Technique for the primary culture of human breast cancer cells and measurement of their prostaglandin secretion

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(Received 20 December 1991/28 April 1992; accepted 18 May 1992)

1. A method is described for the primary culture of human breast tumour cells on feeder layers of the STO mouse embryo fibroblast cell line.
2. The secretion of the prostaglandins E₂ and F₂α from the cells was measured and the results indicate that the secretion of both prostaglandins was dependent on oestrogen-receptor status, with cells from oestrogen-receptor-positive tumours secreting significantly more prostaglandin than cells from oestrogen-receptor-negative tumours.
3. Prostaglandin E₂, but not prostaglandin F₂α, secretion was also significantly greater from cells of tumours from postmenopausal women than from cells of tumours from premenopausal women. Small (<3 cm) tumours secreted significantly more prostaglandin than larger (>3 cm) tumours, and increased levels of prostaglandin were secreted with advancing clinical stage (T₁-T₄).
4. Additional evidence for increased prostaglandin metabolism in oestrogen-receptor-positive tumours compared with oestrogen-receptor-negative tumours was obtained from studies on the uptake of [¹⁴C]arachidonic acid from the cultures. Significantly more labelled arachidonic acid was incorporated into cells from oestrogen-receptor-positive tumours compared with oestrogen-receptor-negative tumours, with the subsequent release of more prostaglandin in response to various stimuli.

INTRODUCTION

It is now well established that tumours can produce greater amounts of prostaglandins (PGs) or PG-like material than the tissues in which they occur [1-4]. The significance of this increased PG secretion, however, remains obscure and there are conflicting reports as to whether these increased levels of PG correlate with other prognostic factors. Poor prognosis in patients with elevated PG levels was first reported by Bennett et al. [5], but from further studies by the same authors [3] it now appears that PGs may produce both undesirable and desirable effects in malignant disease, and that elevated levels of PG-like material in tumours are not necessarily bad, whilst low levels are not necessarily good. Correlation between PGs and favourable prognostic indices have been reported by numerous authors [4, 6-9], whereas no relationship between PGs and established prognostic factors has been reported by others [10, 11]. It is possible, however, that the above discrepancies are attributable more to the techniques and methodologies used rather than to the actual situation occurring in vivo. The most commonly used biochemical prognostic factors in breast cancer are oestrogen receptor (ER) and progesterone receptor status and it is well established that hormone-dependent human breast cancer is characterized by its requirement for oestrogen [12], although the progression to hormone-independence (and subsequent patient mortality) is poorly understood. Primary breast cancer cells are difficult to grow, but in studies using human breast cancer cell lines [13] it has been demonstrated that oestrogen stimulates the growth of the cells, and the suggestion has been made that this role is as a promoter rather than an initiator [14]. The object of the present study was therefore to develop a method for the primary culture of human breast cancer cells for their obvious advantages over cell lines in order to investigate their capacity for PG synthesis and to determine whether this was affected by exogenous oestrogen.

MATERIALS AND METHODS

Tissues

Samples of tumour tissue were obtained from 87 women with early carcinoma of the breast; successful cultures were prepared in 16 premenopausal and 55 postmenopausal cases. In addition, cultures were prepared from 12 benign tumours. The tissues were obtained from the Victoria Infirmary, Glasgow, courtesy of Mr D. Smith, and were transported to...
the laboratory in chilled containers to be used within 1 h of surgery. The patients were staged clinically (T1–T4) and the tumours were grouped according to size (small <3 cm and large >3 cm).

Tissue culture

Primary cultures of human breast tumour cells were grown on feeder layers of the STO mouse embryo fibroblast cell line (a gift from Dr C. M. MacDonald, Department of Immunology, University of Strathclyde) as follows. The STO cells were grown in antibiotic-free GMEM medium composed of Glasgow modification of Eagle’s medium (GMEM × 10, 45 ml), distilled water (400 ml), foetal bovine serum (80 ml), sodium bicarbonate (7.5%, 18.75 ml to yield a final concentration of 18.5 mmol/l) and L-glutamine (2 mmol/l). When the cells reached 70% confluence, they were tested for mycoplasma infection, the medium replaced by fresh medium containing mitomycin C (10 μg/ml) and the cells incubated for 2 h at 37°C in an atmosphere of O2/CO2 (95:5). The cells were then washed, incubated for a further 24 h in medium only and finally removed by trypsinization to be added to multi-well plates at a density of 10^5 cells/cm². After a further 24 h incubation in medium only, the cells were ready for use.

The tumour cells were prepared by washing trimmed tissue with Hanks balanced salt solution (HBSS) and chopping the tissue into small pieces (1 mm) in HBSS. The chopped tissue was incubated at 37°C for 36 h in Dulbecco’s modification of Eagle’s medium (DMEM)/Ham’s medium (1:1, 10 ml) containing collagenase (200 units/ml). The epithelial cells remained as small clumps because of the important ultrastructural markers characteristic of epithelial cells in vivo. The subsequent differences in activity between cells from malignant and benign tumours confirmed that the former contained at least a high proportion of malignant cells. The enriched epithelial cells were finally collected by settlement, resuspended in culture medium based on DMEM/Ham’s medium (composition below) and transferred to individual culture wells containing STO mouse embryo fibroblast feeder cells. The DMEM/Ham’s based culture medium consisted of DMEM (40 ml), Ham’s F10 medium (40 ml), foetal bovine serum (15 ml), sodium bicarbonate (7.5%, 0.5 ml to yield a final concentration of 4.7 mmol/l), L-glutamine (2 mmol/l), penicillin and streptomycin (80 units/ml), insulin (10 μg/ml), epidermal growth factor (10 μg/ml), prolactin (1 μg/ml), hydrocortisone (0.1 μg/ml) and cholera toxin (1 μg/ml). Medium was replaced every 2 days, and after 1 week in culture, appropriate experiments were carried out with or without test substances. All cell cultures were set up in triplicate for 48 h at 37°C in a CO2 incubator. Control incubations with the STO mouse embryo fibroblast cell line were also set up at the same time. After incubation, medium was collected and rapidly frozen to −20°C to await PG analyses. Total DNA per culture well was assayed by the method of Hill & Whatley [15].

Analysis of samples

PG analysis was carried out as described previously [9] using an antibody supplied by the Pasteur Institute, Paris, for analysis of ethyl acetate-extracted samples, with final separation of bound and free prostaglandin E2 (PGE2) being achieved with dextran–charcoal. The precision of the PGE2 assay was 3.7% within assay (precision) and 5.2% between assays (reproducibility), and the detection limit was 5 pg of PGE2. Values for STO feeder cells were subtracted from values for breast cell cultures to obtain net PG synthesis. ER data were kindly provided by Dr R. E. Leake, University of Glasgow, who also obtained tissue samples at the time of surgery (for the method, see [16]).

Uptake and release of [14C]arachidonic acid

Breast tumour epithelial cells were grown as above for 1 week and then cultured for 48 h in the presence of [14C]arachidonic acid (0.1 μCi). Excess radioactive arachidonic acid was removed from the wells by several washes with balanced salt solution. Cells from three wells were then trypsinized and collected for lipid analysis and DNA assay. Fresh medium (1 ml) containing appropriate test substances (oestradiol, progesterone, indomethacin or calcium ionphore A23187) in triplicate at concentrations of 1 μg/ml were added to the remaining wells and culture was continued for another 48 h. Cells were then trypsinized and collected to await lipid and DNA analysis. Separate cultures for STO mouse embryo fibroblast feeder cells were set up as appropriate controls and values obtained from these were subtracted from those of tumour epithelial cell cultures.

Lipids were extracted and separated by t.l.c. using well-established standard techniques involving lipid extraction with chloroform/methanol (2:1), separation of lipids by t.l.c. on Kieselgel G plates with the solvent systems hexane/diethyl ether/formic acid (40:10:1, by vol.) for neutral lipids and chloroform/methanol/25% ammonia/water (60:35:5:2.5, by vol.) for phospholipids. Individual compounds were located by reference to standards, extracted from the
Prostaglandins and breast cancer

Fig. 1. PGE_2 secretion (total, less basal production by STO feeder cells) by cultured cells of benign breast tumours (A, n=12) and malignant breast tumours from premenopausal patients (B, n=15), postmenopausal patients (C, n=54), small tumours (<3 cm, D, n=31), large tumours (>3 cm, E, n=37), ER+ tumours (n=50) and ER- tumours (n=18). Bars represent mean values.

Fig. 2. PGF_2α secretion (total, less basal production by STO feeder cells) by cultured cells of benign breast tumours (A, n=12) and malignant breast tumours from premenopausal patients (B, n=15), postmenopausal patients (C, n=54), small tumours (<3 cm, D, n=31), large tumours (>3 cm, E, n=37), ER+ tumours (n=50) and ER- tumours (n=18). Bars represent mean values.

Fig. 3. PGE_2 secretion (total, less basal production by STO feeder cells) by cultured malignant breast tumour cells according to clinical stage (T_1-T_4). Bars represent mean values.

plates and their radioactivity measured in a liquid scintillation counter.

Statistical analysis

Student's two-tailed t-test for independent comparison was used to test for statistical differences in mean values between the various groups. If the criteria for conducting the t-test were not met, then the non-parametric equivalent, the Wilcoxon rank sum test, was applied to the data. In all cases P values less than 0.05 were considered significant.

RESULTS

Basal PG production by the feeder layer

The production of PGE_2 and prostaglandin F_2α (PGF_2α) by the STO mouse embryo fibroblast cells was found to be 155±33 pg 48 h⁻¹ µg⁻¹ of DNA and 103±6 pg 48 h⁻¹ µg⁻¹ of DNA, respectively. These values were reduced to 76% of the control values in the presence of the PG inhibitor indomethacin, demonstrating that minimal PG synthesis de novo occurred in feeder cells. These mean values were subtracted from the values for PG secretion by the tumour cell cultures.

PG production by tumour cells

Cells isolated from benign tumours had only marginally greater levels of PG activity (Figs. 1 and 2) than the feeder cells. Since the level of PG activity in the cultured malignant cells was considerably greater than in the benign cells, this was taken as indirect evidence that they contained a high proportion of malignant cells. The levels of secretion of PGE_2 and PGF_2α by the cultured breast cancer cells are shown in Figs. 1–4 and it is obvious that PGE_2 is the major compound secreted.
Both menopausal status and tumour size appear to be important factors for PGE₂ secretion from tumour cells and it can be seen from Fig. 1 that the levels of PGE₂ secreted by cells from postmenopausal patients (n = 54) were significantly greater (P < 0.05) than those from premenopausal patients (n = 15), whereas cells from small tumours (n = 31) produced significantly more PGE₂ (P < 0.05) than those from large tumours (n = 37). The clinical stage of the patients was also an important factor for PGE₂ synthesis (Fig. 3), with significantly increased PGE₂ secretion (P < 0.05) occurring from tumour cells with advancing stage [T₁ (n = 6) or T₂ (n = 40) versus T₄ (n = 8)].

PGF₂α secretion appeared in general to mirror that of PGE₂, although the levels secreted were considerably smaller and there were no significant differences with pre- and post-menopausal status (Fig. 2), tumour size (Fig. 2) or clinical stage (Fig. 4). There was, however, significantly increased secretion (P < 0.03) of both PGE₂ and PGF₂α from primary tumour cells of ER-positive (ER+) tumours compared with ER-negative (ER−) tumours (Figs. 1 and 2, respectively).

That all the above increases were due to PG synthesis de novo was confirmed by the fact that in the presence of indomethacin the secreted levels of PG were similar to those for STO feeder cells alone (195 versus 155 pg 48 h⁻¹ µg⁻¹ of DNA for PGE₂). The addition of either oestradiol or progesterone (1 µg/ml) to primary tumour cell cultures appeared to cause an increase in both PGE₂ and PGF₂α secretion from the ER+ tumour cells (Table 1), but only in the case of oestradiol addition to ER+ cells did the results reach significance. Some increased secretion of PG also appeared to occur from cultured cells of the ER− tumours, but in this case the increases did not attain statistical significance (Table 1). The levels of steroid used were considerably higher than those occurring in vivo [17] but are of the order commonly used in studies in vitro.

**Arachidonate metabolism in cultured cells**

Confirmation of increased PG metabolism by ER+ tumours compared with ER− tumours and benign tumours was obtained from the results of studies on the uptake and subsequent release of [¹⁴C]arachidonic acid from primary tumour cell cultures. The average total amounts of [¹⁴C]arachidonic acid incorporated into the total lipid fraction of cultured cells were 24640 ± 3268 d.p.m. 48 h⁻¹ 10 µg⁻¹ of DNA for ER+ tumours (n = 14), 14236 ± 1761 d.p.m. 48 h⁻¹ 10 µg⁻¹ of DNA for ER− tumours (n = 14) and 7663 ± 1299 d.p.m. 48 h⁻¹ 10 µg⁻¹ of DNA for benign tumours (n = 3). The majority of this [¹⁴C]arachidonic acid was incorporated into phosphatidylethanolamine and phosphatidylcholine (Table 2), where it could act as a precursor for PG synthesis. The results in Table 2 are reported as the average percentage incorporation into each compound. The addition of oestradiol or progesterone to the [¹⁴C]arachidonic acid labelled tumour cell cultures stimulated the disappearance of label from the cells (which can be interpreted as release of arachidonic acid, and therefore presumably of PG) significantly (P < 0.05) from ER+ cultured cells but not significantly from ER− cultured cells (Table 3). The calcium ionophore A23187, on the other hand, caused a significant release (P < 0.03) of arachidonic acid from both ER+ and ER− cultured cells.

**DISCUSSION**

Since the initial reports that elevated levels of PGs tended to be associated with a poor prognosis in breast cancer [5], the majority of studies in this area have been with a view to the use of PG measurements for prognosis. Such studies have mainly involved the measurement of basal levels of PGs in tumour tissue and have yielded conflicting results [1−11, 18].

Interest in the role of PGs in breast cancer arose because they are potent stimulators of bone resorption [19] and possess haemodynamic properties [20]. Thus local production of PG by tumours might aid subsequent spread. The recent results of Bennett et al. [3], however, argue against a role for PGs in bone metastasis and these authors also suggest that insufficient prostacyclin will reach the skeleton to have any effect. On the other hand, Meghji et al. [21] have demonstrated that leukotrienes, also formed from the arachidonate cascade,
Table 1. PG secretion by ER+ and ER− breast tumour cells grown in primary culture in the presence and absence of steroids (1 μg/ml, 3.6 μmol/l). Values are means ±SEM. Statistical significance: *P < 0.05 compared with control.

<table>
<thead>
<tr>
<th>Primary culture</th>
<th>Treatment</th>
<th>PGF2α secretion (pg 48h−1 μg−1 of DNA)</th>
<th>PGF2α secretion (pg 48h−1 μg−1 of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER+ tumour (n=14)</td>
<td>Control</td>
<td>2278 ± 471</td>
<td>599 ± 126</td>
</tr>
<tr>
<td></td>
<td>Oestradiol</td>
<td>3311 ± 521*</td>
<td>821 ± 211</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>2932 ± 378</td>
<td>773 ± 186</td>
</tr>
<tr>
<td>ER− tumour (n=14)</td>
<td>Control</td>
<td>1570 ± 460</td>
<td>442 ± 98</td>
</tr>
<tr>
<td></td>
<td>Oestradiol</td>
<td>2139 ± 393</td>
<td>593 ± 143</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>2209 ± 501</td>
<td>641 ± 161</td>
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</table>

Table 2. Incorporation of [14C]arachidonic acid into phospholipids and neutral lipids (as a percentage of total uptake) by primary cultures of breast tumour cells. Values in parentheses show the mean in c.p.m. 48h−1μg−1 of DNA. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol, TG, triacylglycerol; DG, diacylglycerol.

In conclusion, it is highly probable that PGs are simply one of the locally acting growth hormones triggered among the many known growth factors associated with the progression of human breast tumours [12, 13] mainly under the influence of oestrogen and which include platelet-derived growth factor, epidermal growth factor, transforming...
growth factor and insulin-like growth factor. Further study is essential to determine the exact role of the eicosanoids in this process.

REFERENCES


Table 3. Percentage decrease in [14C]arachidonic acid content in cellular lipids of primary cultured breast tumour cells after a 48 h culture period in the presence of oestradiol (1 μg/ml, 3.6 μmol/l), progesterone (1 μg/ml, 3.2 μmol/l) or A23187 (1 μg/ml, 1.9 μmol/l). Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; TG, triacylglycerol; DG, diacylglycerol. Statistical significance: *P < 0.05, †P < 0.03 compared with control.

<table>
<thead>
<tr>
<th>Cellular lipid</th>
<th>Primary culture ...</th>
<th>Decrease in [14C]arachidonic acid content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Oestradiol</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>34.3</td>
<td>48.9†</td>
</tr>
<tr>
<td>PS/PI</td>
<td>15.3</td>
<td>34.2</td>
</tr>
<tr>
<td>TG</td>
<td>35.6</td>
<td>37.4</td>
</tr>
<tr>
<td>DG</td>
<td>24.3</td>
<td>19.4</td>
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