Changes in expression of transforming growth factor beta mRNA isoforms in patients undergoing tamoxifen therapy

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Summary Tumour was obtained from 37 patients with oestrogen receptor-positive breast cancer, before and during treatment with tamoxifen, and examined qualitatively and semi-quantitatively for mRNA of the three mammalian TGF-β isoforms. Levels of TGF-β isoforms were then correlated with tumour response to tamoxifen, as assessed by monthly ultrasound. A high incidence of expression of each isoform was found in tumour material taken both before and during treatment. Semiquantitative assessment of mRNA showed that in the majority of tumours, expression of TGF-βs did not change markedly with treatment, i.e. beyond that which might have been caused by method reproducibility and tumour heterogeneity (variations of < 100% between pre- and post-treatment samples). In those displaying significant variation with treatment, expression of TGF-β_1 and -β_3 increased or decreased in equal numbers, whereas TGF-β_2 expression tended to increase with treatment. Subdividing tumours by clinical response revealed no significant association between changes in expression of TGF-β_1 and TGF-β_3. There was, however, a significant correlation between changes in expression of TGF-β_2 and response (P = 0.018). Thus, of 15 responding tumours displaying substantial changes, 11 showed an increase in TGF-β_2 expression with treatment, whereas none of the non-responding tumours were associated with increased expression. While not providing evidence for a generalised increase in TGF-β expression with tamoxifen treatment, the present study suggests that response to tamoxifen therapy may be associated with an increase in expression of specific TGF-β isoforms in some, but not all, tumours.

Keywords: tamoxifen; transforming growth factor beta

Tamoxifen is widely used in the treatment of postmenopausal breast cancer. Because benefits are usually observed in patients with oestrogen receptor-positive tumours (Stewart, 1989), it appears that the primary effects of tamoxifen are modulated by competitive antagonism of oestrogen at its receptor (Katzenellenbogen et al., 1983; Berthois et al., 1986). However, anti-tumour effects may be mediated secondarily through the action of inhibitory growth factors such as the transforming growth factor betas (TGF-βs) (Butta et al., 1992). TGF-βs have been reported to be inhibitory to breast epithelial cells (Lippman et al., 1987; Travers et al., 1988; Barrett-Lee et al., 1990; Knabbe et al., 1991; Mizukami et al., 1991), and their expression in breast tissues can be modified by tamoxifen (Salomon et al., 1989; Colletta et al., 1990; Sporn et al., 1990; McCune et al., 1992; Butta et al., 1992; Ji et al., 1994). However, TGF-β can also act in a stimulatory fashion (Torre-Amione et al., 1990; Arrick et al., 1992; Arteaga et al., 1993; Chang et al., 1993), and high levels of TGF-β mRNA and protein have been associated with poor prognosis in patients with breast cancer (Dickson et al., 1987; King et al., 1989; Colletta, 1990; Welch et al., 1990; Thompson et al., 1991; Gorsch et al., 1992; Walker and Dearing, 1992; MacCallum et al., 1994; Walker et al., 1994).

Assessments of the role of TGF-βs in the behaviour of breast cancer are also complicated by factors such as (1) TGF-β may be synthesised in one cell type and have its action in another (Lafyatis et al., 1990; Jackowlew et al., 1992); (2) a variety of forms may exist, not all of which are active (Colletta et al., 1991); (3) mRNA and protein levels do not always correlate, and may be differentially regulated (Knabbe et al., 1987; Colletta et al., 1994). There is thus a need to clarify the role of TGF-β in the natural history of

breast cancer and in mediating responses to tamoxifen. To do this we have investigated a group of elderly patients offered primary systemic therapy with tamoxifen before definitive breast surgery. The design of the study has allowed us to (1) observe the effects of tamoxifen treatment on the mRNA isoforms of TGF-β by comparing measurements in the same primary breast cancer both before and after treatment; and (2) to relate changes in expression of TGF-β following tamoxifen treatment with response as measured by monitoring tumour volume during treatment.

Materials and methods

Patients

Thirty-seven patients over 70 years of age and presenting with histologically proven primary breast cancer to the Edinburgh Breast Unit were entered into a study in which primary systemic therapy with tamoxifen was offered. All patients had tumours > 3 cm in size which were ER-rich (> 20 fmol mg^{-1} protein cytosol, Anderson et al., 1989). Therapy (tamoxifen, 20 mg day^{-1}) was administered initially for 3 months, but patients could be electively treated for an extended period of time up to a maximum of 10 months, at the end of which definitive breast surgery was performed. Response to treatment was assessed by monthly ultrasound of the breast (Forouhi et al., 1994), and patients were classified as being responders if there was a decrease in tumour volume of at least 20% between initial biopsy and final surgery; non-responders showed either no change or an increase in tumour volume. By these criteria, 27 responded to tamoxifen and 10 did not. Although the time of treatment was variable, 32 patients showed progressive changes in tumour size. However, one responding patient did not experience a decrease in tumour volume of 20% until 6 months (and therefore, if classified at 3 months, would have been a non-responder), whereas, conversely, a further four patients categorised as non-responders experienced some degree of tumour shrinkage at earlier time points.

Tumours

Tumour tissue was taken before treatment and following primary tamoxifen treatment, snap frozen and stored in liquid nitrogen until extracted for RNA.

RNA Extraction

Tumour RNA was extracted using a modification of the method of Auffrey and Rougeon (1980). Tumour tissue (minimum 0.2 g) was dismembranated and resuspended in 3 M lithium chloride – 6 M urea (6 ml) before being sonicated and left overnight at 4°C to allow nucleic acids to precipitate. Total mRNA was then recovered by centrifugation (15 000 g) and digested in 10 mM Tris/sodium dodecyl sulphate (SDS) (6 ml) containing proteinase K (50 μg ml^{-1}). Protein was removed using 100% phenol (pre-equilibrated with 0.1M Tris, pH 7.4), followed by phenol–chloroform–isoamyl alcohol 25:24:1, v/v/v) and 100% chloroform by centrifugation, with the aqueous phase recovered on each occasion. The RNA was then precipitated overnight in absolute alcohol (2.5 volumes) containing lithium chloride (300 μl, 8 M), and recovered by centrifugation (4000 g, 4°C for 45 min), dried and resuspended in diethyl pyrocarbonate-treated water. RNA content and purity were assessed by spectrophotometry at 260 nm and 280 nm, and aliquots stored at −80°C until assayed.

RNAase protection assay

Probes were prepared as set out in Bartlett et al., (1992), using a Gemini II system (Promega, UK) with full-length transcripts isolated by polyacrylamide gel electrophoresis. Bands eluted from the gel were resuspended in hybridisation buffer for use in the RNAase protection assay. This assay was also carried out according to Bartlett et al., (1992). Sample probe hybrids remaining after digestion with RNAase were denatured and separated by gel electrophoresis. Autoradiographs of the paired RNA samples were assessed by scanning densitometry for changes in expression of the three isoforms occurring during treatment with tamoxifen.

Quantitation

Densitometry results were normalised to actin controls, and changes in expression of TGF-β mRNA defined using several controls. These included (1) replicates of the tumour pairs run on different occasions, to assess reproducibility of measurements (greatest variation ± 68%); (2) different RNA extracts of the same tumour to determine heterogeneity within the tumour sample (greatest variation ± 66%); and (3) three patients studied sequentially over 3–7 weeks without intervening therapy to assess variations associated with no treatment (greatest variation ± 91%). An arbitrary cut off value of ± 100% was chosen to define any changes as significant and attributable to tamoxifen therapy.

Statistical analysis

Analysis of the results generated was carried out using the chi-squared test for trend.

Results

Qualitative analysis

The expression of mRNA for the three isoforms of TGF-β in pretreated and treated tumour samples is shown in Table I. TGF-β$_1$ was expressed in all tumours before treatment, but following therapy, two cancers lost expression (one responding and one non-responding tumour). TGF-β$_2$ was expressed initially by 33 of 37 tumours, and during treatment in 35 of 37 (this included 32 of the tumours which initially showed expression); TGF-β$_3$ expression was detected in 36 of 37 pretreatment and in all post-treatment samples. Therapy had no significant effects on the incidence of expression of TGF-β isoforms within the total group, or between subgroups of responding and non-responding patients.

Quantitative analysis

Limits for normal variations in the expression of TGF-β were determined using several controls (see Materials and methods); changes in excess of ± 100% were regarded as substantial and potentially attributable to tamoxifen treatment. The autoradiograph in Figure 1 shows typical examples of tumours studied before and after treatment with tamoxifen; paired RNA samples being hybridised to a TGF-β$_2$ probe. Patient 1 illustrates substantially increased expression with treatment, patient 2 very little change in expression, and in patient 3, a substantial decrease in level of expression with treatment. As is shown in Table II, the majority of tumours showed no substantial changes with treatment for TGF-β$_1$ and -β$_3$ expression, and those that did were as likely to display an increase as a decrease. For TGF-β$_2$, more tumours showed changes in expression, these being predominantly increased expression after treatment.

When tumours were subdivided into responding and non-responding groups, no significant associations were found between groups for changes in expression of TGF-β$_1$ and -β$_3$ mRNA. However, there was a significant association between increasing expression of TGF-β$_2$ and response ($P = 0.018$, chi-squared test for trend); indeed no non-responding patients showed an increase in TGF-β$_2$, whereas 11 of 27 responding did so.

Discussion

The management of patients with primary systemic therapy provides the opportunity to monitor the effects of treatment
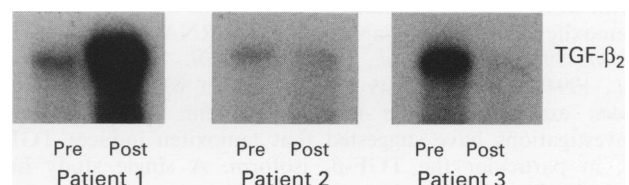

Figure 1 Illustrative autoradiograph. Paired samples of pre- and post-treatment RNA from three patients hybridised to a TGF-β$_2$ RNA probe (600 bp).

Table I Incidence of expression of mRNA for the three isoforms of TGF-β in pre- and post-treatment samples

| | Pre-treatment | | | Post-treatment | | |
	Total (37)	R (27)	NR (10)	Total (37)	R (27)	NR (10)
TGF-β$_1$	37	27	10	35	26	9
TGF-β$_2$	33	26	7	36	26	9
TGF-β$_3$	36	26	10	37	27	10

R, responders; NR, non-responders.

Table II Changes in expression of TGF-β mRNA levels outwith normal variation ($\pm 100\%$) and response to treatment

	TGF-β₁			TGF-β₂			TGF-β₃		
	>	=	<	>	=	<	>	=	<
Total (37)	7	25	5	11	22	4	6	27	4
Responders (27)	6	17	4	11	14	2	5	21	1
Non-responders (10)	1	8	1	0	8	2	1	6	3
χ^2 test for trend	$P = 0.724$			$P = 0.018$			$P = 0.069$		

on individual tumours *in situ* both in terms of clinical response and biological parameters. In this particular study effects of tamoxifen therapy on tumour expression of mRNA for the isoforms of TGF-β have been monitored and correlated with the response of individual cancers. The results show that almost all tumours express each isoform both before and after treatment, and that the majority of tumours do not show substantial changes in TGF-β expression with treatment. However, significant changes in one isoform, TGF-β₂ were more frequently observed with therapy; with a correlation between response to therapy and increasing TGF-β₂ expression.

The high incidence of TGF-β mRNA expression is in keeping with our own previously published work (MacCallum *et al.*, 1994), and that of others (Arrick *et al.*, 1990; Barrett-Lee *et al.*, 1990; Jeng *et al.*, 1993), and is consistent with the high frequency of protein expression in primary breast cancers (McCune *et al.*, 1992; Gorsch *et al.*, 1992; Jhala *et al.*, 1995). Perhaps because of this high expression, TGF-β isoform status did not change with treatment. However, level of expression did appear to vary between pre- and post-treatment samples, and therefore we attempted to quantify the level of expression and relate changes to response to treatment. In order to attribute changes more reliably to the influence of tamoxifen, efforts were made to assess the inherent variability of the methodology, the heterogeneity of the tumours, and the variation associated with sequential sampling of the same cancer without intervening therapy. Results from representative samples suggested that differences of up to 100% could result from repeated assay of different portions of the same tumour. In analysing the results therefore, it was decided to consider variations in excess of 100% as being significant changes.

Using this criterion, in the majority of tumours we were unable to detect major changes in expression attributable to treatment, irrespective of whether the tumour displayed a therapeutic response or not. While, to our knowledge there has been no other published data correlating mRNA expression of TGF-β isoforms with effect of tamoxifen in primary breast tissue, these observations would be consistent with work on breast cancer cell lines, in the majority of which tamoxifen does not apparently affect mRNA levels (Knabbe *et al.*, 1987; Arrick *et al.*, 1992; Jeng *et al.*, 1993; Colletta *et al.*, 1994). This contrasts with studies in which TGF-β has been examined at the level of protein. Most of these investigations have suggested that tamoxifen induces TGF-β, in particular the TGF-β₁ isoform. A single study has examined a small number of individual breast cancers before and after 3 months of treatment with tamoxifen (Butta *et al.*, 1992), and reported increased levels of TGF-β₁ in the stromal compartment of tumours (although that in epithelial cells remained constant). These differences between mRNA and protein expression may result from the synthesis of different isoforms in different cell compartments of tumours (Lafyatis *et al.*, 1990; Jakowlew *et al.*, 1992), and the sequestration of mature protein by fibroblasts (McCune *et al.*, 1992; Dublin *et al.*, 1993).

Although we did not observe a general induction of TGF-β mRNA with tamoxifen therapy, it was of interest that approximately one-third of tumours showed an increase in TGF-β₂ isoform on treatment, and that all these tumours responded to therapy. While this is compatible with tamoxifen's inductive influences on TGF-β protein (Colletta

et al., 1991; Butta *et al.*, 1992; Jordan *et al.*, 1993), it should be noted that the effect on protein is on the β₁ isoform, whereas the mRNA affected in the present study is TGF-β₂. The present study provides evidence of differential regulation of different isoforms of TGF-β, in that only one of the 11 tumours displaying an increase in TGF-β₂ expression also shows an increase in levels of TGF-β₁ mRNA. Similarly, only 4 of the 11 tumours showing increased TGF-β₂ also have increased TGF-β₃ (one tumour showing increased expression of all three isoforms). Interestingly, 17 of 27 tumours in the responding group displayed increased expression of one of the three isoforms of TGF-β compared with one of ten in the non-responding group (increased TGF-β₁ and -β₃).

Given that TGF-βs generally inhibit growth of breast cancer epithelium (Roberts *et al.*, 1985; Moses *et al.*, 1985), the induced response of TGF-β following tamoxifen treatment would be compatible with tumour regression. As a result it has been suggested that TGF-β may mediate tamoxifen anti-tumour properties (Lippman *et al.*, 1987; Knabbe *et al.*, 1991). If this is the case, it needs to be asked why in the present study a substantial proportion of responding tumours do not show increases in TGF-β. There are several potential reasons for this. Firstly, it may be that TGF-βs were induced by tamoxifen, but at an earlier time point than that at which mRNA analyses were performed; indeed if the induction mediates anti-tumour effects it would be expected to precede tumour response. We cannot exclude this possibility as all our patients received at least 3 months' treatment so that evidence of tumour regression could be obtained, and it was only at this time that tumour samples were taken for analysis. However, length of treatment with tamoxifen did not appear to influence results, in that over the comparatively large range of treatment times, there was no evidence for greater induction of TGF-βs at early time points. A further consideration surrounds compartmentalisation of TGF-β between malignant epithelium and stromal elements of the tumour. *In situ* hybridisation studies suggest that the major site of mRNA expression is in the malignant epithelial cells (MacCallum *et al.*, 1995; Auivenin *et al.*, 1995; Walker and Gallacher, 1995), and thus regression of this compartment would diminish the major cellular site of synthesis. However, if TGF-β protein is sequestered by the stromal component (McCune *et al.*, 1992; Dublin *et al.*, 1993) which did not regress on treatment, an impression of up-regulation may be produced at a time when mRNA levels are reduced.

A further potential reason for the non-elevation of TGF-βs in responding tumours is that TGF-βs may not mediate tamoxifen's anti-tumour effects (Ji *et al.*, 1994), which depend on other mechanisms, for example, the presence and concentration of other growth factors or cytokines within the tumour environment (Pepper *et al.*, 1993). Indeed there is accumulating evidence that with progression to more advanced disease resistance to TGF-βs can develop (Schultz and Grant, 1991; Kerbel *et al.*, 1993) in melanoma (Kerbel *et al.*, 1992), colon cancer (Manning *et al.*, 1991) and mouse mammary cancer (Pierce *et al.*, 1995).

The present study illustrates the complexity of investigating the effects of treatment on biological parameters in clinical specimens. Further questions are raised which can only be answered by more in-depth studies, including investigations involving the measurement of mRNA, protein and biological activity at time points preceding clinical response.

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