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The Effect of Endocrine Therapy on Fibroblast Growth Factor-like Activity in Nitrosomethylurea-induced Rat Mammary Tumours

Janet Smith, Surinder K. Chander, Robin Baillie and R. Charles Coombes

Tumour regression following ovariectomy of rats bearing nitrosomethylurea-induced mammary tumours has been well characterised as a model for oestrogen receptor (ER)-positive breast cancer. We have shown that a similar regression response can be induced in these rats by the cytotoxic drug doxorubicin. Conditioned medium (CM) from serum-free explant cultures of the mammary tumours of ovariectomised rats showed a striking increase in its ability to transform NR6 cells compared to that of control or doxorubicin-treated rats ($P = 0.001$, t-test). Activity was also present in CM derived from rat uteri but not in ER-negative tissues such as skin and liver. Activity was further defined as fibroblast growth factor (FGF)-like by its strong affinity to heparin, partial neutralisation by antibodies to acidic FGF (aFGF) and partial co-elution with aFGF on salt elution from heparin. Both aFGF protein and mRNA were detected in tissue preparations of rat tumours and uterus.
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INTRODUCTION

ENDOCRINE MANIPULATION has long been successfully used as a treatment for human breast cancer [1]. The rat nitrosomethylurea (NMU)-induced mammary carcinoma has been extensively used as a model for anti-oestrogen-responsive breast carcinoma, and both ovariectomy and a variety of clinically useful anti-oestrogenic drugs have been shown to induce regression of these tumours [2,3]. Inhibition of oestrogen synthesis is now still the treatment of choice for the majority of breast cancer patients and many of those with metastatic disease [4].

Unfortunately, in the majority of patients endocrine therapy ultimately becomes ineffective and the cancer regrows [5]. This may be due to receptor changes or interaction with growth factors and other factors, rendering cells resistant to endocrine therapy. Cytotoxics such as doxorubicin are often also used in the treatment of breast cancer [6] and also induce tumour regression in the rat Walker mammary model [7].

The fibroblast growth factor (FGF) (of which to date there are seven members), has attracted attention in many disparate fields. Both basic (bFGF) [8] and acidic FGF (aFGF) [9] have been shown to have a wide variety of potent angiogenic effects and they may also be involved in basement membrane breakdown [10]. Both are unusual in that they code no classical signal peptide sequence, leading to speculation of a new secretion mechanism. Indeed, the main mechanism of action may be via sequestration from a basement membrane reservoir or on cell death and rupture, this latter mechanism being an obvious mechanism whereby the wound healing effects of the FGFs could be explained.

Transforming growth factors (TGF; i.e. those growth factors able to induce anchorage-independent growth of certain cell

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lines) have long been implicated in tumour formation and many, including TGF-β and epidermal growth factor (EGF) [11], platelet derived growth factor (PDGF) [12] and kFGF/hst [13] were first identified from tumorigenic tissues or as oncogenes *Int-2* [14], FGF5 [15] and FGF6 [16], although their mode of action is still not completely understood.

One of the problems of detecting such growth factors is that they are highly bioactive and often only present in very small amounts. aFGF, bFGF as well as kFGF/*hst*, but not keratinocyte growth factor (KGF) or *Int-2*, induce anchorage-independent growth of an EGF receptor-defective cell line (NR6) [17] when cultured in soft agar [18,19], whereas TGFα, TGFβ and EGF do not. The use of these cells, in combination with the use of neutralising antibodies and simple biochemical tests, is therefore a convenient and powerful initial step towards characterising FGF activity in a variety of systems.

The majority of FGF have a broad target specificity but of particular importance to breast cancer, which is primarily of epithelial origin, are several FGF which have now been shown to act on epithelial cells. These include bFGF [20] and KGF [21], a 28 kD peptide which is closely related to bFGF. kFGF has been found only in tumorigenic or undifferentiated embryonic tissues [19] and is the protein product of the *hst* oncogene. *Hst* together with *Int-2* may also be of importance in breast cancer as these genes have been found coamplified in some breast tumours [22] and *Int-2* is also the major integration site of the mouse mammary tumour virus (MMTV) [14].

MATERIALS AND METHODS

Rat model

Inbred virgin female (Ludwig/Wistar/Olac) rats treated with NMU [2] were supplied by Olac, Oxon, U.K. Rats bearing mammary tumours between 10 and 20 mm were randomised to experimental groups and tumour size was then measured regularly using vernier calipers. Two independent ovariectomy experiments (10 pairs and five pairs of rats, respectively) were carried out and animals were paired by tumour size and randomised to either ovariectomy or a sham operation (laparotomy). Because tumours are induced spontaneously in this model, tumour sizes can vary significantly from one animal to another. Animals were, therefore, paired as closely as possible according to tumour size but some variation was unavoidable. Animals were killed and autopsied in pairs when the tumour in the ovariectomised rat reached approximately 50% regression (6 days to 2 weeks) with all remaining pairs of rats being killed at 2 weeks.

In doxorubicin treatment experiments, 36 rats were randomised between three treatments: (1) ovariectomy as described above, (2) drug carrier injection and (3) active drug injection. Uterus, liver, mammary tumour, skin and blood samples were taken, and tissues frozen for mRNA and protein analysis. The remainder was dissected immediately for preparation of conditioned medium (CM).

Measurement of oestrogen receptor (ER)

ER was assessed using the dextran-coated charcoal method as described previously [18]. Data were calculated as Scatchard and Woolf plots and subjected to regression analysis. Results were expressed as fmoles ER/mg cytosol protein. Protein (for standardisation) was measured as described by Bradford [24].

Measurement of serum oestradiol and oestrone

Serum oestradiol was measured using a standard radioimmunoassay as described previously [25]. Serum oestrone was measured similarly using oestrone standards, antisera and titrated oestrone (Amersham, Bucks, U.K.) in place of oestradiol.

Preparation of CM

CM was prepared as described previously [18]. Briefly, tissue was removed from newly killed rats and was then microdissected into cubes of approximately 400 μm width which were placed (two pieces per well) into the centre 60 wells of a 96-well tissue culture plate containing 100 μl per well serum-free alpha modification of Eagles medium, supplemented only with penicillin (100 U/ml), streptomycin (0.1 mg/ml) and glutamine (2 mmol/l). Medium was collected after 24 and 72 h of incubation (37°C/5% CO_2), centrifuged (4000 rpm for 15 min at 4°C) and stored at −40°C. Serum-free controls were prepared following the same method and omitting tissue.

In order to ensure that activities measured were related to the source of tissue rather than the number of cells present or to variation in the preparation of conditioned medium, we monitored both cellularity of the source tissue and protein content after preparation of CM. Estimation of the amount of tissue used to prepare CM was achieved both by weighing and by assessing the protein content of the remaining tissue after collection of CM. For estimation of cellularity and cell type, histological sections were made by standard haemotoxylin and eosin (H&E) staining of 4 μm paraffin sections. A measure of cellularity was achieved by counting the cells in ten randomly selected fields and analysing for intergroup variability using an unpaired *t*-test. We also conducted dose–response experiments in which increasing numbers of explants were placed in a well and the activity from each assessed. We observed that at two pieces per well the activity became saturated (data not shown).

Detection of aFGF bioactivity

Growth factor standards (bovine acidic and basic FGF; British Biotechnology, Oxford, U.K.) were diluted to stock solutions of 50 ng/ml and stored in aliquots at −40°C. CM (12 μl per well) or growth factor activities (1–5 ng per well) were assayed by their ability to induce anchorage-independent growth of NR6 cells as previously described [18]. Heat treatment of CM was carried out for 3 min at 100°C, and neutralising antibodies (British Biotechnology) were incubated for 1 h at room temperature with CM before assay. Controls and conditions have been described in detail elsewhere [18]. Briefly, both aFGF and bFGF show a characteristic dose–response curve between the growth factor concentration range 0.1–4 ng and both have been shown to plateau at 4 ng. Downregulation is observed at higher concentrations (5–10 ng) of either aFGF or bFGF, with bFGF yielding a slightly higher plateau level than that of aFGF. Under the conditions described here, EGF alone does not stimulate colony formation of NR6 cells, neither does it have a synergistic effect on the transforming response of the FGF. PDGF, which is heat stable, does have a low NR6 cell transforming ability. The transforming effects of aFGF, bFGF and PDGF are all additive. TGFβ does not have any effect on colony formation either alone or in combination with either aFGF or bFGF, but when PDGF is present, colony formation is inhibited.

Immunoblotting and SDS–PAGE

Protein was extracted from frozen tissue samples of rat tumour and normal tissues, and protein concentration determined as above. Samples (200 μg per track) in loading buffer containing β-mercaptoethanol were reduced for 3 min at 100°C and loaded

on to 18% SDS–PAGE after Laemmli [26]. After electrophoresis, gels were blotted on to nitrocellulose (Hybond-C, Amersham) using a Sartorious dry blot apparatus (Sartoblot-11-S) following the method of Khyse-Anderson [27]. The nitrocellulose blot was then incubated in blocking solution [5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) = 10 mmol/l NaN_3], for 45 min at 37°C, the blotted gel being silverstained to confirm complete and even transfer. After three 5-min washes in 0.1% BSA–PBS the blot was incubated overnight in a sealed plastic bag at 4°C in primary antibody solution (mouse monoclonal anti-aFGF antibody from Upstate Biotechnology Inc., U.S.A.; or polyclonal rabbit anti bFGF, a gift from Andrew Baird, La Jolla, California, U.S.A.) at a concentration of 4 μg/ml in 0.1% BSA–PBS. Antibody staining was visualised by the avidin/biotin-peroxidase method using an ABC vector kit (Vectastains, Vector Laboratories, Peterborough, U.K.) following manufacturer's instructions using 3,3'-diaminobenzidine (0.08% $NiCl_2$ (0.04%) as substrate.

Heparin elution

Samples (1 ml of either conditioned medium, serum-free control, or acidic and basic FGF standards) were incubated with 50 μl sterile heparin-coated acrylic beads (Sigma, Poole, Dorset, U.K.) for 1 h at room temperature on a rotating shaker. The absorbed beads were then removed by centrifugation and washed four times in PBS by centrifugation (retaining the supernatants each time). The washed beads were then sequentially incubated (5 min) with increasing concentrations (0.3, 0.5, 0.9, 1.1, 1.5, 2 and 3 mol/l) of NaCl tissue culture tested (Sigma), with PBS washes inbetween each incubation. Samples were then assayed on NR6 cells in soft agar as described above.

Northern blotting

For northern analysis, total mRNA (20 μg) was denatured by glyoxylation, electrophoresed on 1% agarose gels in 10 mmol/l phosphate (pH 7) and transferred to Hybond-N membrane (Amersham) using 20× standard saline citrate (3 mol/l NaCl/0.3 mol/l sodium citrate). This was followed by standard prehybridisation and hybridisation with ^{32}P-labelled cDNA synthesised by the random primer method [28]. Rat cDNA aFGF probe was a gift from John Fiddes (California Biotechnology Inc., California, U.S.A.). Total mRNA (20 μg) was loaded to each track and blots were probed with an internal marker, glyceraldehyde phosphate dehydrogenase (GAPDH), after probing with aFGF to confirm even transfer.

RESULTS

Activation of NR6-stimulating activity by ovariectomy of rats beraing NMU tumours

In two separate experiments we obtained regression in the majority of ovariectomised tumour-bearing rats. Laparotomy of animals characteristically caused an initial decrease in tumour volume of all rats which was followed by progression so that at 2 weeks the mean tumour volume was 77% of the original size. This was still substantially greater than the mean volume (48% of original size) of tumours in the ovariectomised rat group (Table 1). ER were present in all tumour and uterine tissues and both uterus weight and mean ER were found to be reduced by 50% in ovariectomised rats compared to controls (Table 1).

Explant culture of rat tissues and analysis of CM

We found that rat-derived tissues can be microdissected into small volumes of serum-free medium and successfully maintained for up to 4 days. On explant into tissue culture wells the majority of tissues attach within 6 h of explant, and by 24 h when CM is removed, all but a very few explants were attached (these were excluded when CM was prepared). Replacement of serum-free medium after 24 h does not appear to affect attachment. Although explants were clearly well attached, very little cell outgrowth was observed by 4 days of culture. No significant differences were noted between protein estimations or cellularity of tissue used for CM preparation in the two groups (Table 2) and different batches of CM from the same tissue type showed very little variation with either of these parameters.

Table 1. Analysis of tumour regression; ovariectomy versus laparotomy

	Ovariectomy	Laparotomy
Tumour volume (mm³): start	2498 ± 429	1556 ± 20
Tumour volume (mm³): end	1201 ± 328	1198 ± 368
Number of tumours (*n*)	19	15
Mean reduction in tumour size	52%*†	23%
ER of tumour (fmol/mg protein)	28 ± 2.3†	56.7 ± 23
Uterus weight (g)	0.17 ± 0.03†	0.37 ± 0.02

*Includes three tumours which progressed; removal of these tumours from the regression data gives a mean reduction in tumour size of 60% (mean of progressing tumours was 151.8%). †Significantly different from control value (*t*-test; $P < 0.001$). No differences were found between cellularity of tumours from ovariectomised versus control rats. Uterus weight and ER of tumour and uterus were decreased by ovariectomy. Data are from two separate, paired rat, experiments. Results are expressed as mean ± S.E., unless otherwise stated. $n = 30$ rats.

Table 2. Analysis of rat tissue CM on NR6 cells

	Ovariectomy	Laparotomy
24 h CM tumour	61.1 ± 2.2	31.8 ± 2.3
24 h CM tumour (heat)	23.2 ± 0.3	27.8 ± 0.5
72 h CM tumour	64.7 ± 2.0	20.9 ± 0.9
72 h CM tumour (heat)	21.3 ± 0.7	24.0 ± 1.1
Uterus (24 h)	63.0 ± 3.8	55.1 ± 3.9
Uterus (heat)	22.1 ± 0.5	26.5 ± 0.3
Weight (g/ml cm)*	0.11	0.11
Cellularity (mm²)	63.4 ± 14	67.8 ± 13
Liver 24 h	9.8 ± 1.3	6.6 ± 0.4
Liver 72 h	27.8 ± 0.9	25.7 ± 0.7
Skin 24 h	19.8 ± 1.7	22.2 ± 2.3
Skin 72 h	19.0 ± 2.5	21.2 ± 1.1
Controls		
Serum-free control	24.7 ± 2.7	
1 ng aFGF	48.8 ± 1.8	
Normal mammary gland	26.4 ± 2.5	

Analysis of conditioned medium from rat tumour and non-tumorigenic tissues shows that with the exception of liver, incubation for 24 h or 72 h does not affect the result. Only rat CM from uterus and tumour tissues showed activity. Data expressed as mean ± S.E. of three separate experiments. *Mean weight of tissue used for CM preparation.

Assay of CM on NR6 cells in agar (Fig. 1) revealed a dramatic increase in the activity of CM prepared from tumour tissue of ovariectomised rats, when compared to that of control operated rats, which released barely detectable levels of NR6 transforming activity (P = <0.001, one-tailed t-test). CM from the uterine tissue of both control and ovariectomised rats were also found to transform NR6 cells, and this was slightly higher in the uterine tissue of ovariectomised rats (P = 0.001, one tailed t-test). Normal rat mammary gland CM did not stimulate NR6 cell colony formation (Table 2).

FGF-like activities continue to be released by rat uterine and ovariectomised rat tumour up to 3 days after removal of tissue into serum-free medium (Table 2) and all activities were shown to be heat labile (Table 2), suggesting that the activity was due to one (or more) of the FGF. With the exception of liver CM, which was apparently toxic at 24 h, no differences were observed between the activities of 72-h and 24-h CM. Tissues derived from non-endocrine responsive tissues such as skin and liver did not produce FGF-like activities in either control or ovariectomised rats (Table 2).

Doxorubicin administration induces regression of NMU-induced rat tumours but does not activate high levels of heat labile NR6-stimulating activity in regressing tumours

Administration of the cytotoxic drug doxorubicin also induced regression in the majority of rat tumours (11/12) by an average of 50%, whereas untreated (carrier injected) tumours continued to progress (10/12), and after 4 weeks had increased in volume by 50–100% (Fig. 2). Kruskal–Wallis analysis of tumour size and volume showed that all experimental groups were comparable (P = 0.24) and that the regression induced in these tumours by doxorubicin was comparable to that induced by ovariectomy. In those animals tested, serum oestrone and serum oestradiol were reduced in ovariectomised animals by approximately 2-fold and 4-fold, respectively (Table 3a) when compared to controls, while doxorubicin-treated rats had similar levels to controls. Cellularity was slightly greater in control tumours as opposed to regressing tumours, as also was the protein content of material used to prepare CM. However, cellularity and protein content of CM tumour pieces was comparable between tumours of ovariectomised and doxorubicin-treated rats (Table 3b).

Heat-labile NR6 colony-stimulating activity(s) were released from uterine tissue of control, ovariectomised and doxorubicin-treated rats but not in statistically significant amounts. Levels of FGF-like activity in tumours from doxorubicin-treated rats were significantly lower than in ovariectomised rat tumours (P > 0.001) although significantly greater than controls (P = 0.001).

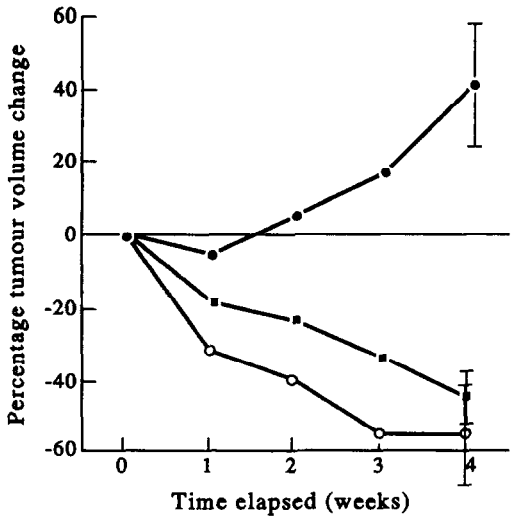

Fig. 2. Percentage change in volume of control (●), doxorubicin-treated (■) or ovariectomised (○) NMU-induced rat mammary tumours. Each point represents the mean of 12 rats. Error bars are S.E. of the mean. Tumour volume was calculated from two perpendicular diameter measurements using the formula: $6\pi (d_1 \times d_2)^{3/2}$ = volume.

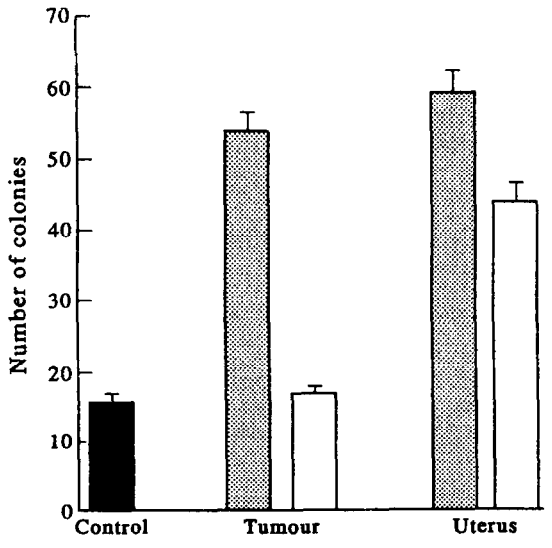

Fig. 1. Stimulation of anchorage-independent growth (AIG) of NR6 cells by 24-h CM demonstrates the presence of FGF-like activity in ovariectomised tumours (▩) but not control rat tumours (□). Control (■) serum-free CM gave a base line stimulation of 10–20 colonies per well. Data represent mean ± S.E. of the mean of six separate experiments including approximately 90 estimations for each point.

Table 3. Comparison of doxorubicin with ovariectomy-induced tumour regression

	Ovariectomy	Doxorubicin	Control
a: Endogenous serum hormone levels			
Oestrone (pmol/l)	67.3 ± 4.9	117.0 ± 18.1	117.8 ± 23.3
Oestradiol (pmol/l)	32.5 ± 12.5	120.0 ± 40.7	137.0 ± 92.7
b: FGF-like activity in conditioned medium			
Tumour	50.4 ± 2.	39.6 ± 2.7	29.2 ± 1.4
n	89	91	89
Uterus	64.9 ± 3.8	70.0 ± 4.6	61.6 ± 4.0
n	60	60	60
Cellularity (mm^2)	47.0 ± 1.6	45.9 ± 1.7	51.3 ± 1.9
Protein (mg/ml cm)	9.5 ± 1.2	9.6 ± 1.4	12.9 ± 1.9

All points are mean ± S.E. NR6 cell stimulating activity in rat tumour and uterus CM derived from rats treated by ovariectomy, doxorubicin or control (carrier injected) 4 weeks after initiation of treatment. All activity was completely removed by heat treatment (100°C/3 min). n = number of estimations.

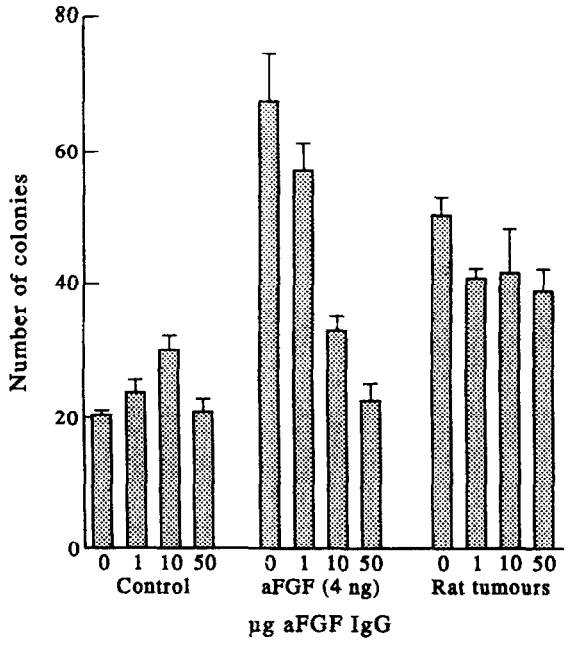

Fig. 3. Partial inhibition of rat tumour CM activity in the presence of increasing amounts (0, 1, 10, 50 μg aFGF IgG) of anti-acidic FGF neutralising antibody compared to standard. Each point represents mean ± S.E. of five replicas.

Acidic FGF in NMU-treated rats

The activity released into serum-free medium by both the uterus and the tumours of NMU-treated rats was heat labile suggesting that activity may be due to the presence of one or more FGF. CM activity of ovariectomised rat uterus and tumour was reduced but not abolished by incubation with 1 μg anti-acidic FGF IgG, and incubation of CM with 10 or 50 μg of the same antibody did not substantially further reduce the activity of this CM (Fig. 3). Slight stimulation of agar-independent NR6 colony formation was observed on addition of the antibody to serum-free medium controls but this was not seen when the highest concentration of antibody (50 μg) was added, and 50 μg of antibody was sufficient to completely abolish the activity of 4 ng aFGF standard.

The presence of aFGF mRNA in rat tumour tissues was detected using northern blotting (Fig. 4a), and in rat uterine and tumour tissues at the protein level by SDS–PAGE and immunoblotting (Fig. 4b). Using densitometry of autoradiographed blots, we found no detectable differences between levels of aFGF mRNA in control and ovariectomised rats, and similarly at the protein level we saw no detectable difference in staining intensity of the 14.5 kDa aFGF dimer bands in either uterine or ovariectomised rat tumour tissue compared to control tumours. No other size bands were identified either by immunoblotting or by northern blotting.

Heparin binding activities

Incubation of tumour CM with heparin-coated sepharose beads and subsequent elution with increasing concentrations of NaCl demonstrated that all three groups of rat tumour produced activity(s) that bound heparin (Fig. 5) and that, in our hands, commercially obtained acidic and basic FGF elute at 0.9–1.1 mol/l NaCl and 1.5 mol/l NaCl, respectively (Fig. 5a). However, the heparin binding activity of CM from control (progressing) tumours (Fig. 5b) was largely eluted from heparin with low (0.5 mol/l NaCl) salt concentration suggesting that this activity was largely due to PDGF activity, although residual activity was present in the 0.9–1.1 mol/l NaCl fractions corresponding to aFGF. Doxorubicin-treated rat tumour CM (Fig. 5c) was found to have low levels of activity at 0.9–1.1 mol/l NaCl corresponding to aFGF and also some activity at 2.0 mol/l NaCl which is more strongly binding than bFGF. As expected, CM from ovariectomised rats (Fig. 5d) had the highest activity with a peak activity co-eluting with aFGF at 0.9–1.1 mol/l NaCl and an additional peak of activity eluting at 2 mol/l NaCl.

DISCUSSION

Many growth factors have been identified as being released by cancer cell lines and have been identified in breast tumours, although the FGF have been much less extensively considered than others.

We have maintained rat tissue explants in serum-free medium for up to 4 days and have analysed this 'conditioned' medium for the presence of heparin binding growth factor activities, finding differences between the tumours of control, and ovariectomised rats and those treated with a cytotoxic drug. The short

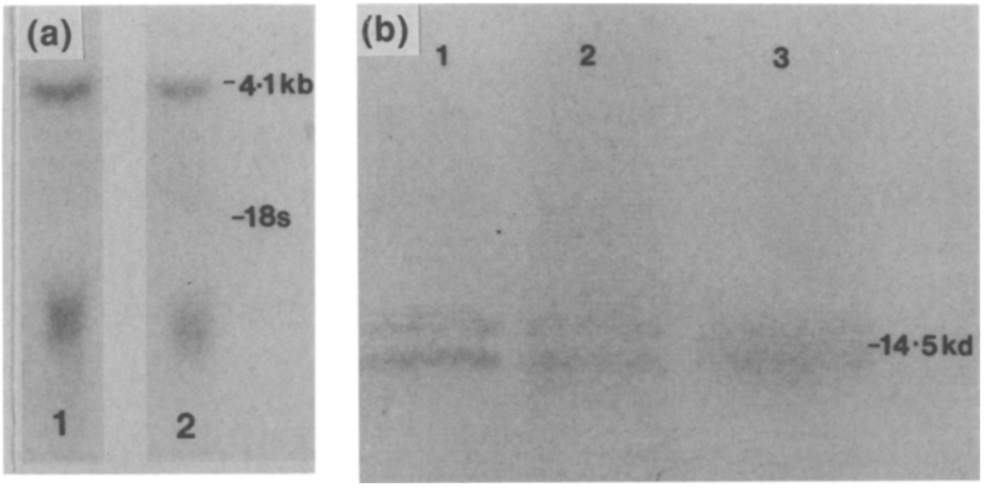

Fig. 4. (a) Hybridisation of rat tumour tmRNA to rat aFGF cDNA in tumours of sham operated (track 1) and ovariectomised (track 2) rat. (b) Immunoblotting of 18% SDS–PAGE gel showing aFGF 14.5 kd doublet in ovariectomised rat tumour (track 1), laparotomised rat tumour (track 2) and uterus (ovariectomised rat) track 3 tissue.

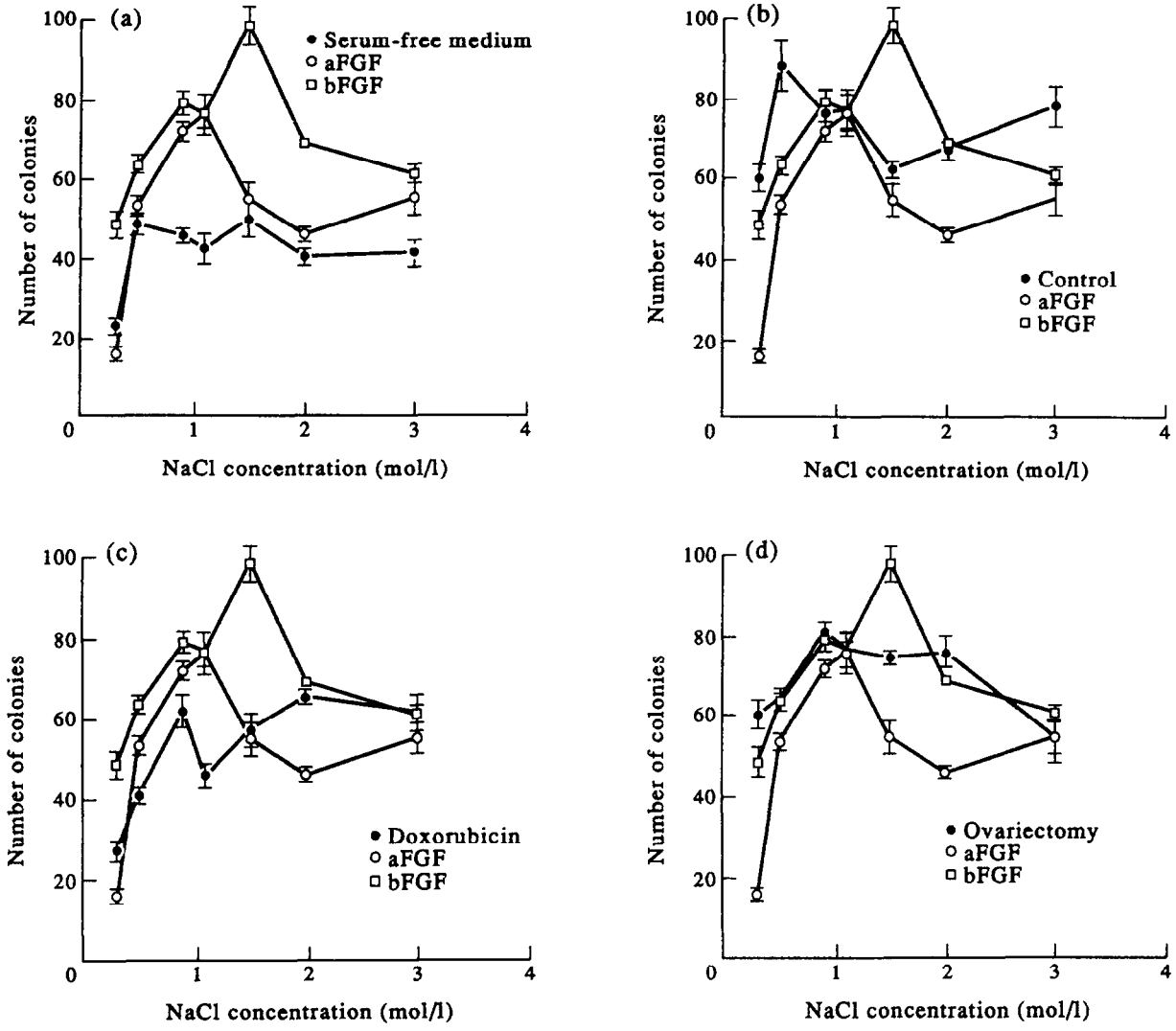

Fig. 5. Sodium chloride elution of heparin bound activity from (a) FGF standards aFGF (○) and bFGF (□) and serum-free control (●); (b) control (progressing) rat tumour CM (●) acidic and basic FGF standards as above; (c) doxorubicin-treated rat tumour CM (●) and standards as above; (d) ovariectomised rat tumour (CM) (●) and standards as above.

time interval and the undissociated nature of these explants means that the composition of growth factors released should closely correspond to the environment of these cells *in vivo*. Although it is likely that cutting of tissue will cause cell rupture and release of intercellular growth factor activities, as will the presence of necrotic cells, such effects appear to be minimal. One explanation for this is that tissues are dissected in petri dishes in quite large volumes (5–6 ml) of serum-free medium thus "washing off" growth factors produced by cell rupture before explants are placed into culture.

Using aFGF antibodies and heparin elution, we have found that a component of the heat labile activity released by ovariectomised tumours is due to the presence of aFGF. We have identified both protein and mRNA in all tumour tissues showing that aFGF is actively synthesised and processed in NMU mammary carcinomas. The differences in CM activity could be partially explained by an increase in release of aFGF by tumours of ovariectomised rats, but heparin profiles of control rat tumours reveal that aFGF may also be released by these tumours, and this may be masked by the joint presence of PDGF and TGFβ (progressing rat tumours release high levels of active TGFβ into their CM [29]) which would suppress colony formation of NR6 cells. However, it must be concluded that an additional factor is present in the tumours of ovariectomised rats. This activity has a high affinity for heparin (eluting at 2.0 mol/l salt), is heat labile and is not abolished by bFGF antibodies. We have found a similar activity in the CM of a subset of human breast cancers (J. Smith, unpublished) and are presently concentrating our efforts on further identifying this factor. The most likely explanation is that it is one of the signal peptides coding FGF such as kFGF, FGF5 or FGF6, but it may also be a novel, altered or embryonic FGF form.

We found no substantial differences in cell numbers of H&E sections between either ovariectomised or control rat tumours although differentiation was noted in the tumours of ovariectomised rats. This is consistent with the findings of Stubbs *et al.* [30] who suggest that tumour control in regressing ovariectomised tumours is mediated both by cell differentiation and a reduction of mitotic index rather than cellularity changes. Their observation that secretion of the heparin-rich basement membrane was increased in ovariectomised tumours provides a mechanism whereby action of increased levels of FGF could be mediated. On the other hand, the mechanism by which tumour regression is induced in doxorubicin-treated rats is mediated

largely, although not entirely, through cell death. Release of low levels of FGF activities in these tumours may, therefore, be a result either of cell disruption or may indicate less extensive changes in the secretion or sequestration of FGF similar to those seen in tumours of ovariectomised animals in response to or as a result of tumour regression. Analysis of rat tumours induced to regress by treatment with anti-oestrogens such as tamoxifen (unpublished data) suggests that the rise in FGF activities in regressing tumours is largely a response to changes in the endocrine environment of the tumour which is likely to lead to tumour differentitation, and this is under further investigation. The role of increased levels of growth factors in tumours is difficult to predict. Many tumours show similar phenomena, often with no obvious pattern or mode of action. Growth factor perturbations may often be the result of changes in the local cellular environment during tumorigenesis and it is these combined effects which are of ultimate importance. The alteration of FGF activities in response to endocrine changes, whether a result or cause of tumour differentiation, provides a tool with which to examine the process by which endocrine therapy is effective in breast cancers and may reveal the mechanisms by which some of these cancers are rendered resistant to such treatment.

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