CLINICAL RESEARCH

Growth factor expression in normal, benign, and malignant breast tissue

MAUREEN T TRAVERS, PETER J BARRETT-LEE, UTA BERGER, YUNUS A LUQMANI, JEAN-CLAUDE GAZET, TREVOR J POWLES, R CHARLES COOMBES

Abstract

Several oncogenes seem to encode certain growth factors that may play a part in regulating cell growth in tumours. To assess whether such factors are synthesised endogenously by tumour cells the amounts of messenger RNA for several growth factors known to be synthesised by cancer cells of the breast *in vitro* were examined in biopsy specimens from 52 malignant and 15 non-malignant tumours of the breast and four samples of normal breast. Transforming growth factor β messenger RNA was significantly more abundant in breast cancers (32 of 42 (76%) having appreciable amounts) than non-malignant breast tissue (five of 13 (38%) having similar amounts). Transcripts for both transforming growth factor α and its receptor, epidermal growth factor receptor, were found more commonly in carcinomas that were negative for oestrogen receptor (64% and 87%, respectively) than in those that were positive (27% and 30%, respectively). Insulin-like growth factor II messenger RNA was present in all 15 samples of non-malignant tissue but was found (in considerably lower amounts) in only 11 of 21 (52%) carcinomas. Epidermal growth factor receptor was also found in all non-malignant breast tissues, compared with 19 of 45 (42%) carcinomas. Platelet derived growth factor A and B chain transcripts coexisted in all normal and benign tissue and most carcinomas.

This differing pattern of expression growth factors in tissue from malignant tumours compared with benign tumours and

normal breast tissue suggests that some growth factors, particularly transforming growth factors α and β , may have an important role in controlling growth of human breast cancers, particularly those that are hormone independent.

Introduction

Growth factors are polypeptides that play a part in normal growth of cells. They are thought to act locally by diffusing through intercellular spaces and binding to specific high affinity cell membrane receptors. The finding that the products of several oncogenes were homologous with certain growth factors and growth factor receptors led to much speculation about their role in carcinogenesis.¹ Recently, several cell lines of human breast cancers have been found to secrete stimulatory growth factors, including transforming growth factor α , which binds to the same receptor as epidermal growth factor^{2,3}; platelet derived growth factor⁴; and the insulin like growth factors I and II.⁵ Another factor, transforming growth factor β , inhibited growth of breast cancer cells *in vitro*.⁶ The finding of receptors for some of these growth factors on breast cancer cells led to the proposal that these growth factors may act in an autocrine manner—that is, they may stimulate or inhibit growth of the cells that secrete them.⁷ The production of growth factors by cells that have receptors for them might, if uncontrolled, result in abnormal cell growth.

Most of this work has centred on the use of cell lines.^{5,8} These may, however, be unrepresentative of breast cancer cells *in vivo* as cells from only about one in 1000 breast cancers will form a clonogenic cell line, the remainder failing to proliferate for more than a few passages. We therefore chose to examine biopsy specimens of breast tissue taken at operation for the presence of messenger RNA encoding the relevant growth factors as its presence implies production by the tumour rather than passive absorption or binding of growth factors synthesised elsewhere.

Materials and methods

PATIENTS AND TISSUE SAMPLES

We collected samples of breast tissue during operation, dissected them free of extraneous material, and snap froze them in liquid nitrogen within

Ludwig Institute for Cancer Research, St George's Hospital Medical School, London SW17 0RE

MAUREEN T TRAVERS, PHD, non-clinical scientist

PETER J BARRETT-LEE, MRCP, clinical scientist

UTA BERGER, MD, clinical scientist

YUNUS A LUQMANI, PHD, non-clinical scientist

R CHARLES COOMBES, MD, FRCP, consultant medical oncologist and senior clinical scientist (also at St George's and Royal Marsden Hospitals)

Royal Marsden Hospital, Sutton, Surrey SM2 5PT

JEAN-CLAUDE GAZET, MS, FRCS, consultant surgeon (also at St George's Hospital)

TREVOR J POWLES, PHD, FRCP, consultant medical oncologist

Correspondence to: Dr Coombes.

five minutes after resection. They were stored in the vapour phase of liquid nitrogen at -196°C for six to 72 months until required. Tissues were examined from 46 primary carcinomas, six lymph node metastases, 15 benign diseases of the breast (11 fibroadenomas and four fibrocystic disease), and four normal breasts. To assess whether differences in the amounts of specific messenger RNAs occurred in carcinomas from different sites in the same patient we assayed both the primary carcinoma and the lymph node metastases in five patients. All samples were stained by routine haematoxylin and eosin and examined histologically by one of us (UB).

The patients were aged from 30 to 85. The carcinomas were clinically staged by the tumour node metastasis criteria of the International Union Against Cancer⁹ as T₁ (six patients), T₂ (20), T₃ (10), or T₄ (three); seven were unstaged. Twenty two patients had histological evidence of metastases of ipsilateral axillary lymph nodes. Histological examination of the primary tumour showed that 36 were infiltrating ductal carcinomas, eight lobular, one was tubular, and one was medullary.

the appropriate plasmid. A scale of the intensity of hybridisation of 1 to 5 was derived, corresponding to 7.8 to 125.0 pg of plasmid DNA.

Northern hybridisation was carried out as described by Thomas.¹⁹ Briefly, polyadenylated RNA was resolved in a 0.8% (w/v) agarose gel containing 2.2 mol formaldehyde/l and blotted on to a Biotodyne A filter. Subsequent prehybridisation, hybridisation, washing, and autoradiography were performed as described above. The size of the transcripts was determined with denatured RNA and DNA markers.

CONTENT OF OESTROGEN RECEPTOR

We measured oestrogen receptor in samples by the dextran coated charcoal technique, a modification of that described by McGuire *et al.*²⁰ If the sample was too small for the conventional assay the receptor was detected visually by immunocytochemical examination with a specific monoclonal

Growth factor transcripts in malignant and benign tumours of breast and normal breast. Figures are numbers (percentages) of tissue samples

Receptor or growth factor	Intensity of hybridisation (scale of 1-5)	Carcinomas positive for oestrogen receptor (n=31)	Carcinomas negative for oestrogen receptor (n=15)	Non-malignant tumours and normal breast (n=19)
Transforming growth factor α	{ 1-5 3-5	8/30 (27) 3/30 (10)	9/14 (64) 0/14	4/18 (22) 1/18 (5)
Epidermal growth factor receptor	{ 1-5 3-5	6/30 (20) 1/30 (3)	13/15 (87) 3/15 (20)	15/15 3/15 (20)
Transforming growth factor α and epidermal growth factor receptor	1-5	2/29 (7)	8/14 (57)	2/14 (14)
Transforming growth factor β	{ 1-5 3-5	28/28 20/28 (71)	14/14 12/14 (86)	13/13 5/13 (38)
Insulin-like growth factor II	{ 1-5 3-5	9/16 (56) 3/16 (19)	2/5 (40) 2/5 (40)	15/15 15/15
Platelet derived growth factor A chain	{ 1-5 3-5	27/27 7/27 (26)	11/11 5/11 (45)	10/10 8/10 (80)
Platelet derived growth factor B chain	{ 1-5 3-5	18/20 (90) 13/20 (65)	12/14 (86) 4/14 (29)	12/12 4/12 (33)

ESTIMATION OF GROWTH FACTOR TRANSCRIPTS

The human complementary DNA probes used were epidermal growth factor receptor (plasmid pHER-A64-1, insert size 1838 base pairs)¹⁰; transforming growth factor α (plasmid sp65-C17N, insert size 1300 base pairs)¹¹; transforming growth factor β (plasmid sp64-BC₁, insert size 1050 base pairs)¹²; insulin-like growth factor II (plasmid pIGF2/8-1, insert size 1150 base pairs)¹³; c-sis (PDGF B chain) (plasmid pL335, insert size 1730 base pairs)¹⁴; and platelet derived growth factor A chain (plasmid pUC-13-D1, insert size 1300 base pairs).¹⁵ The specific inserts were excised with the relevant restriction enzymes and labelled with d-cyditinetriphosphate labelled with phosphorus-32 by a random priming method¹⁶ to a specific activity of approximately 29.6 MBq/ μg DNA. Total cellular RNA was obtained from 0.3-1.0 g of frozen tissue by the guanidinium isothiocyanate method as described by Chirgwin.¹⁷ Polyadenylated fractions were isolated by one passage through oligo (dT) cellulose.¹⁸

For dot blot hybridisation serial dilutions of total RNA that had been denatured by formaldehyde were prepared and applied to a Biotodyne A nylon membrane (Pall Filtration, Portsmouth, United Kingdom) with a Bio-dot manifold (Bio-rad, United Kingdom). In addition, serial dilutions of standard amounts of each recombinant plasmid and of non-homologous RNA were prepared and applied to filters to measure the signal and to determine the extent of non-specific hybridisation in each case. The filters were prehybridised at 42°C for four to six hours in 50% (v/v) deionised formamide, 0.1% (w/v) sodium dodecyl sulphate, 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution with 0.02% (w/v) each of povidone, bovine serum albumin, and Ficoll), 5 mmol edetic acid/l, 0.75 mol sodium chloride/l, and 50 mmol sodium acid phosphate/l, pH 8.3, and denatured sonicated salmon sperm DNA (250 mg/l). They were hybridised overnight at 42°C under the same conditions with the addition of 30-140 MBq of a denatured DNA probe labelled with³² P-d-cyditinetriphosphate/l. After hybridisation the filters were washed with five changes of 2 $\times$ standard saline citrate (20 $\times$ strength: 3 mol sodium chloride/l 0.3 mmol trisodium citrate/l, pH 7) and 0.1% (w/v) sodium dodecyl sulphate at room temperature and two changes of 0.1 $\times$ standard saline citrate and 0.1% (w/v) sodium dodecyl sulphate at 60-65°C.

Autoradiographs were made of the filters at -70°C with Hyperfilm MP (Amersham) and intensifying screens over four to 14 days.

The messenger RNA was measured by comparison with serial dilutions of

antibody as previously described.²¹ In the biochemical assay tissue from a breast carcinoma containing ≥ 15 fmol of receptor/mg cytosol protein was considered to be positive. With the immunocytochemical method staining of more than 10% of the carcinoma cells was defined as a positive result as we have shown that in these cases the tumour cells contain detectable amounts of messenger RNA specifying the oestrogen receptor.²²

STATISTICAL ANALYSIS

Statistical analysis was the χ^2 test with Yates's continuity correction for small numbers or Fisher's exact test.

Results

Dot blot hybridisation was used to determine the amounts of transcripts for several growth factors and for epidermal growth factor receptor in 46 samples of breast carcinoma, 15 of benign tumours of the breast, and four of normal breast (fig 1); the table summarises the results. Thirty one of the 46 carcinomas and five of seven fibroadenomas were positive for oestrogen receptor.

Transcripts for epidermal growth factor receptor were found in all of the samples of non-malignant tissue and in 19 out of 45 (42%) carcinomas. In the samples of malignant tumours however, epidermal growth factor receptor messenger RNA was more often associated with lack of oestrogen receptor, only six of 30 tumours positive for oestrogen receptor containing transcripts for epidermal growth factor receptor compared with 13 of the 15 tumours negative for the receptor ($p < 0.001$). Northern blot analysis of both benign and malignant breast tissue showed three distinct bands hybridising with the epidermal growth factor receptor probe at 10.0, 6.4 and 4.8 kilobases (fig 2). The presence of epidermal growth factor receptor protein was confirmed by immunocytochemistry with monoclonal antibody epidermal growth factor-R1²³ in 15 carcinomas expressing epidermal growth factor receptor messenger RNA. The staining was localised in the cell membrane and cytoplasm of tumour cells. Immunoreactivity to epidermal growth factor

receptor was not found in cryostat sections of 10 carcinomas that did not contain epidermal growth factor receptor messenger RNA.

Transforming growth factor α messenger RNA was detected in four of 18 samples of non-malignant breast tissue and in 17 of 44 carcinomas. Messenger RNA for both transforming growth factor α and its receptor, epidermal growth factor receptor, were found together in 10 of 43 cancers, and, of these, eight were negative for oestrogen receptor ($p < 0.001$). Northern blot analysis showed transcripts of 4.8 and 2.2 kilobases for transforming growth factor α in several malignant tumours. The 4.8 kilobase transcript was more abundant in all tumours examined except one, which contained a greatly increased amount of the 2.2 kilobase messenger RNA (fig 2). The importance and prevalence of this transcript are unknown.

The 2.5 kilobase transforming growth factor β messenger RNA (fig 2) was, in contrast, found in all tissues but was present in higher amounts (intensity of hybridisation $\geq 3+$) in carcinomas (32/42, 76%) than in normal or benign tissue (5/13, 38%) ($p < 0.05$). The degree of lymphocytic infiltration assessed in cryostat sections on an arbitrary scale of 1-6 in 40 carcinomas was not related to the amount of transforming growth factor β messenger RNA ($\chi^2 = 3.98$, $p = 0.55$). Similarly, the amount of transforming growth factor β messenger found in four lymph node metastases was not related to the volume of normal lymph node tissue in the sample. The abundance of transforming growth factor β messenger RNA in carcinomas was not related to their content of oestrogen receptor or to the presence or absence of messenger RNAs for the other growth factors studied.

Insulin-like growth factor II transcripts were found in all 15 normal and benign tissues but in only 11 of 21 carcinomas ($p < 0.005$). In non-malignant breast tissue the amount of insulin-like growth factor II messenger RNA was much higher (intensity of hybridisation $> 5+$) than that of other growth factors; the amount of insulin-like growth factor II messenger RNA in carcinomas was generally low (hybridisation intensity $< 3+$).

Platelet derived growth factor A chain and B chain (c-sis) transcripts were present in all normal and benign breast tissues. The amount varied somewhat in carcinomas, but there did not seem to be any difference between carcinomas that were positive or negative for oestrogen receptor.

FIG 1—Dot blot analysis of total RNA from primary breast cancers. Serial dilutions of plasmid DNA (125-7.8125 pg); (A) or total RNA (20-0.125 µg); (B and C) were spotted on to Biotrans A membrane and hybridised to probe labelled with ^{32}P -d-cytidine triphosphate. (A) Scale of intensity of hybridisation; dilutions of standard (in this case epidermal growth factor receptor plasmid) hybridised with labelled epidermal growth factor receptor complementary DNA probe. (B) Dot blot of RNA from five primary breast cancers hybridised to epidermal growth factor receptor complementary DNA probe; tracks 1-3 show positive hybridisation, 4 and 5 are negative. (C) Dot blot of RNA from five primary breast cancers hybridised to transforming growth factor α complementary DNA probe; only track 4 shows positive hybridisation. Concentration of RNA at each dilution given to the right.

FIG 2—Northern blot analysis of RNA from primary breast cancers. Polyadenylated RNA (4 µg) was resolved on formaldehyde/agarose gels blotted on to Biotrans A filters and then hybridised with probes (transforming growth factor α , transforming growth factor β , and epidermal growth factor receptor) labelled with ^{32}P -d-cytidine diphosphate. Size of bands showing hybridisation, determined by reference DNA and RNA markers given in kilobases to the right of tracks.

Platelet derived growth factor B chain was undetectable in four of 34 samples (12%) of carcinoma. Lymph node metastases from five patients contained similar amounts of platelet derived growth factor B chain as the primary tumours. Northern hybridisation showed three bands that hybridised with the A chain probe, at 2.9, 2.4 and 1.8 kilobases, and a single band that hybridised with the platelet derived growth factor B chain (c-sis) probe, at 4.0 kilobases.

We examined the relation between the presence of messenger RNA for each growth factor and the histological type of tumour, menopausal state of the patient, and T or nodal state. There was no significant correlation independent of oestrogen receptor state and, in particular, between epidermal growth factor receptor messenger RNA and lymph node state at presentation ($\chi^2 = 0.26$, $p = 0.61$, $n = 36$). Owing to the small numbers of patients studied we could not determine whether the presence of growth factor messenger RNA and prognosis or response to treatment are related.

Discussion

Our results show differences in the expression of growth factors and their receptors, as measured by the production of messenger RNA, exist between breast carcinomas positive and negative for oestrogen receptor and between non-malignant breast tissue and breast carcinomas.

Only tumours containing epidermal growth factor receptor messenger RNA had immunoreactive epidermal growth factor

receptor protein; this confirms previous reports that the presence of this receptor, or in our study its messenger RNA, is inversely related to the presence of oestrogen receptor in breast tumours.^{3 24 25} Epidermal growth factor receptor transcripts were, however, found in all samples of non-malignant breast tissue examined.

Because of the presence of transcripts for epidermal growth factor receptor it was important to determine whether messenger RNA for a ligand of this receptor, transforming growth factor α , was also synthesised in breast tissue. A correlation, though not an absolute one, had been shown previously between the presence of messenger RNA for epidermal growth factor receptor and that for transforming growth factor α in renal and squamous cell carcinomas.²⁶ We found transcripts for transforming growth factor α in both normal and malignant breast tissue but more commonly in tumours that were negative for oestrogen receptor (64%) than in tumours that were positive (27%) or non-malignant breast tissue (22%). Transcripts for transforming growth factor α and its receptor, epidermal growth factor receptor, coexisted more commonly in carcinomas negative for oestrogen receptor, suggesting a possible autocrine mechanism for the growth of these tumours independently of hormone. This finding of low frequency expression of transforming growth factor α in tumours positive for oestrogen receptor is at variance with in vitro observations, which suggest that oestrogen stimulates proliferation of cells positive for oestrogen receptor by inducing synthesis of transforming growth factor α .²⁷

Transforming growth factor β messenger RNA was detected in all normal, benign, and malignant tissue; the amount of transforming growth factor β transcripts was, however, significantly higher in malignant than non-malignant tissue. The reasons for this are unclear as in vitro work suggests an inhibitory role for transforming growth factor β in breast cancer cells. The higher amounts in cancer cells may perhaps play a part in suppressing the immune response to the tumour.²⁸

We also considered the contributions of other cell types to the increased amounts of transforming growth factor β messenger RNA in cancers. A high amount of transforming growth factor β message was seen in normal human lymphocytes; no correlation was found, however, between the degree of lymphocytic infiltration and expression of transforming growth factor β .

In a few patients in whom we obtained two samples (one from the primary carcinoma and one from a lymph node metastasis) the amounts of transforming growth factor α , transforming growth factor β , epidermal growth factor receptor, and platelet derived growth factor B chain messenger RNA in the two samples were identical or within one point on the intensity scale.

Insulin-like growth factor II showed a completely different pattern of expression from transforming growth factor β in that its messenger RNA was found in non-malignant tissue in high amounts and in only half of the carcinomas, in which it was present at much lower amounts. This growth factor is of particular interest as both tumour and stromal cells contain insulin-like growth factor II receptors²⁹⁻³¹ and it could therefore act in both an autocrine and paracrine fashion. Expression of this growth factor is uncommon in lines of breast cancer cells⁸ and, as we have shown, in breast carcinomas but high in normal tissue and benign tumours of the breast. This may indicate that the main site of synthesis of these transcripts is the stroma rather than the tumour itself.

These findings pose several important questions. Firstly, the site of production of these growth factors must be identified; breast tumour tissue contains both normal and tumour cells, and therefore growth factors may not necessarily be synthesised by the carcinoma cells. Studies currently under way, of in situ hybridisation, a technique in which the radioactive complementary DNA probe is hybridised directly with messenger RNA in histological sections, may help to resolve this problem. Another important question is the influence of the menstrual cycle on the expression of growth factor genes. We have shown that expression of oestrogen receptor is switched off in the luteal phase of the menstrual cycle,³² and serum hormones may therefore regulate epidermal growth factor receptor and transforming growth factor α or expression of other growth factors in normal breast cells. Other studies carried out in vitro suggest that antioestrogens may exert their effects on tumour

growth through modulating the synthesis of growth factors.⁷ We are currently determining whether this occurs in vivo by examining the amounts of growth factor transcripts in sequential biopsy specimens from patients having endocrine treatment. Finally, by studying more patients a single growth factor or profile of growth factors in the primary breast cancer may possibly be identified that can predict the clinical course of the disease and the likely response to treatment.

We thank R Derynck, A Ullrich, and Genentech Inc for the transforming growth factor α , transforming growth factor β , epidermal growth factor receptor, and insulin-like growth factor II complementary DNA probes; and R Dickson and C H Heldin for the c-sis and platelet derived growth factor chain A complementary DNA probes, respectively. We thank Alison Lundie and Elaine Morris for their help in preparing the manuscript.

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(Accepted 18 January 1988)