by centrifugation at 1200 g for 10 min and the plasma aliquots were stored at −70 °C.

Standard venepuncture

Blood samples were collected from healthy volunteers using a tourniquet by venepuncture with a 21 gauge needle and vacutainer blood collection system (Becton and Dickenson, Oxford, UK). Blood was collected into EDTA tubes and plasma was immediately separated by centrifugation at 1200 g for 10 min, and plasma aliquots were stored frozen at −70 °C. Thus, trauma during venepuncture was the only difference in the procedure for obtaining plasma samples from normal volunteers.

The effect of immunosuppressive drugs: the allograft recipients

Blood samples from 52 patients (mean age ± SEM 44.2 ± 2.0 years; 35 males, 17 females; 44 Caucasian, 8 non-Caucasians) undergoing renal transplantation at Manchester Royal Infirmary were obtained by standard venepuncture procedure as part of normal clinical follow up and monitoring of graft function. Samples were separated within 3 h by centrifugation at 1200 g and storage at −70°C. Thirty-two patients were receiving cyclosporine-based immunosuppression with a mean drug trough level of 156 ng/ml, range 64–275 ng/ml (monotherapy or combined with steroids and azathioprine), and 20 patients received tacrolimus monotherapy with a mean drug trough level of 13 ng/ml, range 8–36 ng/ml. In the total patient group, the methods of venepuncture and sample preparation were kept constant (and were typical of those employed
widely in clinical practice) to allow comparison of the effects of immunosuppressive drugs on TGFβ1 levels.

**Acid/urea activation of plasma TGFβ1**

The method was based on the R&D Systems (Abingdon, UK) procedure from the QuantiKine Immunoassay kit. To 100 μl of plasma, 100 μl of 2.5 N acetic acid/10 M urea were added, mixed and incubated for 10 min at room temperature. The samples were neutralized by adding 100 μl of 2.7 N NaOH/1 M HEPES and added to the assay plate within 15 min of activation.

**Acid activation of plasma TGFβ1**

One-and-a-half microlitres of 6 M HCl was added to 100 μl of plasma and incubated for 15 minutes at room temperature. Samples were neutralized to pH 7.0–7.4 with ~3.0 μl of neutralizing buffer (50% vol/vol 1 M HEPES/6 M NaOH).

**Immunoassay for TGFβ1**

The in-house assay has been described in detail [13]. Briefly, the capture antibody was a monoclonal to TGFβ1,2,3- (Genzyme, Framingham, USA, clone 1D11) and the detection antibody was a chicken polyclonal antibody specific to TGFβ1 (R&D Systems), followed by peroxidase conjugated donkey anti-chicken Ig (Jackson Labs, Strach, Luton, UK). Signal was generated by the addition of Ameerlite substrate (Johnson and Johnson Clinical Diagnostics, Ascot, UK) and read on a Microlumat LB96P luminometer (EG&G Berthold, Leod, UK). Data was analysed using Microwin software.

**Assay for β thromboglobulin (βTG)**

An ELISA for βTG was employed using the principle of competition between soluble βTG in plasma and solid phase βTG coated onto an ELISA plate for a limited amount of anti-βTG antibody added to the samples. Purified βTG (Novabiochem Ltd, Nottingham, UK) was coated onto ELISA plates at 200 ng/ml in 100 μl of 0.1 M sodium bicarbonate/carbonate buffer, pH 9.6 for 16 h. After washing, the plates were blocked with 5% bovine serum albumin (BSA) for 1 h. Plasma samples were diluted 1:25 with assay buffer (5% BSA in PBS with 0.05% Tween 20) and mixed with an equal volume of rabbit anti-βTG antibody (at a dilution of 1:2000) and added to the plate. βTG standards covering the range 3.0–600 ng/ml were included and the assay was calibrated against the WHO 1st International Standard Preparation for βTG (83/501). After incubation for 4 h, the plates were washed and incubated with peroxidase-conjugated anti-rabbit IgG (Jackson Labs) (dilution 1:8000) for 2 h. Following washing, ABTS substrate was added and the absorbance was measured at 405 nm in a multi-well spectrophotometer and results calculated using Softmax software.

**Statistical methods**

Non-parametric data sets were analysed by Mann–Whitney U-test for differences in median. Data sets showing a normal distribution were analysed for differences in mean by students t-test or paired t-test. A correlation between TGFβ1 and βTG was assessed using linear regression and Pearson’s correlation test. Significance was attributed to analyses with \( P < 0.05 \).

**Results**

The effect of venepuncture

Ten healthy normal individuals volunteered to undergo two venepunctures on the same occasion using the special procedure (to avoid platelet activation) applied to one arm and the standard method with vacutainer (to replicate normal trauma causing platelet activation) to the other. The subsequent processing of the samples was identical to allow investigation of the effect of venepuncture trauma only. The samples were analysed for the presence of TGFβ1 and βTG, and the results are shown in Table 1 and Figure 1. Eight of 10 samples taken under conditions that avoid platelet activation showed undetectable levels of TGFβ1 (<100 pg/ml) following acid/urea activation by the in-house assay (Figure 1a). In contrast, all the paired samples taken by standard venepuncture had measurable levels of TGFβ1 (median 7.70 ng/ml, interquartile range 5.87–13.64 ng/ml). In parallel measurements of βTG (Figure 1b) in the same samples, the median value of βTG in the special samples was 0.71 μg/ml.

**Table 1. TGFβ1 and βTG results on 10 normal blood samples taken on the same occasion by two different venepuncture methods**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Special venepuncture</th>
<th>Standard venepuncture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TGFβ1 pg/ml</td>
<td>βTG μg/ml</td>
</tr>
<tr>
<td>1</td>
<td>&lt;100</td>
<td>0.194</td>
</tr>
<tr>
<td>2</td>
<td>&lt;100</td>
<td>0.580</td>
</tr>
<tr>
<td>3</td>
<td>9280</td>
<td>4.979</td>
</tr>
<tr>
<td>4</td>
<td>&lt;100</td>
<td>0.930</td>
</tr>
<tr>
<td>5</td>
<td>&lt;100</td>
<td>0.661</td>
</tr>
<tr>
<td>6</td>
<td>&lt;100</td>
<td>1.442</td>
</tr>
<tr>
<td>7</td>
<td>&lt;100</td>
<td>0.480</td>
</tr>
<tr>
<td>8</td>
<td>&lt;100</td>
<td>0.763</td>
</tr>
<tr>
<td>9</td>
<td>&lt;100</td>
<td>0.460</td>
</tr>
<tr>
<td>10</td>
<td>2440</td>
<td>1.700</td>
</tr>
</tbody>
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(interquartile range 0.53–1.19 µg/ml) compared with a median value of 3.39 µg/ml (interquartile range 2.27–4.33 µg/ml) in the standard samples, a significant 4.8-fold increase (P = 0.0029 by the Mann–Whitney U-test). However, these values for βTG clearly indicate that despite the precautions taken with the special venepuncture procedure, the βTG values at 0.71 µg/ml represent a >20-fold increase above the level deemed to be present in plasma where no platelet activation has occurred, but ~20-fold less than is found in serum [12].

The two special samples that had detectable TGFβ1 also had the highest levels of βTG (4.98 and 1.70 µg/ml) and their paired standard samples showed the lowest increases in βTG (Figure 1b), indicating either a significant in vivo platelet activation in these normal individuals or a greater sensitivity to platelet activation on venepuncture. By expressing the results from the standard venepuncture as TGFβ1:βTG ratio, the spuriously high TGFβ1 levels are corrected for degree of platelet activation (Table 1).

**The effect of immunosuppressive drugs on platelet-released TGFβ1 and βTG**

TGFβ1 and βTG were measured in plasma samples taken by standard venepuncture from renal transplant patients on either cyclosporine- or tacrolimus-based immunosuppressive therapy and the results are shown in Figure 2. There was a significant relationship between TGFβ1 and βTG in the sample population when analysed by Pearson’s correlation test, r = 0.751, P < 0.0001.

There was no correlation between the platelet count and either TGFβ1 (Pearson correlation r = −0.067, P = 0.634) or βTG (r = 0.025, P = 0.959) in these samples (data not shown).

There was a significantly higher mean concentration of TGFβ1 in samples from patients on cyclosporine therapy compared with patients on tacrolimus (28 090 ± 26 860 pg/ml vs 7173 ± 10 610 pg/ml, respectively; students t-test P < 0.002) (Figure 3a). Mean βTG values were also higher in samples from patients on cyclosporine therapy compared with those on tacrolimus (8.14 ± 5.45 µg/ml vs 3.66 ± 3.32 µg/ml, respectively, students t-test P < 0.002) (Figure 3b). However, when the TGFβ1 values were corrected for the degree of platelet activation by factoring with βTG, there was no significant difference in plasma TGFβ1 between patients on cyclosporine or tacrolimus (4117 ± 2993 pg/µg βTG vs 2971 ± 658 pg/µg βTG, respectively; P = 0.294) (Figure 3c).

There was no difference in the mean absolute platelet count between patients on cyclosporine- or tacrolimus-based therapy (295 ± 84 vs 258 ± 72 × 10³/µl, respectively; P = 0.113) (Figure 3d).

**Fig. 1.** The effect of venepuncture procedure on the detection of (a) TGFβ1, and (b) βTG in paired plasma samples from normal subjects following deliberate sample activation. Values are represented as mean ± 1 SD, with values below detection limit designated as zero.

**Fig. 2.** Correlation of TGFβ1 levels and βTG levels in plasma samples from 52 transplant patients after deliberate sample activation. Pearson’s correlation test, r = 0.751, P < 0.0001.
Fig. 3. Plasma values (mean ± SD) in patients on cyclosporin [■] or tacrolimus [■]. (a) TGFβ1; P < 0.002 students t-test. (b) βTG; P < 0.002 students t-test. (c) TGFβ1 per µg βTG; P = 0.294, not significant, students t-test. (d) Platelet count P = 0.113, not significant, students t-test.

Discussion

This report documents a median plasma TGFβ1 level of 7.7 ng/ml (interquartile range 5.87–13.64 ng/ml) following acid/urea activation of the plasma samples taken by standard venepuncture from healthy controls. This is consistent with an aggregated mean of 8.1 ng/ml from other reported normal plasma values of 3.8 ± 2.9 ng/ml [11], 12.2 ng/ml (2.2–19.3 ng/ml) [9] and 8.2 ng/ml (4.0–18.9 ng/ml interquartile range) [14], where standard venepuncture has been used.

The one study carefully established to avoid platelet activation during venepuncture reports 4.1 ± 2.0 ng/ml (range 2.0–12.0 ng/ml) [3]. However, we have demonstrated that this plasma TGFβ1 level is artefactually generated by activating platelets during the standard venepuncture procedure and therefore does not represent the in vivo circulating pool of TGFβ1. Eight of 10 paired samples from healthy volunteers show <100 pg/ml TGFβ1 when precautions to reduce platelet activation are introduced. The two normal samples with the detectable level of TGFβ1 have the highest level of βTG, an accepted measure of platelet z granule
secretion [12], consistent with either in vivo platelet activation or activation despite the special procedure. Furthermore, dual measurement of TGFβ1 and βTG in 52 plasma samples from allograft recipients taken by standard venepuncture shows a significant positive correlation, indicating that the degree of platelet activation either in vivo or during venepuncture determines the level of detectable TGFβ1 following activation of the sample.

Thus, deliberate sample activation prior to measurement of TGFβ1 simply reveals the quantity of latent TGFβ1 released from platelets during venepuncture. For each subject, the TGFβ1 level represents the product of their intrinsic platelet stability, the degree of trauma that these platelets suffer during venepuncture and their total platelet count. In addition to these variables, further modulation of platelet biology in vivo by disease, drug therapy, surgery, haemodialysis or ethnic influences may need to be controlled. We contend that the evidence we have provided in this study supports the hypothesis that normal plasma TGFβ1 levels are undetectable (<100 pg/ml) if procedures are taken to minimize platelet activation.

It is valid to ask whether immunosuppressive drugs used in transplantation, in particular cyclosporine and tacrolimus, promote the expression of TGFβ1. It is conceivable that part of their immunosuppressive action may be mediated through the potent immunosuppressive properties of TGFβ1. Additionally, their chronic nephrotoxicity may be driven through the profibrotic expression of TGFβ1. Therefore, we quantitated the levels of plasma TGFβ1 in transplant patients on cyclosporine or tacrolimus using routine venepuncture and deliberate activation of the samples prior to measurement. Under these conditions, patients on cyclosporine appear initially to have higher plasma levels of TGFβ1, compared with patients on tacrolimus. However, there are clearly increased levels of platelet activation as evidenced by increased βTG levels in the cyclosporine samples; the apparent high plasma TGFβ1 levels in patients on cyclosporine merely result from greater platelet degranulation during venepuncture. This is not surprising since cyclosporine augments platelet reactivity [15,16] whereas tacrolimus does not [17,18]. Therefore, expressing the plasma results as TGFβ1 per unit of βTG identifies no difference in the plasma total TGFβ1 levels in patients on cyclosporine or tacrolimus. Thus, any study claiming to show a difference in TGFβ1 levels between groups or treatments that relies on deliberate activation of the latent TGFβ1 must control for the level of platelet activation in the sample as has been previously recommended [3]. Failure to include proper controls for platelet activation as a source of circulating TGFβ1 has resulted in a claim that cyclosporine stimulates in vivo TGFβ1 expression [5]. Yet this effect is arguably no more than that demonstrated in this paper, i.e. that cyclosporine renders platelets more sensitive to secretion of α granule proteins such as βTG and TGFβ1 when subjected to the stress of venepuncture. Similarly, a study suggesting that ethnic differences in rates of ESRF are dependent on overexpression of TGFβ1 has not controlled for differential platelet activation between the groups, resulting in artefactual secretion of TGFβ1. It is established that patients in ESRF on chronic haemodialysis show enhanced platelet activation [19]. Therefore, this apparent ethnic difference in expression of TGFβ1 may simply reflect uncontrolled effects on platelet activation caused by different patterns of haemodialysis and prescription of anti-hypertensive drugs [20].

In order to ensure meaningful data on circulating TGFβ1 levels, the ideal strategy is the adoption of the rigorous procedures necessary to avoid platelet activation during venepuncture as described by workers in the field of platelet biology [12]. To date, only one study of TGFβ1 has adopted these precautions [3] and they claim that there is a low level of circulating TGFβ1 from a non-platelet derived pool. These authors advocate strongly the use of a platelet release marker correction factor, which we have demonstrated does control for spurious effects of drugs causing release of TGFβ1 into the blood sample. Concentrating on producing ‘platelet poor plasma’ overlooks the variable platelet secretion of TGFβ1 that has already happened during venepuncture.

We suggest that the quality and validity of interpretation of most of the clinical data on circulating TGFβ1 levels is seriously flawed due to the failure to control for the level of platelet activation in the blood samples. The future for measurement of clinically relevant pools of TGFβ1 must be in the development of assays that quantify the products of in vivo activated TGFβ1 which may circulate as TGFβ1 complexes. Stable complexes of TGFβ1−endoglin have already been described and similar complexes with other natural antagonists may exist. Understanding the role of TGFβ1 in disease pathology is an important goal that is not being helped currently by the assay of a spuriously high platelet-derived pool of TGFβ1, as if it represented a real in vivo circulating pool of TGFβ1.

References

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Received for publication: 29.3.00
Accepted for publication: 26.5.00