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Original Article

The Role of Serum TGF-β Isoforms as Potential Markers of Osteoporosis

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Abstract. Osteoporosis is a major public health problem characterized by low bone mineral density (BMD) that presently has no biochemical test useful for its diagnosis. The cytokine TGF-β has been postulated to play a role in controlling bone density by regulating the fine balance between bone matrix deposition by osteoblasts and its resorption by osteoclasts. We explored whether measurement of serum levels of different TGF-β isoforms could be useful as a clinical tool in osteoporosis. We measured the concentration of TGF-β1 antigen using the BDA19 capture sandwich enzyme-linked immunosorbent assay (ELISA), TGF-β2 antigen concentration using a Quantikine sandwich ELISA kit and TGF-β3 antigen concentration using a modified version of the TGF-β1 Quantikine sandwich ELISA kit. Subjects were 41 women with osteoporosis (with nontraumatic vertebral fracture or lumbar spine BMD *Z*-score <-1.5 SD) and a total of 199 control women from different sources. Serum concentrations of TGF-β1 and TGF-β2 were similar in all groups. However, detectable levels of TGF-β3 (>0.2 ng/ml) were found in 35 of 41 patients with osteoporosis (median 7.2 (5.2–8.9) ng/ml) compared with 11 of 36 controls or 24 of 89 healthy women of unknown bone density. Differences among the groups could not be accounted for by age, weight, medications, use of hormone replacement therapy or the presence of osteoarthritis. Using the optimal cut-off of $\geqslant$2 ng/ml, the test was able to detect an individual with low spine BMD (*Z*-score <-1.5) with a sensitivity of 84% and a specificity of 53%, with similar results for the femoral neck. The odds ratio for osteoporosis associated with a positive test at this level was 5.93 (95% CI 2.41–11.59), and 4.1 (95% CI 1.66–10.11) using the WHO cut-off of *T*-score <-2.5. Serum TGF-β3 concentration is raised in osteoporotic women and the test appears to have potential as a marker for osteoporosis. The underlying mechanisms and the relationships between TGF-β3 and bone turnover and fractures remain to be explored.

Keywords: Bone mineral density; Osteoporosis; TGF-β

Introduction

Osteoporotic fractures, which affect 1 in 3 women in developed countries, are a major cause of mortality and morbidity leading to massive health care costs (an estimated $14 billion in the USA) [1]. Since these fractures are difficult to treat once they have occurred, it is important to prevent or treat the early asymptomatic phase with an increasing range of useful therapies. Accurate inexpensive screening methods to detect the disease before clinical fractures occur would have major implications, because at present the asymptomatic phase of osteoporosis is difficult to detect. Bone densitometry is currently the best clinical tool available to predict fracture risk and select patients for treatment, but its expense and poor performance as a screening tool have limited its widespread use other than for case-finding. Biochemical markers of bone turnover and formation have shown to be promising in predicting fractures in the elderly up to 2 years before the event, and there is a 2-fold increased risk of fracture in individuals with high levels [2]. However, disappointingly, these same markers are of no clinical use in estimating the bone density of an individual or discriminating between those with and without osteoporosis. Thus, there is an urgent

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need for new serum or urine markers that can select diagnostic or risk groups. One potential marker is the pluripotent cytokine transforming growth factor-β (TGF-β). Evidence for the role of TGF-β in regulating bone mineral density (BMD) comes from a number of observations: it is present at high concentration in bone [3,4], it promotes osteoblast proliferation [5] and their subsequent differentiation [6], and also decreases osteoclast activity by several mechanisms [7,8]. The rate of bone formation is altered in TGF-β1 knockout mice [9], and administration of TGF-β corrects the bone density deficiency in elderly mice with osteoporosis [10] and in rats with suspension-induced osteoporosis [11]. Mutations in the gene encoding human TGF-β1 have also been associated with low BMD [12].

TGF-β protein is found in a wide variety of tissues in addition to bone, including blood. There are three closely related TGF-β isoforms (β1, β2 and β3) with isoform-specific distribution patterns [13]. To date only the β1 isoform has been detected at levels in excess of 1 ng/ml in human blood [14,15]. While there are a few isolated reports of both TGF-β2 and TGF-β3 being measured in human sera, for example by Kopp et al. [16] who detected low levels (<0.2 ng/ml) in sera from cancer patients, and by Danielpour and Roberts [17] who examined an unspecified number of sera during development of their SELISA, there has been no systematic evaluation of the levels of TGF-β2 or TGF-β3 in sera from normal individuals. The concentration of circulating TGF-β has been correlated with a number of disease states, including atherosclerosis [18], certain types of cancer [19] and fibrosis [20].

Using novel assays for TGF-β isoforms in serum, we have investigated whether the circulating concentration of different isoforms of TGF-β is altered in women with osteoporosis and whether these assays might be useful as a screening test.

Subjects and Methods

Selection of Subjects

The first group consisted of consecutive women attending osteoporosis clinics within the rheumatology department at St Thomas' Hospital or Chingford Hospital, London, who had an age-corrected lumbar spine bone mineral density (LS-BMD) more than 1.5 standard deviations below the age-specific mean (Z-score), equivalent to a future fracture risk of 4-fold, or who had already suffered a nontraumatic vertebral fracture. BMD scans were performed using a Hologic QDR1000/W dual-energy X-ray absorptiometry (DXA) machine (Hologic, Waltham, MA) at the lumbar spine between L1 and L4, with a reproducibility in our hands of 1.2%, and 2% at the femoral neck. All the women in the osteoporosis group also met the current WHO definition of osteoporosis, i.e., had a *T*-score below 2.5 standard deviations (SD) of the peak mean [21]. The second group were control subjects of similar age with normal LS-BMD (within 1 SD of the age mean and with a *T*-score > -2.5 SD) recruited from a larger group of women participating in a general population survey from Chingford [22]. For the third group, blood samples were also obtained from a random selection of healthy volunteer women of unknown bone density and medication history, but of similar age to the women with osteoporosis, who responded to an advertisement requesting a small blood sample.

After preliminary analyses had demonstrated a difference among the groups, an additional group was used. This consisted of 70 women (age range 28–86 years) referred to the osteoporosis clinics with a wide range of known bone density levels measured on the same Hologic QDR 1000 machine, used to estimate the performance of the TGF-β3 assay as a diagnostic screening tool in a clinical setting.

Preparation of Blood Samples

Nonfasting peripheral blood samples were taken and allowed to clot, spun for 15 min and the serum supernatant aliquoted and frozen at −20 °C until analyzed. Once the sample had thawed, analysis was performed within 1 h and all assays were performed on separate aliquots of serum that had been frozen only once (at the time of serum preparation). All samples had less than 2% hemolysis. Those performing the assays were masked to the clinical details.

Assays for TGF-β

Active plus acid-activatable latent (a+l) TGF-β1 in serum samples was assayed using the BDA19 capture sandwich ELISA described previously [14]. This assay detects both the active form of TGF-β1 and the acid-activatable latent precursor found in human plasma. It does not detect the platelet large latent TGF-β1 complex (which requires urea as well as acid for activation). This assay detects the same concentration of (a+l) TGF-β1 in serum as in platelet-poor plasma prepared from the same blood sample. The BDA19 capture ELISA using current batches of BDA19 antiserum (R&D Systems) shows no detectable cross-reactivity to other recombinant growth factors tested, including TGF-β2 and TGF-β3, although earlier batches of BDA19 antibody showed a small degree of reactivity to recombinant TGF-β3 [14].

Total TGF-β2 antigen in serum samples was assayed using the TGF-β2 Quantikine ELISA kit (R&D Systems) in accordance with the manufacturer's instructions.

Total TGF-β3 antigen in serum samples was assayed using a modification of the TGF-β1 Quantikine ELISA kit (R&D Systems); details are given in the Appendix. This assay detects the same concentration of TGF-β3 in serum and platelet-poor plasma prepared from the same blood sample. In a pilot study, only 2 of 10 individuals had detectable TGF-β3 in serum, but for these individuals the concentration of TGF-β3 was similar in

serum and plasma. This is consistent with reports that TGF-β1 is the predominant form in human platelets [23], which we confirm here. The intra-assay coefficient of variation (CV) for the assay was 6.2% and the inter-assay CV was 7.9% using 16 aliquots of a sample containing ~10 ng/ml recombinant TGF-β3. The concentration of TGF-β3 in replicate aliquots of serum did not change by more than 10% following five cycles of freeze-thawing. The detection limit of the assay was 0.2 ng/ml, equivalent to a sensitivity to 0.02 ng/ml allowing for the 10-fold dilution necessary during acid–urea activation [24].

Statistical Analysis

Measures of BMD and levels of TGF-β2 were approximately normally distributed and therefore summarized as means and standard errors. Differences among groups were compared either by the Student's unpaired t-test (for two groups) or ANOVA (for more than two groups). However, the concentrations of TGF-β3 antigen in the whole population show a significantly skewed distribution, which remains significantly non-normal even after logarithmic or boxcox transformation. Consequently these distributions are described by the median and interquartile range, necessitating the use of nonparametric tests: a Mann–Whitney U-test (for two-group comparisons) or a Kruskal–Wallis test (for more than two groups). Correlations were computed based on ranks (Spearman's rank test). Frequency data were assessed using the chi-squared test. In all cases $p<0.05$ was taken to indicate significance. For statistical analysis, TGF-β3 concentrations below the detection limit of the ELISA (<0.2 ng/ml) were estimated to be 0.1 ng/ml. Receiver operating characteristic (ROC) curves were calculated to define the optimal cut-off point for TGF-β3 levels as a potential diagnostic for different BMD definitions of osteoporosis [22].

Results

The women with osteoporosis had a mean spine BMD 1.7 standard deviations below the age-standardized mean (mean T-score = -3.2) and 38% also had a radiographically detectable vertebral fracture. They were slightly younger than the control women, and as expected were significantly lighter and had an earlier menopause (Table 1). The BDA19 assay was used to measure the concentration of TGF-β1 in serum, because this assay does not detect platelet TGF-β1 [14]. This assay therefore provides a proxy measure for the circulating concentration of TGF-β1 in plasma. The concentration of (a+l) TGF-β1 was 4.8 (2.0–6.1) ng/ml in the control group and 2.4 (1.0–6.5) ng/ml in the osteoporosis group, which was not a statistically significant difference (p = 0.22; Mann-Whitney U-test).

The serum samples from the osteoporotic women contained detectable but very low levels of TGF-β2 antigen (0.13 ± 0.01 ng/ml; mean ± standard error), not significantly different from the individuals with normal bone density (0.15 ± 0.01 ng/ml; p = 0.23, Student's unpaired t-test). We found detectable levels (>0.2 ng/ml) of TGF-β3 in the serum of 35 of 41 individuals in the osteoporotic group compared with only 11 of 36 women in the control group (problems with 4 samples meant they were unanalyzable) and 24 of 89 of the volunteer women.

Table 1. Characteristics of the groups studied

	Controls	Osteoporotic patients
Number	40	41
Age (years)	60.3 (2.8)	56.8 (6.0)
Age at menopause (years)	49.4 (4.4)	46.0 (12.8)
Weight (kg)	68.7 (12.6)	59.2 (8.0)
LS-BMD (g/cm^2)	0.97 (0.14)	0.70 (0.21)
LS Z-score	+0.54	−1.66
LS T-score	−0.69	−3.2
FN-BMD (g/cm^2)	0.75 (0.11)	0.63 (0.11)
FN Z-score	+0.06	−1.19
FN T-score	−0.88	−1.98
HRT (n)	7 (17%)	12 (30%)

Values are means (standard errors). 'Weight' is reported because height was not measured for the calculation of body mass index. 'LS-BMD' is the lumbar spine bone mineral density and 'FN-BMD' is the femoral neck bone mineral density. 'LS Z-score' and 'FN Z-score' represent the age-corrected bone mineral densities for lumbar spine and femoral neck, respectively. 'HRT' is the number of women in each group currently taking hormone replacement therapy. With the exception of HRT, no more than two women in the whole study were taking any prescription medication.

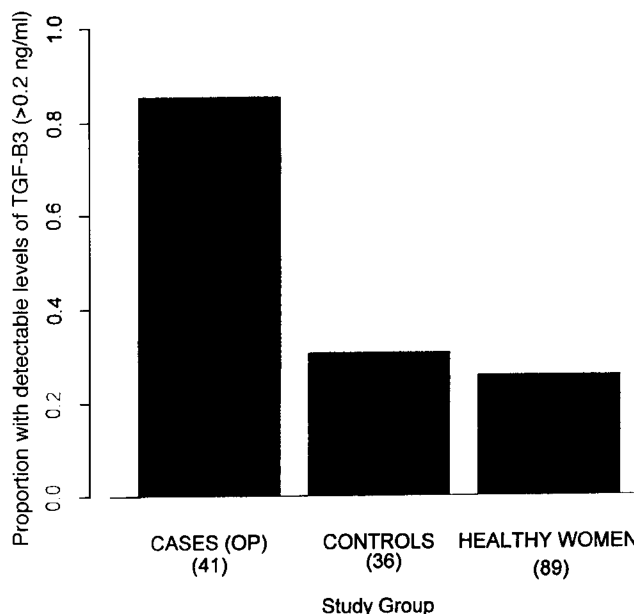

Fig. 1. The proportion of subjects with detectable levels of TGF-β3 (>0.2 ng/ml) in each of the three study samples: the osteoporosis (OP) group (n = 41), the control group (n = 36) and the set of healthy women (n = 89). The proportion of subjects with detectable levels of TGF-β3 is significantly higher in the osteoporosis group than in all the controls put together (p<0.001). There is no difference between the controls and the set of healthy volunteer women (p=0.34).

Table 2. Sensitivity and specificity estimates using the pooled data (n = 141)

TGF-β3 (ng/ml)	Lumbar spine BMD					
	Z-score −1.50 SD		*Z*-score −1.00 SD		*T*-score −2.5 SD	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
>1.0	0.837	0.485	0.872	0.484	0.811	0.447
>2.0	0.837	0.535	0.830	0.537	0.792	0.506
>3.0	0.674	0.576	0.809	0.579	0.680	0.532

Sensitivity and specificity for different TGF-β3 thresholds as a diagnostic test for osteoporosis using milder and more prevalent definitions of osteoporosis.

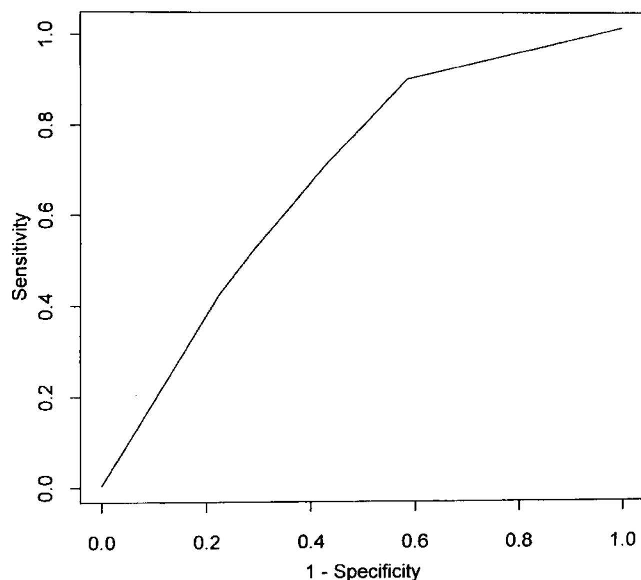

Fig. 2. ROC curves for the performance of a positive test for TGF-β3 at or greater than 2 ng/ml to detect individuals with osteoporosis (defined as 1.5 SD below the age mean for BMD of the lumbar spine), based on 141 women of known bone density.

The median levels (and ranges) were 7.2 (5.2–8.9) ng/ml in the osteoporotic group, 2.4 (0.1–5.9) ng/ml in the control group and 1.6 (0.1–4.6) ng/ml) in the volunteer women ($p<0.001$, Kruskal–Wallis test; (Fig. 1). There was no significant difference in serum TGF-β3 concentration between the women with osteoporosis who had had a previous fracture (6.9 (4.8–9.1) ng/ml; $n=15$) and those who had not (7.2 (5.3–8.7) ng/ml; $n=26$; $p = 0.83$, Mann–Whitney U-test). Adjustment for any differences in age, weight, menopause status, hormone replacement therapy (HRT) or use of any prescription medication did not influence the results. Within the osteoporotic subjects with detectable levels of TGF-β3 we were unable to detect any significant correlation of TGF-β3 levels with BMD at the spine ($r=0.003$, $p=0.98$), and only a weak correlation at the hip site ($r=0.32$, $p=0.17$), before or after age correction. The results were similar in the control group. In neither cases nor controls was there a correlation between TGF-β3 levels and age, weight, smoking status or menopause. Current HRT users had slightly higher levels of TGF-β3, but exclusion of these individuals did not affect the results.

To assess the clinical usefulness of TGF-β3 as a diagnostic test in a clinical setting and to increase the power of our study we added to our sample a further 70 women, of whom 68 were all analyzable with full BMD and isoform data, with a range of bone densities so that ROC curves could be calculated on the pooled group of 141 women of known bone density and TGF-β3 levels to determine the optimal TGF-β3 cut-off limits for different levels of bone density. Using a definition of osteoporosis of LS-BMD (Z-score) below −1.5 SD (regardless of the presence of vertebral fractures), a cut-off of 2 ng/ml TGF-β3 had the best performance, with a sensitivity of 84% and a specificity of 53% (Fig. 2). For the 68 of the 70 additional subjects BMD at the femoral neck (FN) had also been measured, and the test characteristics using FN-BMD were the same, being 84% and 53% respectively. Selecting a milder (and more prevalent) definition of osteoporosis of Z-score < -1 SD (equivalent to a 2.5-fold increased fracture risk), the sensitivity was 61% and the specificity 62%. The test also had similar characteristics using the age-independent WHO criteria (T-score < -2.5 SD): sensitivity and specificity 79% and 51% for LS-BMD and 58% and 73% for FN-BMD. The risk of having osteoporosis (using the definition Z-score < -1.5 SD) associated with a positive blood test of 2 ng/ml or more was 5.93 (95% CI 2.41–11.59), and 4.1 (1.66–8.86) for the WHO definition of osteoporosis (T-score < -2.5 SD).

We conclude that elevated serum concentrations of TGF-β3 are associated with the presence of osteoporosis. However, we were unable to detect any significant correlation between the serum concentration of TGF-β3 and BMD (at either the femoral neck or lumbar spine, before or after age correction) in either the control group or the group with osteoporosis.

Discussion

Women with osteoporosis have significantly elevated levels of TGF-β3, which has previously been believed to be present only at very low levels in human serum. An individual with a TGF-β3 level of 2 ng/ml or more has an approximately 4- to 6-fold increased risk of having osteoporosis as diagnosed by different cut-offs of BMD measurements. The elevated levels of TGF-β3 among women with osteoporosis could not be explained by

differences in age, weight, menopause, arthritis, HRT use or medications. TGF-β1 levels were not significantly different among the groups despite the smaller coefficient of variation for the assay compared with that for TGF-β3.

Our observations are consistent with the substantial body of evidence implicating TGF-β in the regulation of osteoblast and osteoclast activity. However, most of the available data suggest that TGF-β activity promotes mineralization [5–9,11], and low levels might be expected in osteoporosis. This apparent paradox would be resolved if the serum concentration of TGF-β3 were inversely related to the TGF-β3 concentration in the bone tissue. This is not an unlikely postulate, since the level of TGF-β activity in bone tissue appears to be regulated by extracellular matrix localization, particularly binding to the TGF-β binding protein LTBP [25]. Although speculative, it is possible that elevated serum TGF-β3 levels may then reflect failure to retain TGF-β3 within the bone matrix, resulting in reduced bone tissue TGF-β activity that may contribute to the development of pathologic low BMD and osteoporosis.

The hypothesis that elevated circulating concentrations of TGF-β3 results from failure to retain TGF-β in the bone matrix is further supported by recent work on the mechanism of action of heparin. Chronic use of this sulfated proteoglycan results in demineralization of the bone and reduced BMD [26,27]. Studies have now shown that an important action of heparin is to release TGF-β complexes from their interaction with matrix proteins [28,29]. Thus heparin may act by reducing the bone matrix reservoir of TGF-β. It is unclear where any released TGF-β protein may go, but one possibility is loss of the TGF-β into the circulation. However, it is not currently known whether TGF-β3 binds efficiently to LTBP or fibrillins in bone. The lack of a clear linear relationship between TGF-β3 and BMD, whilst possibly being related to small sample sizes and scale differences, may also be explained by this ‘overspill’ effect, since the serum TGF-β3 concentration may be a relatively poor proxy measure of bone TGF-β3 levels. The cross-sectional nature of the study makes it difficult to separate cause and effect and, as fracture cases were prevalent, we could not look at time since fracture.

Although one can speculate that TGF-β3 activity may play a role in regulating bone turnover, such a mechanism is unproven. It is plausible that elevated levels of TGF-β3 in circulation may result from loss of bone matrix and be a consequence of the demineralization process. However, since our findings that the serum concentration of TGF-β1 and TGF-β2 are not increased in osteoporosis, despite TGF-β1 being present at high levels in bone and being of undoubted importance in bone metabolism suggest that the elevation in TGF-β3 concentration is unlikely to be due to nonspecific release of the protein during demineralization. It is unclear why only the concentration of the β3 isoform should be altered in the serum of osteoporotic patients, but may involve feedback loops. Although most work has focused on TGF-β1, no clear qualitative differences have emerged between the different isoforms regarding their effect on bone cell activity, although in several models TGF-β3 appears to have a 3- to 10-fold greater potency than the other two isoforms and binds more strongly to fetal bone cells [30]. Recent preliminary studies have also shown that administration of TGF-β3 to animals with bone fractures increases speed of healing [31]. Further bone work on TGF-β3 knockout models would also be helpful to judge whether TGF-β3 has a major role in bone or is acting primarily as a passive marker.

Bone densitometry is currently the method of choice in determining risk of osteoporotic fracture [32]. BMD testing is, however, far from perfect; it lacks sufficient sensitivity and specificity for use as a general screening tool for fracture prevention, it is affected by artifacts in the elderly, and relatively few patients have ready access to the machines [32]. This has encouraged the search for other diagnostic approaches including serum and urine markers of bone formation and resorption. Partly because variation in levels of these markers between individuals is large, existing assays have not been sufficiently discriminatory for diagnostic use on their own or have not been proven to be useful as an adjunct to BMD measurements. For example, in a study comparing a number of existing bone markers as a screening test, using 35 osteoporotics and 208 controls, only one marker (urinary deoxypyridinoline) was significantly different (by 30–40%) between the two groups, with an optimal sensitivity of approximately 60% [33]. Early reports suggested such markers may be able to discriminate fast and slow bone losers and predict bone loss, although recent studies have been unable to confirm this [34]. Risk factor profiles, whilst useful in epidemiologic studies, have not been shown to be sensitive or specific enough in identifying individuals with low BMD or asymptomatic fracture to justify their widespread use as a surrogate for or addition to BMD [35,36]. Thus there is currently no simple screening method that replaces the need for a bone density measurement and that could be widely applicable to the general population. The results for TGF-β3 show it is not ideal as a screening test and the current test is labor-intensive and costs about $8–10 in reagents. Its use as a rapid commercial assay in conjunction with and/or comparison with existing bone turnover markers needs to be investigated further. Due to inadequate quality and quantity of stored samples, direct comparison with bone markers was not possible in the current study.

In conclusion, whilst the mechanism for the increase in TGF-β3 in the serum of osteoporotic patients is still speculative and it remains to be seen whether TGF-β3 has any future prognostic value for fractures or rates of bone loss, measuring the serum concentration of TGF-β3 may be useful in diagnosing existing preclinical osteoporosis as a surrogate or supplementary test for bone densitometry and in understanding the complex pathways involved in osteoporosis.

Appendix. An assay for TGF-β3 in human plasma

We have developed a novel assay method for measuring total TGF-β3 antigen in human plasma using commercially available reagents. This assay utilizes the capture agent of the Quantikine TGF-β1 assay (R&D Systems), which captures both TGF-β1 and TGF-β3 following treatment with acid and urea. Bound TGF-β3 was then detected using the anti-TGF-β3 specific polyclonal antiserum BDA48 (R&D Systems). This assay detects recombinant human TGF-β3 (R&D Systems) in the concentration range 0.4 ng/ml to 25 ng/ml (Fig. A1a) but does not detect TGF-β1 or TGF-β2 at concentrations up to 100 ng/ml (ΔA450 < 0.1% compared with 100 ng/ml TGF-β1). TGF-β1 can, though, interfere with the capture of TGF-β3 by competing for capture sites (Fig. A1b). However, inclusion of at least 10 μg/ml of TGF-β1 specific neutralizing antibody (BDA19; R&D Systems) abolishes the interference caused by 100 ng/ml TGF-β1 (Fig. A1b). This is confirmed by our observation that where individuals have detectable levels of TGF-β3 in plasma (which contains <5 ng/ml TGF-β1), we detect the same level in serum (which contains >50 ng/ml TGF-β1). Consistent with this observation of similar levels of total TGF-β3 antigen in plasma and serum, we did not detect TGF-β3 in lysed human platelets (Fig. A1c).

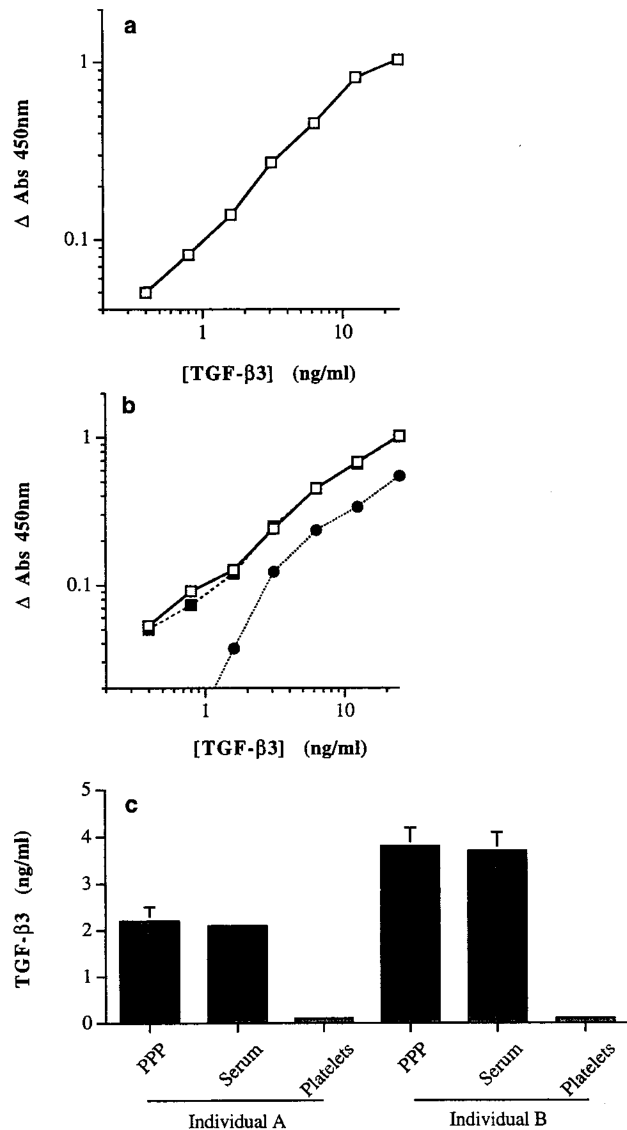

Fig. A1. **a** Detection of standard solutions of recombinant human TGF-β3, presented as the difference in absorbance at 450 nm after the color reaction, between the standard solution and the blank (Δ Abs 450 nm). The concentration of TGF-β3 resulting in half-maximal Δ Abs 450 nm was 16 ± 3 ng/ml (n = 3). The sensitivity of the assay was between 0.1 and 0.4 ng/ml. **b** Effect of TGF-β1 on the detection of TGF-β3. Standard solutions of TGF-β3 were detected significantly less well in the presence of 100 ng/ml TGF-β1 (*filled circles*) than in the absence of TGF-β1 (*filled squares*). However, TGF-β3 standard solutions incubated in the presence of 100 ng/ml TGF-β1 and 25 μg/ml anti-TGF-β1 neutralizing antibody (*open squares*) were detected normally. **c** TGF-β3 antigen (mean ± standard error) in platelet-poor plasma (PPP), serum and platelets prepared from a single bleed from each of two individuals (A, 46-year-old man; B, 42-year-old woman). Bloods for eight other individuals were analyzed in a similar way, but <0.5 ng/ml TGF-β3 was detected in PPP, serum or platelets from these individuals.

Measurement of TGF-β antigens in complex biologic fluids is often complicated by the presence of binding proteins, although these difficulties are usually reduced or eliminated when an extraction procedure or activation procedure is used prior to assay. To determine whether binding protein effects influenced the measurement of TGF-β3 in human plasma, we performed two further experiments. In the first experiment, recombinant human TGF-β3 was spiked into 10 different plasma samples that were then assayed before and after addition of the TGF-β3. In this experiment the recovery was 98 ± 4%. In the second experiment we took two different samples with detectable levels of TGF-β3, serially diluted the sample (after acid–urea activation) and measured the amount of TGF-β3 present at four separate dilutions. Provided that the measured concentration of TGF-β3 was greater than 63 pg/ml, the calculated concentration of TGF-β3 antigen varied by less than 15% in all cases tested (CV = 7.6%, n = 7).

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