Pergamon

European Journal of Cancer Vol. 32A, No. 9, pp. 1566–1573, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

PII: S0959-8049(96)00115-3

Original Paper

Multiple Forms of TGF-β_1 in Breast Tissues: a Biologically Active Form of the Small Latent Complex of TGF-β_1

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The aim of this study was to investigate whether breast cancer growth *in vivo* could be due to a failure in the activation of TGF-β_1, a growth factor which has been shown to affect the development of normal breast tissue. Tissue samples of 40 breast carcinomas and the normal adjacent tissue from 37 (henceforth referred to as 'adjacent tissue'), as well as 13 specimens of benign lesions, were included in this study. The specimens were used *in vitro* to produce conditioned medium (CM), and this was examined for TGF-β_1 activity by measuring growth inhibition of the mink lung epithelial cell line CCL-64. Immunoblotting and electrophoresis were used to detect the presence of TGF-β1 in CM and homogenised tissue samples. We demonstrated that the majority of TGF-β_1 in breast cancer conditioned medium was biologically active, in direct contrast to CM prepared from benign disease specimens. Furthermore, active TGF-β_1 was also identified in CM prepared from adjacent tissue, suggesting an important early role for this growth factor in the spread of this disease. Three distinct breast cancer related (BCR) molecular weight species of TGF-β_1 (12.5/25 kDa, 50 kDa and 95 kDa) were identified. Both the 50 kDa and 95 kDa bands immunoprecipitated by an anti-TGF-β_1 antibody were also immunoreactive with anti-TGF-β_1 binding protein antibodies suggesting that the 50 kDa band may comprise at least part of the previously described small latent complex of TGF-β_1. However, using the CCL-64 cell assay, we were able to demonstrate that the 50 kDa TGF-β_1 BCR protein was biologically active whereas the large (95 kDa) TGF-β_1 BCR latent complex protein was not. Adjacent tissue was more likely to contain the 50 kDa form than the tumour tissues ($P < 0.05$). Similarly, the 50 kDa molecule was also more common in patients who had oestrogen receptor (ER) negative tumours (compared with ER positive patients; $P < 0.05$) and in those who had received tamoxifen treatment prior to surgery ($P < 0.01$). In all of these cases, the increase in the incidence of the small active complex form was accompanied by a decrease in the incidence of the high molecular weight complex (95 kDa). We confirmed that, *in vitro*, the 95 kDa TGF-β_1 BCR can be proteolytically cleaved to yield a 50 kDa TGF-β_1 BCR. Finally, we observed a correlation between the presence of the 50 kDa complex protein and reduced levels of plasminogen activator (PA), which was significant in ER negative patients ($P < 0.05$) and tamoxifen-pretreated patients ($P < 0.01$). This suggests that the secretion of this active TGF-β_1 protein may provide breast tumours with a mechanism whereby they can escape oestrogen dependence, and may provide an explanation for the common problem of tamoxifen resistance.

Key words: TGF-β_1, breast cancer, proliferation, latent complex, oestrogen receptor
Eur J Cancer, Vol. 32A, No. 9, pp. 1566–1573, 1996

INTRODUCTION

BREAST CARCINOMA is the most common solid tumour of women in the Western world. An effective treatment regime for patients with oestrogen receptor (ER) positive tumours involves the use of endocrine therapy. Unfortunately, in the majority of patients, endocrine therapy ultimately becomes ineffective and the cancer regrows [1]. This process may be mediated by changes in growth factor secretion or by loss or gain of their receptors.

TGF-β_1 is one of the best characterised and widely distributed members of the large family of potent small peptide growth factors known as the TGF Betas and has often been found in neoplastic and malignant tissues [2]. TGF-β_1 has been shown to be a powerful growth inhibitor of many cell

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Received 4 Sep. 1995; revised 8 Feb. 1996; accepted 12 Feb. 1996.

types including both endothelial and epithelial cells [3] suggesting a potential positive role in tumour control, although in breast cancer cell lines their effect is unclear. However, TGF-β_1 has also been shown to be mitogenic for a variety cell types [3] and indeed many of the additional reported functions for TGF-β_1, including angiogenesis, immunosuppression and promotion of extracellular matrix secretion, suggest a less benign role for this molecule in tumourigenesis [4].

TGF-β is most usually found to be secreted in the latent (inactive) form in which it is synthesised, both by cell lines [5] and in non-pathogenic tissues of both adult and developing organisms [6]. It is then cleaved at its target site under a tightly controlled mechanism which *in vivo* probably involves proteolysis [7], although *in vitro* treatments with acid, alkali or heat [6] will also effect cleavage and activation. The TGF-β_1 small latent complex consists of the mature dimer of TGF-β (25 kDa) bound, by non-covalent bonds, to a precursor remnant or latency associated protein (LAP). TGF-β_1–LAP has been reported to have a molecular weight of between 40 and 42 kDa [8] and 62 kDa depending on the extent of its glycosylation [9]. The small latent complex binds by means of disulphide bonds to betaglycan which acts as a TGF-β_1 binding protein and the small latent complex becomes a large latent complex consisting of the dimeric precursor (linked by disulphide bonds) and the binding protein. The size of this large TGF-β_1 precursor has been shown to vary with tissue type [10] between 125 and 190 kDa.

In the human breast, TGF-β_1 protein is present both intracellularly and extracellularly in most active mammary epithelia in normal [11], benign and malignant human breast tissues [11–14]. Normal mammary epithelial cells show a strong growth inhibition [15] and differentiative [16] response to TGF-β_1 suggesting a developmental role for this growth factor in the normal mammary gland. In patients with malignancy, TGF-β_1 protein is observed at the advancing edges of primary tumours [13], and there seems to be a correlation between decreased TGF-β_1 mRNA synthesis [14], active TGF-β_1 protein [12] and breast tissue malignancy as measured by a reduction in lymph node metastasis [14]. These apparent anomalies between protein and mRNA synthesis can be partly reconciled by the fact that the TGF-β_1 mRNA is known to regulate its protein product [4]. Similarly, data from breast cancer cell lines suggests that, although most of these cells synthesise and release both active and latent TGF-β_1, the majority of ER+ cell lines are insensitive to its growth inhibitory effects, although in ER positive cell lines oestrogens [17] and antioestrogens, such as tamoxifen, can be shown to cause TGF-β_1 synthesis and induction [18] *in vivo*.

Thus, from previous studies, we know that TGF-β_1 is present in human breast tissues and that, under certain conditions, breast cancer cell growth can be inhibited by TGF-β_1. The aim of this study was to investigate whether breast cancer growth *in vivo* could be due to a failure in the processing of the TGF-β_1 molecule leading to the unavailability of biologically active TGF-β_1 peptide in the tumour itself. There is now considerable evidence [19] to suggest that, although post-transcriptional controls may play a role [20], an important level of control for TGF-β action is post-translational. We have therefore analysed both conditioned medium (CM) and tissue protein from breast tissues of benign and malignant breast disease for the presence of active and latent TGF-β_1 moieties in order to examine the importance of this process in breast cancer. We have used a combination of biochemical and bioassay approaches to determine the extent of TGF-β_1 activation in these samples and to characterise the proteins involved. In order to gain an insight into the mechanism of TGF-β_1 activation in the human breast, we have also determined plasminogen activator levels in these same tissues.

MATERIALS AND METHODS

Patient tissues

Tissue samples were collected from patients attending St George's Hospital, London, U.K. as follows: from the breast carcinomas of 40 patients, from histologically normal tissue adjacent to the breast cancer of 37 of these patients (henceforth referred to as 'adjacent tissue') and from the lesion and histologically normal adjacent tissue of 13 benign breast disease patients (4 reduction mammoplasty, 1 virginal mammary hyperplasia, 4 fibroadenoma, 4 fibrocystic disease). The breast cancers of these 40 patients were divided into one of four subtypes depending on the histopathology of their biopsies as assessed by a pathologist at St George's Hospital, London. As expected, the majority of malignancies (31/40) were infiltrating ductal carcinomas. Of these, approximately half (15/31) were ER negative (slightly over the national average) with 3 samples of unknown ER status. Tissue adjacent to the cancerous lesion was collected from 28/31 of these patients. Of the remaining samples, 7/40 were lobular carcinomas (4/7 were ER+) and adjacent tissue was collected from all of these. The two remaining samples were 1 ER− medullary carcinoma and 1 ER+ intraductal carcinoma.

Samples were divided in two and half were snap-frozen in liquid nitrogen for protein and histological analysis and the rest placed into serum-free medium and dissected within 1 h of removal from the patient for preparation of conditioned medium as previously described [21]. Histological status, tumour grade and TNM status were obtained from the case notes and ER status was assessed either by Oestrogen Receptor Immunochemical Assay (ERICA) or Dextran Coated Charcoal (DCC) depending on the sample size as described previously [22, 23].

Preparation of conditioned medium

Tissue was collected into serum-free medium (alpha-Eagles Minimal Essential Medium) immediately after surgery and pathological assessment, and was then removed directly for preparation of CM as described by Smith and coworkers [22]. CM was removed after 24 and 72 h incubation and was stored in aliquots at −40°C until required for TGF-β bioassay.

Assay for TGF-β biological activity

Mink lung epithelial cells (CCl-64) were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine at 37°C 5% CO_2 until subconfluent (24–48 h). After trypsinisation, cells were washed with assay medium (DMEM with, 1% fetal calf serum, 25 units/ml penicillin, 25 μg/ml streptomycin, 10 mM HEPES buffer and 2 mM L-glutamine) and recovered by centrifugation (1000 rpm/3 min). Cell number was determined with a haemocytometer. To assay for TGF-β activity, cells were seeded at a density of 10^4 cells/well into a 96-well plate. TGF-β standards, samples purified from gels, or conditioned medium were added to attached cells after 2 h and plates incubated for a further 22 h. Tritiated thymidine (0.5

μCi/well) was then added to each well and the plates incubated for a further 6 h. Cells were washed twice with 1 mM unlabelled thymidine in PBS and then fixed in 80% methanol for 2 × 5 min. After 4 washes in deionised water, samples were processed for scintillation counting as described previously [23].

Preparation of total protein

Total protein was prepared from frozen tissue samples of 40 patients with malignant lesions and 37 adjacent non-malignant tissues. Of the frozen tissue 0.2 g were homogenised in 1 ml phosphate buffered saline (PBS, pH 7.2) and stored in aliquots at −40°C until required, as described previously [11, 18]. Protein concentrations were determined using the Bradford method [24].

Antibodies

Rabbit polyclonal antiserum to mature TGF-β_1 (CC A1/30) was a gift from Drs Sporn (NIH, Bethesda, U.S.A.) and Ellingsworth (Celtrix, Palo Alto, U.S.A.). Polyclonal rabbit antisera to TGF-β_1 latency associated protein (Ab 96-1, here designated anti-TGF-β_1-LAP) and TGF-β_1-binding protein (Ab37, here designated anti-TGF-β_1-BP) were gifts from Professor C.-H. Heldin (Ludwig Institute, Uppsala, Sweden).

Immunoblotting and electrophoresis

Native and reducing SDS–PAGE gels were prepared and run using the method of Laemmli [25] and as described previously [26]. Transfer and immunodetection were performed as previously described [2] except that visualisation of bands was achieved using enhanced chemiluminescence (ECL, Amersham, U.K.). Dot blotting was carried out by spotting standards (100 and 50 ng of TGF-β_1, British Biotechnology, U.K. or TGF-β_1-BP, a gift from Professor C.-H. Heldin, Uppsala, Sweden) or test samples (10 μl aliquots of 50 or 95 kDa bands purified by elution) onto nitrocellulose filter strips under suction. Antibody binding and detection were as for immunoblotting.

Immunoprecipitation

Immunoprecipitation was carried out following the method of Harlow and Lane [26]. Briefly, samples (100 μg total protein) were incubated with rabbit IgG (100 μg) for 1 h at 4°C in NET-gel buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% nonidet P-40, 1 mM EDTA, 0.25% gelatin and 0.02% Na azide). *Staphylococcus aureus* protein-A (60 μl) was then added and the samples incubated for a further 1 h at 4°C after which they were centrifuged at 12000 rpm and the supernatant removed and stored. The pellet was then washed twice with NET-gel buffer and once in 10 mM Tris–HCl (pH 7.5), and the washes collected and added to the supernatant. Immunoprecipitation of TGF-β_1 was then carried out by incubation (1 h/4°C) of the supernatant/wash mix with 8 μg antibody (cc A1/30), followed by further incubation (1 h/4°C) with protein-A (30 μl of a 10% (w/v) bacterial cell wall suspension: equivalent to 1.2 mg IgG/ml). The sample was then pelleted and washed as above. The pellet was then assayed by SDS–PAGE after resuspension in 1× loading buffer and immunoblotted (see Results section).

Protein band elution

Protein bands were separated using native PAGE and located by staining with Coomassie blue for 10 min (0.25% Coomassie brilliant blue (Sigma, U.K.) in 25% isopropanol/10% glacial acetic acid fixative made up in deionised water). Gels were then destained for 45–60 min in 7% glacial acetic acid and rinsed in deionised water.

The bands were then cut from the gel using a clean scalpel blade and incubated overnight with agitation in 0.192 M glycine/25 mM Tris/0.1% SDS (pH 8.3) in siliconised glass bottles at room temperature. Samples were then separated on agarose rod gels and purified using the method of Wu and colleagues [27]. Extracted samples were then lyophilised and stored at −40°C and assayed within 1 month. Samples were reconstituted in sterile PBS pH 7.2 prior to use in the bioassay system or acid/protease treatments described below.

Acid and protease treatment of eluted bands

To test for differences in electrophoretic mobility of the bands eluted, the eluted bands (50 μl) were treated with (a) an equal volume (50 μl) of 1 M HCl pH 1.5 for 30 min at room temperature followed immediately with an equal volume of 1M NaOH pH 12.5; (b) as in (a) but followed by 30 min with 0.3 units Plasmin (Sigma, U.K.); or (c) 30 min at room temperature with plasmin alone (0.3 units). Treated samples were then loaded on to SDS–PAGE gels and immunoblotted as described above.

Plasminogen activator assay

Total protein samples prepared as described above were assayed for plasminogen activator activity on casein/agarose gels as follows. Gel preparations were prepared with 1% agarose and 0.6% dried milk (both in 0.1 M Tris–HCl pH 8.0) with 0.03 units plasminogen added to one of the preparations. To prevent setting, agarose preparations were kept in a water bath at 42°C until they were poured into 9 cm plates and allowed to set. Wells were then created using the end of a 5 ml pipette. Five microlitre samples of urokinase or plasmin standards were added to the wells and the plates incubated overnight at 37°C. Radii of lysis were measured with a clean ruler and the data expressed as mm/μg total protein.

Statistical methods

Clinical analysis and verification of conditioned medium were carried out as described previously [21]. Analysis of samples containing low, medium or high molecular weight bands was carried out using a Chi squared test unless the sample size was less than or equal to 10 when Fisher's exact test was employed.

RESULTS

TGF-β bioactivity in breast cancer conditioned medium

CM prepared from 15 malignant biopsies and 12 biopsies from the non-malignant tissue margin adjacent to a malignant lesion were assayed for the presence of TGF-β activity by measuring their ability to inhibit the proliferation of the mink lung epithelial cell line CCL-64 (Figure 1a). To determine the levels of latent compared with active TGF-β present, we measured TGF-β bioactivity in both heat treated and heat untreated samples of CM. TGF-β activity was found at similar amounts in CM from both cancer and adjacent tissues. Heat treatment revealed a very slight increase in TGF-β activity in CM from malignant tissues which could be attributable to the presence of latent TGF-β protein. No corresponding increase in inhibition was observed in the CM from adjacent tissues.

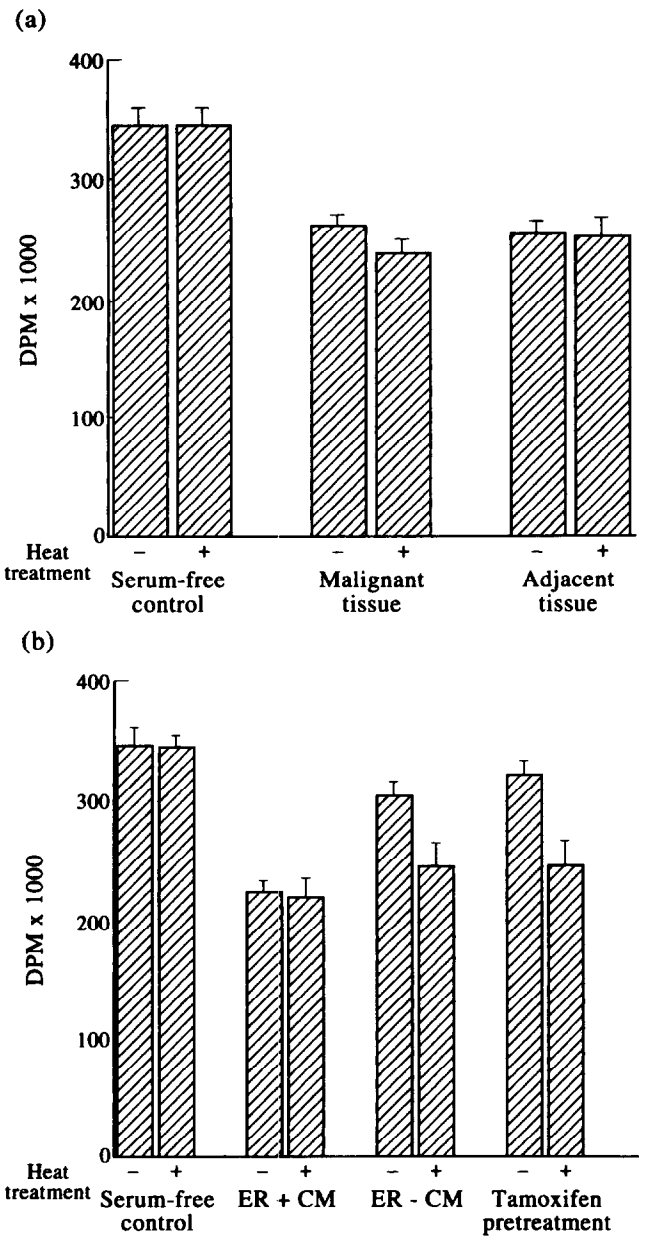

Figure 1. Inhibitory effect on CCL-64 growth of (a) breast cancer and breast cancer adjacent tissue biopsy conditioned medium (CM) compared with serum-free control medium before and after heat treatment. The presence of heat stable TGF-β-like inhibitory activity in both types of CM and the presence of low levels of latent TGF-β-like activity in CM of malignant but not adjacent tissue, which is released by boiling are demonstrated. (b) CM from malignant breast carcinomas showing the effect of oestrogen withdrawal on TGF-β latency. Significant levels of latent TGF-β-like activity are revealed after heat treatment of ER– and tamoxifen-pretreatment CMs but not from CM derived from ER+ cancers. *$P < 0.05$ by chi squared analysis (with Yate's correction). Data expressed as disintegrations per second (DPM) × 1000 as determined by scintillation counting.

When CM from malignant tissue was subdivided on the basis of ER status of the cancer (Figure 1b), it was found that this latent TGF-β component was due to there being significantly less activity in the ER negative samples ($P < 0.05$) prior to heat treatment, compared with that from ER positive cancers. Similarly, when we compared CM from tamoxifen-pretreated patients with CM samples from those who were not treated with tamoxifen, we also observed a lower level of activity prior to heat treatment ($P < 0.05$). In both these cases, the activities after heat treatment were similar.

However, immunoblotting (Figure 2) with the CC 1-30 anti-TGF-β_1 antibody revealed a single band of MW 50 kDa in both malignant and adjacent CM samples, suggesting that the mature (12.5/25 kD) peptide was absent or present at levels too low to account for the inhibitory activity observed in these CM samples.

Mature TGF-β_1 and TGF-β_1–LAP are differentially distributed in human breast tissues

In order to gain a better understanding of the distribution of the 50 kDa and mature peptide forms of TGF-β_1, we analysed total protein from 40 breast cancer biopsies and 37 matched adjacent and histologically normal human breast tissues for immunoreactivity with anti-TGF-β_1 antibody (CC A1/30). Gel electrophoresis of these samples allowed the identification of three distinct groups of TGF-β_1 related molecular weight species (Table 1) which were all immunoreactive with the CC A1/30 antibody directed against the mature TGF-β_1 peptide. We also investigated some of these samples

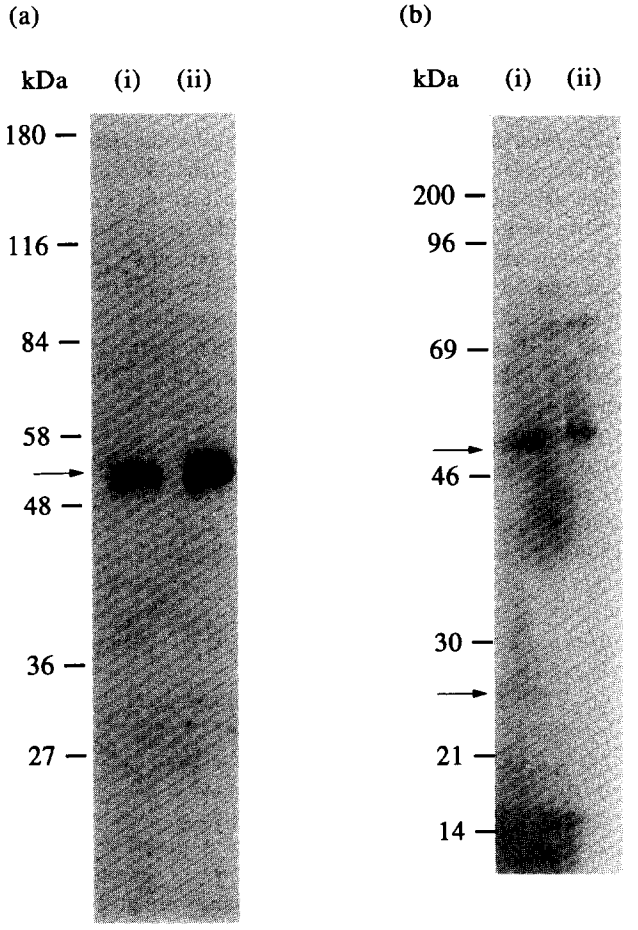

Figure 2. Detection of 50 kDa TGF-β_1 BCR by immunoblotting with the anti-TGF-β_1 antibody CC 1-30 (8 μg/ml) in (a) conditioned medium from adjacent (track (i)) and malignant (track (ii)) breast tissue biopsies (100 μl/well); and (b) whole tissue preparations of adjacent (track (i)) and malignant (track (ii)) breast tissue biopsies (100 μg/well). The position of the molecular weight markers is indicated on the left of the figures.

Table 1. Distribution of mature TGF-β_1 and the high (kDa) and intermediate (kDa) TGF-β_1 forms in malignant and adjacent malignant tissues of patients with breast cancer. Bands were detected by immunoblotting of 10% SDS–PAGE gels using the anti-TGF-β_1 antibody: CC A1/30. Three species of bands were identified on the basis of molecular weight as follows: (1) low kDa or mature TGF-β_1; (2) an intermediate kDa form; and (3) a high kDa form

	12.5/25 kDa TGF-β_1	50 kDa TGF-β_1 BCR	95 kDa TGF-β_1 BCR
All cancers			
Malignant tissue	24/40* (60%)	8/40 (20%)	6/40 (15%)
Adjacent tissue	22/37 (59%)	15/37 (41%)	1/37 (27%)
P value	>0.1	<0.05	<0.1
ER status (cancers)			
ER+	10/18 (56%)	1/18 (6%)	3/18 (17%)
ER−	11/18 (61%)	7/18 (39%)	0/18 (0)
P value	>0.1	<0.05	>0.1
Tamoxifen treated			
Pretreated	0/6 (0)	4/6 (67%)	0/6 (0)
Non-treated	24/34 (71%)	4/34 (12%)	6/34 (18%)
P value	>0.005	<0.01	>0.1

*Number positive/total examined.

by immunoprecipitation with CC A1/30. Electrophoresis of the immunoprecipitated pellet revealed the presence of the same three molecular weight species (data not shown) suggesting that these breast cancer related (BCR) TGF-β_1 proteins were restricted to these three forms. However, not all forms were present in each sample (Table 1). Under reducing conditions (β-mercaptoethanol and SDS only), we detected both 12.5 kDa and 25 kDa (dimer) forms of mature TGF-β_1, indicating some resistance to complete reduction. This was overcome by addition of 8 M urea to the reducing buffer which induced complete reduction of the 25 kDa form to the monomer 12.5 kDa form. Under these conditions, we found detectable levels of mature TGF-β_1 (low MW form) in 24 of 40 (60%) of the malignant tissues assayed, and in 22 of 37 (59%) of the adjacent tissue specimens. Interestingly, we were unable to identify mature TGF-β_1 (12.5/25 kDa) in any of the samples obtained from patients who had been treated with tamoxifen prior to their surgery and this observation was statistically significant ($P < 0.005$, Table 1). However, we found no correlation in these samples between oestrogen receptor status and detectable levels of mature TGF-β_1, indeed presence of mature TGF-β_1 was equally divided between ER+ (56%) and ER− (61%) patient samples (Table 1).

A TGF-β_1 related protein of approximately 50 kDa was observed in the malignant tissue of 8 of 40 patients (20%). Of these, 7 of 8 (88%) were found to be from ER− tumours, which was a statistically significant excess ($P < 0.05$) and represented the presence of this protein in 39% of all ER− tumours examined. However, we also found the 50 kDa protein in 15 of 37 (41%) adjacent tissue samples and this excess in adjacent samples was also significant at the 5% level (Table 1). The most striking observation was that 4 of 6 (67%) of the tamoxifen-pretreatment group were found to have this 50 kDa protein in their tumours. This was significant ($P < 0.01$) compared with only 4 of 34 (12%) samples, from patients not treated with tamoxifen, having this protein at detectable levels.

In a few patients, we found a high molecular weight TGF-β_1 related band of approximately 95 kDa. This 95 kDa TGF-β_1 related protein was restricted to malignant tissue samples, except for one case, where we also detected its presence in the adjacent tissue. None of the ER negative or tamoxifen-treated patients had this high MW protein band.

The 50 kDa TGF-β_1 related protein cross-reacts with antibodies to two other TGF-β_1 associated proteins and is biologically active

In order to gain insight into the identity of the breast cancer related (BCR) 50 and 95 kDa forms of TGF-β_1, we performed a dot blot antibody screen with a panel of antibodies raised against TGF-β_1 related proteins. We found that, although the controls confirmed the specificity of the antibodies to their antigens, both the 50 kDa and 95 kDa TGF-β_1 BCR proteins displayed immunoreactivity to the Ab 96-1 and Ab 37 antibodies, suggesting that they contained elements of both the TGF-β_1-latency associated protein (LAP) and the TGF-β_1-platelet binding protein (BP) in addition to the TGF-β_1 component.

In order to further characterise the TGF-β_1 BCR proteins, we partially purified both 50 and 95 kDa forms by elution of bands cut from Commassie stained SDS–PAGE gels. Both forms were assayed on CCL-64 mink lung epithelial cells, but only the 50 kDa form was found to have dose-dependent inhibitory activity which was comparable with, but not as potent as, that induced by (nanogram) equivalent amounts of the TGF-β_1 control (Figure 3). Heat treatment did not induce significant additional activity suggesting that the 50 kDa TGF-β_1 BCR protein is biologically active.

Proteolytic cleavage of high MW (95 kDa) TGF-β_1 BCR yields the 50 kDa TGF-β_1 BCR protein

Mature TGF-β_1 is thought to be generated from the inactive form *in vivo* by means of proteolytic cleavage of a large latent complex. In addition, *in vitro* treatment of latent TGF-β_1 forms with either acid or heat treatment has been shown to generate bioactive mature peptide, although probably via a different cleavage site. In order to investigate the relationship between the three TGF-β_1 BCR proteins identified, we subjected elution purified 95 kDa TGF-β_1 BCR to both heat and acid treatments in combination with plasmin proteolysis. CC

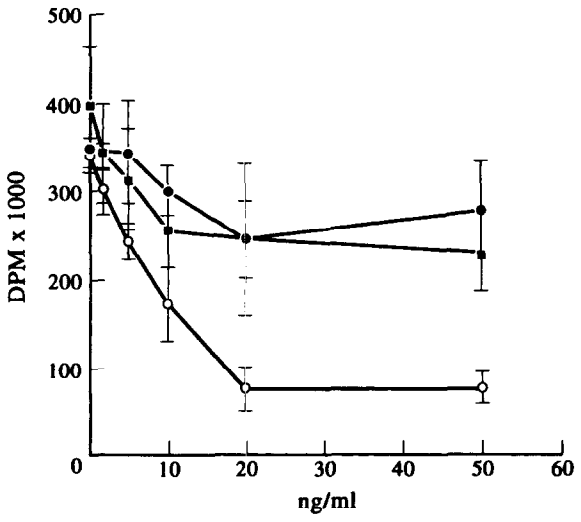

Figure 3. Dose-dependent inhibition of CCL-64 cells by TGF-β_1 (○), elution purified 50 kDa TGF-β_1 BCR (●) and heat treated elution purified 50 kDa TGF-β_1 BCR (■) demonstrating the absence of latent activity in the 50 kDa TGF-β_1 BCR.

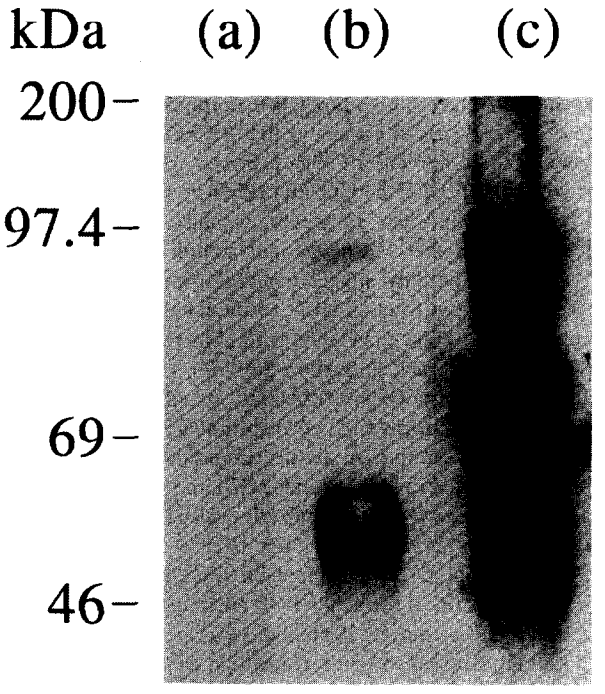

Figure 4. Elution purified 95 kDa TGF-β_1 BCR treated with plasmin and immunoprecipitated with CC 1-30 anti-TGF-β_1 antibody was immunoblotted with (i) (lane a) 10 μg/ml normal rabbit serum, (ii) (lane b) 100 μg/ml anti-TGF-β_1-LAP antibody 96-1 and (iii) (lane c) anti-TGF-β_1-BP antibody (Ab 37). Lane B demonstrates the proteolytic conversion of the 95 kDa to the 50 kDa TGF-β_1 BCR. Both 50 kDa and residual 95 kDa proteins show immunoreactivity with both TGF-β_1-LAP and TGF-β_1 (CC 1-30) antibodies after plasmin treatment. Lane C reveals an additional or intermediate degradation product at 75 kDa which is immunoreactive with both anti-TGF-β_1-BP and anti-TGF-β_1 antibodies but not with anti-TGF-β_1-LAP. The position of the molecular weight markers is indicated on the left of the figure.

A1-30 immunoreactive mature TGF-β_1 (12.5 kDa under reducing conditions) was observed after cleavage of the 95 kDa TGF-β_1 BCR with (a) heat, (b) acid, (c) acid followed by plasmin or (d) plasmin alone. A 50 kDa protein was observed after cleavage of the 95 kDa form with plasmin with or without pretreatment with acid. CC A1-30 (anti-TGF-β_1) immunoprecipitation of this 95 kDa protein after plasmin treatment (Figure 4) revealed the presence of two CC 1-30 immunoreactive degradation proteins in addition to residual 95 kDa protein. Only the 50 kDa protein generated from the 95 kDa TGF-β_1 BCR was immunoreactive with Ab 96-1 (anti-LAP) suggesting that it was identical to the 50 kDa TGF-β_1 BCR. Immunoblotting with anti-BP TGF-β_1 antibody (Ab 37) revealed a further protein of intermediate size (75 kDa) which was not seen *in vivo* and which probably represents a partially proteolysed form of the TGF-β_1–BCR (95 kDa), this protein showed no immunoreactivity to TGF-β_1–LAP antibody.

Plasminogen activator activity is increased in ER+ breast cancers

In order to assess the possible role of plasmin as an *in vivo* activator of TGF-β_1 peptide and the 50 kDa TGF-β_1 BCR, we analysed the same patient tissues for the presence of plasminogen activator (PA) using a radial caseinolysis assay. Higher levels of PA activity were observed in malignant (0.057 + 0.012 mm/μg protein) compared with adjacent tissues (0.03 + 0.008 mm/μg protein: $P < 0.01$), although we also observed elevated levels of PA in fibrocystic (benign) lesions (0.133 + 0.098 mm/μg protein) compared with their adjacent tissue (0.04 mm/μg protein), which was of a level comparable with that seen in the adjacent tissue of the breast cancer specimens.

We also found significantly higher levels of PA activity in ER+ compared with ER− cancers ($P < 0.05$, Figure 5), and higher levels in those patients who had not been treated with tamoxifen compared with those who had. Therefore, the 50 kDa TGF-β_1 BCR was inversely correlated with PA activity suggesting that the primary source of the 50 kDa TGF-β_1 BCR may not be generated by plasmin cleavage of higher MW TGF-β_1 BCR forms.

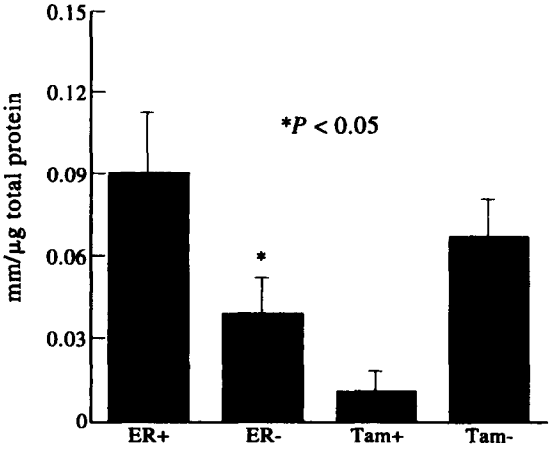

Figure 5. Levels of plasminogen activator activity detected in malignant breast tissues demonstrating the reduced levels observed in ER− cancers and tamoxifen-treated patients. Values shown represent mean and standard deviation from three replicate experiments from 14 ER+, 17 ER−, 5 Tam+ (tamoxifen-treated) and 29 Tam− (no tamoxifen treatment) patients. Statistical analysis was by Student's *t*-test. Samples of 5 μl tissue homogenate were used and corrected for total protein content using the Bradford microassay.

DISCUSSION

We identified three forms of breast cancer related (BCR) TGF-β_1, all of which show immunoreactivity to the TGF-β_1 specific antibody CC A1-30. We demonstrated that one of these TGF-β_1 BCR forms is the 25 kDa mature TGF-β_1 dimer [27]. In agreement with previous reports [13, 17], this active form of TGF-β_1 was identified in approximately 60% of breast carcinomas. However, we also showed that CM prepared from these same patient samples contained CCL-64 inhibitory activity suggesting biologically active TGF-β_1 secretion, but we were unable to detect the mature 25 kDa dimer form in these CM by immunoblotting, suggesting that it was not secreted and therefore not responsible for the inhibitory activity observed. Instead, we observed a single 50 kDa MW band form which corresponded with a 50 kDa form of TGF-β_1, identified in tissue preparations. This 50 kDa protein identified in breast cancer CM and tissue preparations was immunoreactive with antibodies raised against two separate TGF-β_1-related proteins, the latency associated protein (LAP) and the β-2 microglobulin platelet binding protein (BP), in addition to the CC A1-30 antibody raised against the mature TGF-β_1 dimer. Both the 50 kDa form and a higher (95 kDa) molecular weight form, which was also immunoreactive with the LAP and BP antibodies, persisted when electrophoresed under stringent reducing conditions. Assay of elution purified samples of this 50 kDa TGF-β_1 BCR demonstrated that it was biologically active, and therefore could not be the small latent complex protein previously described [9, 28], although it may be a mutated or uniquely cleaved version of this protein. The 95 kDa TGF-β_1 BCR was found to be biologically inactive after purification by gel elution, although it yielded the biologically active 50 kDa TGF-β_1 BCR, in addition to mature TGF-β_1 when experimentally cleaved with the protease plasmin, suggestive of the 60 kDa TGF-β_1 complex obtained by Lamarre and coworkers [28] from plasmin treated betaglycan. It is also possible that our 95 kDa latent complex may be a dimer [29] of the 50 kDa TGF-β_1 BCR, although the presence of an additional (75 kDa) band in plasmin treated samples, not observed *in vivo*, argues against this.

We identified a clinical correlation between the presence of the 50 kDa precursor form and oestrogen receptor negative tumours, samples derived from patients treated pre-operatively with tamoxifen and non-malignant tissues adjacent to malignant samples. Although very little latent bioactivity was observed in either breast cancer or adjacent tissue CM, we did observe low levels of residual heat labile latent TGF-β and lower plasminogen activator activity in the ER- and tamoxifen tumour groups. This finding may reflect the differences in fibroblast growth factor production in these tumours [21], which may directly affect PA synthesis and release. Alternatively, these data also suggest a role for oestrogen-mediated regulation of TGF-β_1 latency either directly via oestrogen-mediated downregulation of PA [30] or by post-translational modification of TGF-β_1 [12, 17–19]. Therefore, although we cannot entirely exclude the presence of small amounts of either TGF-β_2 or TGF-β_3 in these breast cancer CMs (which would not be detected by the TGF-β_1-specific CC A1-30 antibody), we must conclude that the predominant source of TGF-β inhibitory activity in breast cancer CM was due to the 50 kDa TGF-β_1 BCR. The additional latent component observed in ER– and tamoxifen-treated tumours may be due to the presence of a combination of latent TGF-β forms, and/or to the presence of small amounts of 50 kDa TGF-β_1 BCA dimer or the 95 kDa TGF-β_1 BCR form at levels below detection.

The presence of a secretable, biologically active small TGF-β_1 complex (the 50 kDa TGF-β_1 BCR) in 39% of ER– and 67% of tamoxifen-treated tumours suggests an important role for TGF-β_1 in the maintenance of human breast malignancies, which is consistent with earlier studies and provides a possible explanation for the poor prognosis of patients whose tumours have escaped steroid control. The presence of this protein in 41% of biopsies adjacent to tumours suggests a role in tumour progression and metastasis. The 50 kDa TGF-β_1 BCR may therefore represent a mutant or naturally occurring TGF-β_1 complex, which is independent of both oestrogen and plasminogen activator for its activation. It has been shown previously [31] that the small latent complex can bind the mannose-6-phosphate receptor which is believed to be involved in the activation of latent TGF-β_1 [31]. The small active complex may be activated via the same mechanism. If this protein does have the stability of the small latent complex [32, 33], it may also prove to play an important role in metastatic breast disease.

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Acknowledgement—This work was funded by grant number SP10988 from the Cancer Research Campaign.