Transforming Growth Factor-β1 Circulates in Normal Human Plasma and Is Unchanged in Advanced Metastatic Breast Cancer


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ABSTRACT

A method has been developed to determine true plasma transforming growth factor β (TGF-β) levels by using the platelet α granule-specific marker, platelet factor 4, to correct for the TGF-β contributed by platelets degranulated ex vivo. TGF-β levels were measured on acid-ethanol extracts of human plasma using isoform-specific sandwich enzyme-linked immunosorbent assays. Normal human subjects had 4.1 ± 2.0 ng/ml TGF-β1 (range, 2.0–12.0; n = 42), <0.2 ng/ml TGF-β2, and <0.1 ng/ml TGF-β3 in their plasma. There were no significant changes with age or with hormonal status, but any given individual showed fluctuations of up to 3-fold in measured plasma TGF-β levels due to unknown factors. Of 28 patients with advanced metastatic breast cancer, 2 had greatly elevated TGF-β1 levels, while the rest were in the normal range. The presence of physiologically significant levels of TGF-β1 in the plasmas of normal human subjects may indicate previously unsuspected endocrine roles for this peptide, while TGF-β2 and TGF-β3 appear to act only in a local autocrine/paracrine fashion.

INTRODUCTION

The TGF-βs are multifunctional cytokines whose properties include being highly potent inhibitors of epithelial cell growth (1). The presence of one or more isoforms in essentially every epithelial tissue probably reflects a critical role in regulating normal epithelial homeostasis. TGF-β1 null mice show hyperproliferation in the basal layer of the epidermis (2), and epidermal tumors derived from TGF-β1 null keratinocytes show more rapid malignant progression than their wild-type counterparts, suggesting that dysregulation of TGF-β1 function may enhance the carcinogenic process (3). Similarly, colon carcinoma cells transfected with antisense TGF-β1 show a more aggressive phenotype when grown in nude mice than do their untransfected counterparts (4). The observation that the anti-estrogen tamoxifen increases TGF-β isofrom expression in breast cancer cells in vitro and locally in vivo in human breast tumors is also consistent with a potential role for TGF-βs in preventing tumor progression (5, 6).

In later phases of tumor progression, TGF-β overexpression may actually enhance tumorigenesis through paracrine stimulation of the tumor stroma and inhibition of the immune surveillance system. Transfection of a rat prostatic carcinoma cell line with TGF-β1 resulted in larger, more metastatic tumors (7). Furthermore, three immunohistochemical studies (8–10) have shown a correlation between increased staining for TGF-β1 in human mammary carcinomas and disease progression, although it is not clear whether this is causally related to progression or represents a failed homeostatic response. Clearly, changes in local TGF-β levels may be important during carcinogenesis in a number of organ systems, with potentially different effects at different stages in the process. We therefore wished to develop a method for accurate determination of circulating TGF-β levels to evaluate their potential usefulness as prognostic indicators, as disease markers, or as intermediate biomarkers of chemopreventive or chemotherapeutic efficacy.

Human platelets contain large amounts of TGF-β1 in the α granules (~2500 molecules/platelet (11)). Thus measurements of circulating TGF-β levels must be made on plasma, not serum. Since it is extremely difficult to prepare plasma without any platelet degranulation, it is not clear whether previously determined levels of TGF-β in the plasma of normal subjects might not have been derived from platelets degranulated ex vivo in the course of sample handling (12–14). To circumvent this problem, we have developed a method to correct for the platelet contribution by measuring levels of the specific platelet α granule protein, PF4. We now present our results for normal controls and for patients with advanced metastatic breast cancer.

MATERIALS AND METHODS

Collection of Blood and Preparation of Plasma

Plasma TGF-β levels were determined in 42 apparently healthy subjects, ranging in age from 20 to 60 years, and included 17 males, 14 premenopausal females, 6 pregnant fe-

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2 The abbreviations used are: TGF-β, transforming growth factor β; PF4, platelet factor 4; βTH, β-thromboglobulin; SELISA, sandwich enzyme-linked immunosorbent assay.

3 L. M. Wakefield, unpublished data.
males, and 5 postmenopausal females. Postmenopausal women were not on estrogen replacement therapy, and anyone taking drugs that might affect platelet activation was excluded.

The method for drawing blood and preparing plasma was designed to minimize platelet degranulation. A good venipuncture and blood withdrawal with no stasis was important for the quality of the samples. Typically, an 18- or 19-gauge butterfly needle was inserted into the antecubital vein. A tourniquet was only used when necessary to dilate the vein, and was removed following insertion of the needle. For normal volunteers, vacueters and syringes were not used, as these cause turbulence that can increase platelet degranulation. The first 4 ml of blood were allowed to flow into a serum separator tube, were clotted for 2 to 3 h at room temperature, and used to prepare serum. If there is any excess trauma from the venipuncture, these first few milliliters of blood are typically more degranulated than subsequent tubes due to the release of tissue factor and ideally should be discarded if serum is not required. The next 4.5 ml of blood were collected into a pre-chilled Diatube H tube (American Bioproducts Co., Parsippany, NJ) containing 0.5 ml of a platelet degranulation inhibitor cocktail comprising citrate (3.2%), theophylline (15 mM), adenosine (3.7 mM), and diprydiamole (0.2 mM) (15). Samples were mixed by gentle inversion and immediately placed on a slurry of ice and water. Within 1 h of collection, they were spun for 30 min at 1200 × gmax in a refrigerated centrifuge. The top two thirds of the plasma supernatant were withdrawn, taking care not to go near the platelet interface, and samples were aliquotted and stored at −70°C. In some experiments, 0.5 ml 1.5% sodium-EDTA was substituted for the platelet inhibitor cocktail and did not result in significantly greater platelet degranulation, provided plasma was prepared promptly and carefully. All plasma samples were typically assayed for TGF-β within 3 months of collection, as a slow but significant increase in assayable TGF-β occurred on long-term storage (a 3 ng/ml increase over 20 months). This may reflect release of covalently bound TGF-β from α₂-macroglobulin (16).

Blood was also collected from 28 patients with advanced metastatic breast cancer. All had bidimensionally measurable Stage IV disease, and the group included both women with estrogen receptor-positive (22/28) and estrogen receptor-negative tumors (6/28). Most patients had received prior hormonal therapy (22/28), or chemotherapy (23/28), or both (19/28), and 18 of 28 patients had prior radiation therapy. However, all patients had been off all therapies for at least 1 month at the time of sampling. The blood was collected using a variety of procedures depending on the constraints imposed by the condition of the patient. Needles from 18 to 21 gauge were used; for some patients tourniquets had to be used throughout the collection, and syringes or vacueters were used in many cases where good gravity flow could not be achieved. For several patients, blood was drawn from an in-dwelling central line. Plasmas were prepared and stored as for the normal subjects.

Preparation of Samples for TGF-β Assays

Preliminary experiments indicated that TGF-β could not be measured accurately in whole plasma or serum using the standard growth inhibition assays, quantitative radioreceptor assay, or TGF-β SELISA assays, as sample dilution curves were not parallel to the standard (14). To eliminate interfering components, all samples were extracted with acid-ethanol prior to assay. This procedure activates any latent TGF-β complexes, so no distinction can be made between latent and active TGF-β. Biologically inactive TGF-β covalently bound to α₂-macroglobulin is not extracted. In detail, 0.5 ml of plasma or serum was extracted overnight with four volumes of acid-ethanol containing protease inhibitors (95% ethanol, 2% HCl, 1 mM (2-aminoethyl)-benzensulfonyl fluoride, and 5 mg/ml Pepstatin A). All steps were performed at 4°C. Extracts were clarified by centrifugation for 20 min at 10,000 × g, and the supernatant was dialyzed extensively against three changes of 4 ml HCl in Spectrapor 3 dialysis tubing (molecular mass cutoff = 3.5 kDa). It is important to leave airspace in the dialysis bag or sample is forced out by the large increase in volume (>2X) that occurs on dialysis. The increase in volume on dialysis was determined and corrected for by weighing the samples. The dialysate was then clarified at 10,000 × g for 10 min, and aliquots were lyophilized and stored dry at −70°C.

Prior to assay, lyophilizates were resuspended in 1/4 volume of SELISA solubilization buffer (4 mM HCl, 0.1% BSA, 150 mM NaCl), vortexed vigorously, and rocked overnight. The resuspended sample was clarified in a microfuge and assayed immediately. At all steps following dialysis, losses of TGF-βs were reduced by using tubes siliconized with Sigmacote (Sigma Chemical Co., St. Louis, MO). In preliminary experiments, 125I-labeled TGF-β was added to plasma and serum samples to monitor recoveries at each step. Recoveries were as follows: clarified extract, 64 ± 6%; clarified dialysate, 65 ± 7%; resuspended lypophilize (4-fold concentration), 50 ± 13%. Recoveries were essentially identical for TGF-β1 and β2, and for plasma and serum. An overall recovery factor of 50% was used to correct all subsequent samples for handling losses. Recoveries dropped precipitously if lypophilizates were resuspended in smaller volumes (6 ± 1% for a 10-fold concentration factor).

TGF-β and Platelet Marker Assays

Platelet Markers. Platelet a granule markers platelet factor 4 (PF4) and β-thromboglobulin (βTH) levels were determined in whole plasma or serum using commercial Asserochrom ELISA kits (American Bioproducts Co.). Platelet marker levels appeared to be stable over several months in samples stored at −70°C (data not shown).

TGF-βs. TGF-β1 and TGF-β2 were quantitated independently in acid-ethanol extracted samples using isofrom-specific SELISA assays, as described previously (14). There is <0.1% cross-reactivity of TGF-β1 in the TGF-β2 assay, while in the TGF-β1 assay, there is 0.5% cross-reactivity with TGF-β2 and 5% cross-reactivity with TGF-β3. The TGF-β1 SELISA can be done with commercially available reagents (17). Samples were assayed for TGF-β3 using a newly developed TGF-β3 SELISA assay. In some experiments, TGF-βs were also assayed using a growth inhibition bioassay, measuring 125I-labeled deoxyuridine incorporation in CCL64 mink lung epithelial indicator cells (18). All three TGF-β isoforms are

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active in this assay. A pan-specific TGF-β neutralizing monoclonal antibody (AB 1835–01; Genzyme, Cambridge, MA) was used to reverse the inhibitory effect and confirm specificity. The different isoforms were identified using purified immunoglobulin fractions from a turkey polyclonal antibody specific for TGF-β1 and a rabbit polyclonal antibody specific for TGF-β2 raised in this laboratory (18). The combination of these two antibodies showed <0.2% cross-reactivity with TGF-β3.

Correction of Measured Plasma TGF-β Levels for the Contribution from Degranulated Platelets

The platelet contribution to the measured plasma TGF-β levels was corrected by using the platelet α granule markers PF4 or βTH as indicated below. Both proteins are found exclusively in the α granule and so their presence in plasma is a sensitive indicator of platelet degranulation (19). PF4 has a short half-life in vivo when compared to βTH; therefore, elevated PF4 generally indicates ex vivo degranulation, whereas elevated βTH reflects both ex vivo and in vivo degranulation (19). We chose to use PF4 as the marker in the present studies to avoid correcting for potentially significant in vivo degranulation. However, we obtained essentially identical results on all samples using either marker (data not shown).

For the normal controls, the ratio of TGF-β:PF4 in platelets was determined by measuring it in serum, where the platelets are fully degranulated, and assuming that the plasma contribution of TGF-β was negligible in comparison. Subsequent measurements showed mean plasma TGF-β to be ~4 ng/ml compared with ~85 ng/ml for serum, validating the assumption. A mean ratio (‘R’) of 0.017 ± 0.006 ng TGF-β/1U PF4 was determined (range, 0.008–0.030, n = 33). The measured plasma TGF-β was then corrected for the platelet contribution using the measured plasma PF4 level (‘P’) as follows: TGF-β_corrected = TGF-β_measured - R × P. For the normal controls, each plasma value was corrected using the autologous value for R determined from the matched serum. This allowed for interindividual variation in the ratio and increased the accuracy of the correction. For the breast cancer patients, whose sera were not collected, measured plasma TGF-β levels were corrected using the mean value of R determined on the normal population above.

Since true plasma TGF-β levels were found to be relatively high (~4 ng/ml), the platelet contribution to the measured plasma TGF-β and hence the correction factor were generally quite small. For normals, the contribution of platelet-derived TGF-β to measured plasma TGF-β values was 12 ± 13% (mean ± SD, n = 42; range, 1–46%), while for the present group of cancer patients, it was actually lower (5 ± 4%; range, 0–16%), despite what were anticipated to be suboptimal collection conditions. In the patient group, only two subjects showed measured (uncorrected) plasma TGF-β values that were above the normal range. Matched PF4 values were low (5 and 19 IU/ml; corresponding to 0.1 and 0.3 ng/ml platelet-derived TGF-β), indicating that the elevation was genuine and not due to platelet degranulation during sample acquisition.

The similar platelet contributions in our normal and patient populations suggests that, unlike clinical measurements of PF4 and BTH (19), the measurement of plasma TGF-β is relatively insensitive to the blood collection procedure. However, sample handling after the blood has been drawn (see Fig. 1) is still important in determining the quality of the sample. We now only routinely make PF4 measurements on samples showing elevated TGF-β levels (>8 ng/ml, which is 2 SDs above the mean for normal subjects). In experiments with other patient populations (data not shown), where samples have not been handled optimally due to practical constraints, PF4 determinations have been helpful in assessing the extent of ex vivo platelet degranulation.

RESULTS

Validation of PF4 Correction Method. The data in Fig. 1 show the results of applying the platelet marker correction method to deliberately mishandled plasma samples. These were derived from consecutive tubes drawn from a single individual following a single venipuncture. Plasma in tube 1 showed some degranulation due to a traumatic needle stick (5% of total platelets degranulated), while some of the platelet interface was deliberately included when the plasma supernatant was withdrawn. Tube 3 was handled under near-optimal conditions. Measured plasma TGF-β1 levels were corrected for the contribution due to platelet degranulation using the PF4 correction procedure described in “Materials and Methods.” Following correction, all three samples gave similar values. Gray columns, measured TGF-β1; solid columns, corrected TGF-β1. Results are mean ± SD (n = 3).

Fig. 1 Validation of PF4 correction procedure using deliberately mishandled plasma. Three successive tubes of blood were drawn from one subject following a single venipuncture. Plasma was prepared and assayed for TGF-β as described in “Materials and Methods,” except that the needle was deliberately not inserted smoothly, resulting in increased platelet activation in tube 1, while for tube 2 some of the platelet interface was deliberately included when the plasma supernatant was withdrawn. Tube 3 was handled under near-optimal conditions. Measured plasma TGF-β1 levels were corrected for the contribution due to platelet degranulation using the PF4 correction procedure described in “Materials and Methods.” Following correction, all three samples gave similar values. Gray columns, measured TGF-β1; solid columns, corrected TGF-β1. Results are mean ± SD (n = 3).

TGF-β1 Levels in Normal Subjects. Matched measured and corrected plasma TGF-β1 levels are shown in Fig. 2
Normal pregnant subject had very low serum TGF-β1 (14.7 ± 1.4 ng/ml) reflecting a low platelet content of normal subjects. Plasma TGF-β1 levels were measured and corrected for the platelet contribution as described in "Materials and Methods." Lines connect matched measured and corrected values for each individual.

Estrogen and androgens have been shown to regulate TGF-β production in various target tissues both in vitro and in vivo, and hormonal ablative therapies cause local tissue induction of TGF-βs in the human prostate and breast (6, 20–23). We therefore investigated the effect of hormonal status on circulating TGF-β levels. The data in Fig. 3 show no significant difference in plasma TGF-β1 levels in men, premenopausal women, pregnant women, and postmenopausal women. Combining all groups, plasma TGF-β levels were 4.3 ± 1.4 ng/ml, while serum TGF-β levels were 3.7 ± 1.4 ng/ml.

Fig. 3 Effect of hormonal status on plasma TGF-β1 levels in normal human subjects. Plasma TGF-β1 levels were measured and corrected for the platelet contribution as described in "Materials and Methods."
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shown), the SELISA assay is probably more accurate than the bioassay for plasma TGF-β determinations, though the bioassay can clearly give clinically useful results (12, 13). However, it remains possible that a fraction of the TGF-β measured by the SELISA assay may not be biologically active. In all samples tested, a combination of anti-TGF-β1 and anti-TGF-β2 antibodies gave as much or more neutralization of plasma TGF-β activity in the bioassay as did the pan-specific monoclonal antibody that recognizes all isoforms (Fig. 5). This confirms that none of the samples contained significant levels of TGF-β3, as also shown by the TGF-β3 SELISA.

TGF-β Levels in Women with Advanced Metastatic Breast Cancer. There is immunohistochemical evidence for increased expression of TGF-βs in metastatic breast cancer tissue (8–10). To determine whether this might cause a systemic elevation of TGF-βs, we measured plasma TGF-β levels in 28 women with advanced metastatic breast cancer. The data in Fig. 6 show that circulating TGF-β levels were in the normal range for 26 of 28 patients, while 2 patients showed genuinely elevated plasma TGF-β1, even after correction for platelet degranulation. One of these patients also had detectable TGF-β2 (0.7 ng/ml), as did a second patient with normal TGF-β1 levels. No other patients had detectable TGF-β2. There was no obvious correlation between the elevated plasma TGF-β1 and any disease or treatment parameter in these patients.

DISCUSSION

In common with many other growth factors, TGF-βs have been assumed to have highly local autocrine or paracrine actions. We have developed a method that allows us to measure true plasma TGF-β levels by correcting for the confounding contribution of TGF-β from platelets degranulated ex vivo. The demonstration of physiologically significant levels of TGF-β1 in normal human plasma suggests that this isoform may have some hitherto unsuspected endocrine role. We have also shown that the predominant TGF-β isoform in human plasma is TGF-β1, since TGF-β2 and β3 were generally undetectable, representing <5% of the total TGF-β.

Many cells respond to active TGF-βs in the pg/ml range (1). Thus the presence of ng/ml concentrations of TGF-β in the plasma represents a large reservoir of this cytokine, in considerable excess over the normal biological activity range. TGF-βs are secreted by platelets and most cell types in biologically latent forms that must be activated before exerting their biol-
similar plasma TGF-β levels, which suggests that the source tissue is probably not responsive to sex hormones in terms of TGF-β production. Human platelets are the most concentrated source of TGF-β in the body, and TGF-β might leak from circulating aging platelets in vivo. However, this is unlikely to be the origin of plasma TGF-β1 since there is no correlation between plasma TGF-β1 levels and platelet count, as reflected in the serum TGF-β1. Furthermore, the plasma TGF-β1 levels measured in this study would correspond to a degranulation of 5% of the total platelet content of the body, and β-thromboglobulin levels were not equivalently elevated (data not shown). Quantitatively the most extensive pool of TGF-β in the body is bone. Bone has ~300 μg TGF-β/kg compared with ~100-fold less in most soft tissues (1), and is thus a plausible source of plasma TGF-β.

Recent studies with TGF-β1-knockout mice have indicated that the null phenotype can be rescued by maternal transfer of TGF-β1 transplacentally, or postnatally via the milk (31). Immunohistochemical analysis shows similar staining patterns for TGF-β1 in many tissues of both the null and the wild-type pups derived from heterozygous mothers. These data suggest that some of the characteristic tissue distribution patterns previously observed for TGF-β1 may be established by local sequestration of the factor, rather than by local synthesis, since both extracellular and intracellular staining for TGF-β1 were seen in null pups (31). Latent TGF-β has been shown to bind to the extracellular matrix in mesenchymal cells (32), and active TGF-β binds to a wide range of extracellular proteins (33–35). It is possible that tissue TGF-β1 may be in equilibrium with the plasma reservoir, and that tissue-specific distribution patterns can be established and maintained in part by regulated local expression of molecules that bind TGF-β1. This may explain some of the discrepancies frequently observed between in situ hybridization and immunohistochemical localization of TGF-β1 (36, 37). The balance between local synthesis and sequestration in determining local TGF-β1 levels may vary among tissues, whereas human TGF-β2 and TGF-β3 must derive entirely from local synthesis. Thus, while TGF-β2 and β3 may act exclusively as local autocrine or paracrine regulators, TGF-β1 may also be involved in long-range endocrine interactions between distant tissues (Fig. 7).

Three studies (8–10) have shown a correlation between increased immunohistochemical staining for TGF-β1 in mammary carcinomas and disease progression, so we wished to determine whether plasma TGF-β levels might be a useful marker for advanced disease. In the present study, only 2 of 28 women with advanced metastatic breast cancer showed elevated plasma TGF-β1 levels, and this did not correlate with any obvious parameter of disease status. One of these two patients had an exceptionally large volume of metastatic disease in both liver and bone, which may have contributed to the elevated TGF-β. However, the other patient with elevated plasma TGF-β had only moderate volume bone disease, as did most of the patients in this study group. All of the rest of the patient group had plasma TGF-β levels in the normal range. This suggests that secretion of TGF-β by the tumor does not contribute significantly to the total TGF-β in the circulation, and that TGF-β1 is not elevated systemically through any ongoing in vivo platelet

![Plasma TGF-β1 levels in women with advanced metastatic breast cancer.](image)

**Fig. 6** Plasma TGF-β1 levels in women with advanced metastatic breast cancer. Plasma TGF-β was measured and corrected for the platelet contribution as described in "Materials and Methods." Dashed line, 2 SDs above the mean value of plasma TGF-β for normal controls.
activation, which has been proposed to occur in patients with clinically active cancer (38).

A recent study has shown that elevated plasma TGF-β is strongly predictive of susceptibility to fibrotic complications following bone marrow transplantation in breast cancer patients (12). In that study, more patients had elevated plasma TGF-β levels than we have observed here, which could be a result of the induction chemotherapy given to that patient cohort. However, our results agree in showing no obvious correlation between circulating TGF-β levels and tumor burden. In contrast to the situation with breast cancer, nearly 80% of patients with hepatocellular carcinoma were shown to have elevated plasma TGF-β levels (13). This was proposed to derive from the tumor, since surgical resection decreased the plasma levels. Since we have shown that normal circulating TGF-β levels are relatively high, it probably takes a large lesion or one showing a very high rate of TGF-β synthesis to impact on plasma TGF-β levels.

It has been proposed that development of agents that increase endogenous production of TGF-βs may represent a new approach to chemoprevention of cancer (39). Since elevated plasma TGF-β seems to be associated with a predisposition to abnormal fibrogenesis (12), it will be important to design these agents specifically to increase TGF-β isoform expression locally in target tissues, without any systemic elevation. We are currently using the method described here to assess the effect of chemopreventive agents such as tamoxifen and synthetic retinoids on circulating TGF-β levels in women with breast cancer.

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