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Short Term Organ Culture of Human Breast Tumour Tissue and its Application in Studies of Steroid Metabolism*

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Abstract—*A method of organ culture capable of maintaining explants of human breast tumours for 3 days, as evidenced by comparison of the tumour histology before and after culture, is described. In general, survival was good, although cultures of scirrhous tumours were less successful. A commercial culture medium, Ham's F10, was used without serum addition but with the addition of insulin. Tumour tissue was cultured with 4-[^{14}C]-cholesterol (11 cases), 4-[^{14}C]-pregnenolone (3 cases) and 1β,2β-[^{3}H]-testosterone (3 cases). The existence of desmolase and aromatase enzyme systems in breast tumour tissue is suggested by the separation of neutral, acidic and phenolic fractions from cholesterol and pregnenolone cultures. Metabolites have not yet been identified. Further evidence of aromatase activity is given by the formation of [^{3}H]-water from 1β,2β-[^{3}H]-testosterone.*

INTRODUCTION

ALTHOUGH steroid hormones, especially oestrogens, have been implicated in breast cancer, the mechanism of their involvement is not clear. Based on the results of endocrine therapy, two distinct types of tumour have been postulated, hormone-dependent and hormone-independent. Recently it has been suggested that the latter type may be more capable of transforming steroid precursors to oestrogens, thus freeing the tumour from dependence on circulating oestrogens and explaining the lack of success of endocrine therapy in many cases [1, 2].

Various workers have investigated the metabolism of radioactive steroids by human breast tumour tissue [1–8]. Most have used C_{19} precursors, but Abul-Hajj has reported results from experiments using pregnenolone [9]. The metabolism of cholesterol by human breast tumours has hardly been investigated, although it is the only sterol always available to the tumour tissue irrespective of the endocrine status of the patient. It seems important, therefore, that it should be investigated. Adams and Wong [5] have shown the existence of a desmolase enzyme system in breast tumour homogenates and Dao *et al.* [8], working with microsomal preparations from breast tumour, identified several C_{19} steroids as metabolites of 4-[^{14}C]-cholesterol.

The majority of studies with breast tissue have used incubation methods, either with slices or homogenates. Organ culture has not generally been used, although the method has distinct advantages. The anatomical relationship between cell types is maintained, and the physiological reactions occurring *in vivo* are more likely to be reproduced *in vitro* by intact cells [10].

Human breast tumour tissue was first successfully maintained in short term culture by Cameron and Chambers in 1937 [11], but the technique still proves difficult. Part of the difficulty arises from the fact that epithelial cells and fibroblasts are maintained in close proximity and the latter tend to oppose the growth of the former, possibly by depriving them of nutrients or hindering cell division [12, 13]. Benign breast tumours, along with medullary and intraductal carcinomas, do not usually present problems, but the scirrhous carcinoma, rich in fibroblasts, can be difficult to maintain [13] and most breast tumours are of the scirrhous type [10].

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Using a modified version of the watch-glass technique described by Fell [14], and with the histological appearance after culture as criterion, we have successfully cultured most types of tumour tissue. This may be due to the ability of the method to limit in some way the outgrowth of undifferentiated cells and to promote organization [10]. The length of time of culture, 3 days, may also be important.

This paper describes the culture procedure and some preliminary results of steroid metabolism by cultured human breast tumours.

MATERIALS AND METHODS

4-[^{14}C]-Cholesterol (54.5 mCi/mmol) and 4-[^{14}C]-pregnenolone (55 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, England, and 1β,2β-[^{3}H]-testosterone (40.0 Ci/mmol) from New England Nuclear, W. Germany. Purity was checked by thin layer chromatography on silica gelimpregnated glass fibre (ITLC, Gelman Hawksley, Northampton, England), followed by scanning in a radiochromatogram scanner (Packard, Model 2200).

Radioactivity was counted in a liquid scintillation counter (Packard Tri-carb) using Picofluor (Packard) as scintillator. Counting efficiency was 90% for [^{14}C] and 50% for [^{3}H].

Solvents (B.D.H., Poole, England) were of analytical grade and were distilled before use.

A stock solution (1 mg/ml) of insulin (Sigma London Chemical Co., Poole, England) was prepared by dissolving the hormone in 0.005 M HCl [12]. It was kept at 4°C.

Organ culture

Cultures were maintained for 3 days, the radioactive steroid being present for the final 24 hr only. A commercial culture medium (Ham's F10, Flow Laboratories, Irvine, Scotland) was used, to which was added insulin (10 μg/ml), L-glutamine (10 μg/ml) and, to avoid bacterial infection, crystamycin (Glaxo Laboratories). All procedures were carried out in a laminar flow hood using aseptic techniques.

Freshly removed breast tumour tissue, kept at 4°C, was freed of fat and obvious necrotic tissue and placed on sterile filter paper soaked in Ham's F10, resting on an upturned sterile 200 ml beaker. A glass plate (7.5 cm × 5 cm) with 3 cover slips (2.2 cm × 2.2 cm) forming a U-shape glued to it was placed over the tissue, which was thus surrounded by the cover slips. Pressure was applied to the plate and the tissue was cut by sliding a sterile skin graft knife blade (Eschmann) under the glass so that the tissue was cut uniformly to the thickness of the cover slips. Vented culture Petri plates (Flow Laboratories) were used for the cultures. Each compartment contained a stainless steel grid and 0.8 ml of culture medium. Tissue slices, prepared as described, were cut into small explants (2 mm × 2 mm × 0.8 mm) using a scalpel blade and one explant was placed on each grid. Two Petri plates were usually filled, each plate containing 25 explants. The tissue was in contact with the medium through surface tension at the grid. Culture was carried out at 37°C in a humid atmosphere of O_2/CO_2 (95/5%). A sample of tissue was placed in formalin for histological examination.

The medium was changed after 24 hr and 48 hr. The contents of cells damaged in setting up the culture were thus removed. The 48 hr replacement medium contained radioactive steroid and was prepared the day before required and left overnight at 4°C. Medium (43 ml), containing bovine serum albumin (BSA) fraction *V* (2% w/v), was added to the steroid (10 μCi) dissolved in ethanol (0.2 ml) in a sterile tube. BSA helps the steroid to remain in solution. Completeness of solution was checked by counting an aliquot for radioactivity before addition to the culture, which was continued for a further 24 hr. The tissue was then removed, washed with phosphate buffered saline (PBS), pH 7.4, and placed in formalin for histological examination. The medium was pipetted from the Petri plate chambers into the sterile bottle containing the tissue washings. The chambers and grids were also washed with PBS and the washings added to the same bottle.

Extraction and fractionation of culture medium

Protein was precipitated by adding acetone (4 vols). After filtering and washing the precipitate with acetone, the solvent was removed under reduced pressure. The aqueous solution was diluted to a known volume, adjusted to pH 1.0 with conc. HCl and extracted with diethyl ether (5 × 1 vol). The ether extract was washed with water (3 × 0.1 vol) (thereby removing phenol red present in Ham's F10 medium), dried over anhydrous sodium sulphate and evaporated to dryness. The residue was dissolved in 90% aqueous methanol (50 ml) and the solution extracted with pentane (3 × 50 ml) to remove unchanged 4-[^{14}C]-cholesterol. The aqueous methanol solution was evaporated to dryness and the residue taken up in 70% aqueous methanol (50 ml). This was extracted with *n*-hexane (3 × 50 ml) to remove other

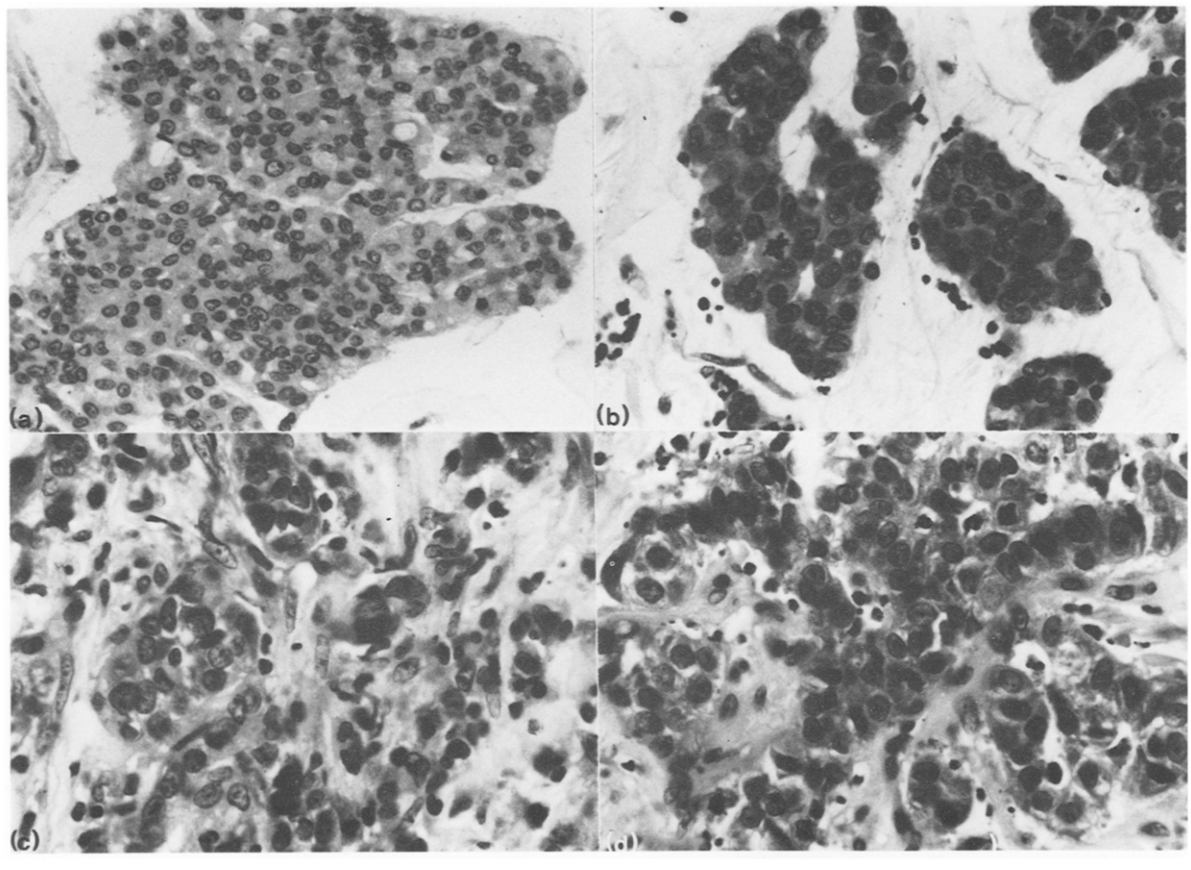

Fig. 1. *Histological appearances of breast carcinomas, stained haematoxylin and eosin.* (*a*) *and* (*b*): *the same case before and after culture respectively. A mitosis is present in the post-culture section* (× 130). (*c*) *and* (*d*): *another case before and after culture respectively, showing the good preservation of the carcinoma cells* (× 200).

sterols and lipids. The aqueous methanol solution was evaporated to dryness. The residue was dissolved in diethyl ether (50 ml) and extracted successively with concentrated carbonate solution [15] (4 × 10 ml) and 2 M NaOH (4 × 10 ml). Each total extract was backwashed with diethyl ether. The extracted ether solution was washed with water to remove alkali, dried and evaporated, giving the neutral fraction. Both the carbonate and the NaOH extracts were acidified to pH 1.0 with conc. HCl and extracted with diethyl ether (4 × 20 ml). After washing with water, drying and evaporating to dryness, acidic and phenolic fractions were obtained.

Aromatase activity

A modified version of the method of Thomson and Siiteri [16] was used to provide evidence of aromatase activity. Breast tissue was cultured as described and after 48 hr medium containing 1β,2β-[^{3}H]-testosterone was distributed between the 50 compartments of two Petri plates. After a further 24 hr, the medium was removed and extracted with chloroform (3 × 1 vol). The aqueous phase was distilled, the distillate collected and its radioactive content determined.

RESULTS

Histological details of the tumours studied before and after culture are given in Table 1. In general, the histological appearance of the cancer cells, the stroma, the lymphocyte infiltrate and the non-neoplastic breast structure, if present, is well preserved during the culture period. In every case where carcinoma is present, at least some areas of the tissue show live cancer cells in the same relation to the stroma as they had before culture. There is in all cases, however, increased evidence of cell death, revealed by pycnosis or karyorrhexis, and, in some cases, areas of massive cell death with lysis of nearly all the cells. This failure to survive in some areas is more frequent in cultures of scirrhous carcinomas. Mitotic figures in the carcinoma cells are definitely less frequent in the cultured specimens than in the original tissues. Where mitoses are present in the post-culture sections, they are also present, and in greater numbers, in the pre-culture sections; in other cases mitoses are observed before, but not after, culture. This reduced frequency of mitoses and a slight increase in the frequency of isolated necrotic cells distinguish the most successful post-culture sections from the pre-culture ones. Examples of tumour histology before and after culture are shown in Fig. 1.

The results of fractionating the medium into neutral, phenolic and acidic fractions after culture are shown in Table 2. After culture with 4-[^{14}C]-cholesterol, 80–95% of the recovered radioactivity is found in the pentane fraction (not shown) and represents unchanged cholesterol. Conversion into neutral, phenolic and acidic metabolites is suggested by the presence of radioactivity in these fractions. 4-[^{14}C]-Pregnenolone was metabolized to a greater degree, though much of the radioactivity in the neutral fraction may represent unchanged substrate. The hexane fraction (not shown) contained 30–40% of the recovered radioactivity and there was greater transformation to phenolic and acidic material than from cholesterol. In control experiments with boiled tissue and 4-[^{14}C]-cholesterol as substrate, no radioactivity was found in the neutral, phenolic or acidic fractions.

DISCUSSION

Our intention was to develop a simple culture procedure capable of maintaining human breast tumours for a short time period, thus enabling metabolic studies to be undertaken in conditions more representative of those found *in vivo* than is possible using techniques such as incubations of tissue homogenates.

The organ culture method described shows a satisfactory degree of success in maintaining explants over a 3-day period, as shown by the histological results. We believe this success to be influenced by the short culture period, the method of cutting explants, rigorous asceptic techniques and the use of insulin at levels within the physiological range as a component of the culture medium.

Explant survival is influenced by the method of preparation; a considerable degree of tissue crushing has been observed in explants prepared by mechanical means [12]. However, cut as described, we found very little evidence of crushing.

It has been claimed that addition of various components such as enzymes, co-factors, serum or hormones results in greatly improved survival of breast tissue in culture. Bearing in mind that the complexity of a medium is not necessarily related to its ability to carry out its desired function [17], we have attempted to keep our medium as simple as possible, since in any metabolic study the presence of somewhat arbitrary additives at unphysiological levels is to be avoided if the culture is to remain comparable with the *in vivo* conditions.

Insulin has been claimed to be responsible for an increase in DNA synthesis [18], for

Table 1. *Details of histological findings before and after organ culture*

Case no. and age (if known)	Histology	Before culture				After culture			
		Fibrosis	Hyperchromatism	Mitoses	Lymphocyte infiltration	Necrosis	Mitoses	Necrosis	Remarks
1 52 yr	Undifferentiated infiltrating carcinoma	++	++	++	+++	+	++	++	Good preservation: one small area of cell death
2 48 yr	Infiltrating carcinoma: modest tubule formation	+	+	+	++	±	−	+	Excellent preservation: only occasional cell death
3 47 yr	Benign dysplasia: small duct papillons	+	−	−	−	−	−	−	Appearances unchanged in culture
4 38 yr	Undifferentiated infiltrating carcinoma	+	+++	+++	++	+	−	+++	Extensive cell death, fibrous stroma and cell debris remaining: small area of normal breast tissue
5 41 yr	Loose fibrous tissue, some ducts. No carcinoma	−	−	−	−	−	−	−	Appearances unchanged
6 24 yr	Pericanalicular fibroadenoma	−	−	−	−	−	−	−	Appearances unchanged
7 —	Fat and fibrous tissue	−	−	−	−	−	−	−	Appearances unchanged
8 41 yr	Fat and fibrous tissue	−	−	−	−	−	−	−	Appearances unchanged
9 —	Undifferentiated infiltrating carcinoma	++	+++	+++	+	+	+	++	Good preservation

10 65 yr.	Infiltrating carcinoma with moderate tubule differentiation	+++	+	+	+	−	−	+	Good preservation
11 50 yr	Undifferentiated infiltrating carcinoma	++	+	−	+	−	−	++	Some areas of dead cells: other areas of live carcinoma cells
12 57 yr	Infiltrating carcinoma: slight tubule formation	+++	+	−	−	−	−	++	Most areas well preserved: cell death in some areas
13 —	An intraduct carcinoma: dilated ducts, some with sweat gland metaplasia	+	−	−	−	−	−	−	Dilated ducts well preserved: no carcinoma seen
14 33 yr	Undifferentiated infiltrating carcinoma	++	+	−	+	−	−	±	Appearances almost unchanged
15 —	Infiltrating carcinoma with slight tubules differentiation	++	+	−	+	−	−	−	Appearances almost unchanged
16 50 yr	Undifferentiated carcinoma in sheets and columns	+	++	+++	±	−	+	−	Infiltrating columns with similar appearances to before culture
17 48 yr	Undifferentiated infiltrating carcinoma	++	+	+	+++	±	−	−	Appearances almost unchanged

Table 2. Metabolism of steroids by breast tumour tissue

Substrate	Case no.	Counts in fraction as % of total counts recovered in medium after culture: Neutral	Phenolic	Acidic
	Malignant cases			
Cholesterol 4C-[^{14}C] 10 μCi	1	0.27	0.13	0.11
	2	2.5	0.27	0.49
	9	0.39	0.04	0.04
	10	1.75	0.26	0.57
	11	0.68	0.04	0.02
	12	0.34	0.04	0.06
	13	0.43	0.05	0.11
	17	0.31	0.07	0.07
	Non-malignant cases			
	3	0.3	0.11	0.14
	7	0.13	0.01	0.01
	8	0.17	0.01	0.02
Pregnenolone	Malignant case			
4C-[^{14}C] 10 μCi	4	47.0	0.41	0.14
	Non-malignant cases			
	5	52.0	0.82	0.5
	6	56.0	0.62	0.18
		DPM in aqueous as % DPM recovered in medium		
	Malignant cases			
Testosterone 1β-2β-[^{3}H] 100 μCi	14		0.54	
50 μCi	15		0.37	
25 μCi	16		0.83	
10 μCi	Control		0.27	

growth stimulation [19] and for maintaining ribosome function [20]. This has prompted its incorporation as a permanent ingredient of serum-free culture media. Wellings and Jenkoft [21], on the other hand, found no beneficial effects of its incorporation into the culture medium. In our experience, from cultures not reported here, there was evidence of very poor survival and metabolism without the addition of insulin, and it was therefore added to the culture medium.

At one time it was assumed that serum was essential for the growth and survival of mammalian cells in culture [22, 23]. Serum has been suggested to have a protective action on the tissue, possibly by acting as a physiological buffer [24], and to influence adhesion to the growth surface [25]. There are, however, several problems associated with its use [24], not the least of which is its chemical heterogeneity. The fact that so many hormones are present in serum makes its use in the present work undesirable. It has been shown that it is possible to maintain mammalian cells in short term organ culture using certain chemically defined media [26, 27]. Ham's F10 medium was used in this work, without any experimentation into alternatives, since it does not contain cholesterol. In any work involving radioactive cholesterol, the risk of isotope dilution by endogenous tissue cholesterol is high. This problem would be amplified by using a medium containing cholesterol. After culture, the tissue was washed but not extracted. Early work had shown that it retained very little radioactivity and it was felt more important to use the tissue for histological studies.

Cultures 14, 15 and 16 (Table 2) present strong independent evidence for the existence during culture of an active aromatase enzyme system in breast tumour tissue. Aromatization of 1β,2β-[^{3}H]-testosterone leads to the production of tritiated water by stereospecific tritium loss at positions 1β and 2β [28–30]. Tritium may also be lost to the water by two other processes unrelated to aromatization, namely ketoenol tautomerism, affecting the tritium at position 2β, and tritium–hydrogen exchange

between steroid and aqueous medium. The pH of the medium largely rules out the former, which only occurs to any extent under alkaline conditions, but the latter represents a genuine source of error. A control experiment was therefore performed to estimate the degree of tritium–hydrogen exchange. 1β,2β-[^{3}H]-Testosterone was dissolved in medium and left for 36 hr before being treated as in the testosterone experiments. The percentage of counts in the distillate, although high, is lower than that obtained after culture of the breast tumour tissue with 1β,2β-[^{3}H]-testosterone (Table 2) and it was concluded that aromatization took place during culture.

The results obtained from cultures of breast tumour material with cholesterol and pregnenolone are summarized in Table 2. Although the figures for the amount of radioactivity in each of the fractions are not quantitative, they are presented in order to give some idea of the amount of metabolism that has occurred. In all cases, radioactivity is present in the neutral, phenolic and acidic fractions. Despite the care taken during the partition procedures, we are aware that cross-contamination may occur, so radioactivity in the phenolic fraction may not be associated entirely with phenolic compounds. However, the demonstration of an active aromatase enzyme system in cultured breast tumours makes it reasonable to assume that some of the radioactivity in the phenolic fraction is associated with true phenolic compounds. Moreover, the purification procedure of Tetsuo *et al.* [31], which separates phenolic steroids from others, has been applied to some of the phenolic fractions; radioactivity has always been found in the fraction designated phenolic by Tetsuo *et al.* (unpublished observations). It should be remembered that cultures grown using boiled tissue gave no radioactivity in neutral, phenolic or acidic fractions.

Work is in progress to identify metabolites using mass spectroscopy and the results will be published later.

Organ culture is a valuable technique, providing as it does an environment more representative of the *in vivo* situation than is possible with other methods. Although our results on the metabolism of cholesterol by human breast tumours are not definitive, they show that this method of organ culture can usefully be applied to the study of steroid metabolism.

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