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IN VITRO METABOLISM OF TESTOSTERONE BY BREAST MICROCYSTS

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Summary—Breast microcysts are considered to be a normal finding in the adult female breast without any increased risk of developing carcinomatous change. Breast cyst fluid contains steroids but no studies have been reported on the ability of breast microcysts to metabolise steroid hormones. It was, therefore, the aim of this study to identify the metabolites formed on incubation of radiolabelled testosterone with microcysts. In all instances dihydrotestosterone and androstenedione were formed. Oestrogens were not identified. This study, therefore, provides evidence for the presence of 5α-reductase and 17-oxidoreductase enzyme systems in breast microcysts.

INTRODUCTION

Human breast microcysts, which by definition are less than 3 mm in maximum diameter, have been shown to be lined by apocrine epithelium [1]. Microcysts occur commonly and are considered to be a normal finding in the adult female breast. They do not predispose to subsequent development of breast cancer [2]. Some microcysts enlarge into macrocysts (>3 mm in diameter) which may be associated with a higher risk of breast cancer [2], although this is still the subject of much controversy. Nothing is known of the ability of microcysts to metabolise steroid hormones. In this study we investigated the ability of microcysts to metabolise testosterone *in vitro*.

EXPERIMENTAL

Samples

Five microcysts were carefully dissected from four breast excision biopsy specimens proven histologically on frozen section to be benign. The cysts were immediately frozen in liquid nitrogen and stored at −70°C prior to analysis.

Chemicals

[1,2,6,7-^{3}H]Testosterone (80 Ci/mmol) was purchased from Amersham International plc, Amersham, England, and purified just before use by thin-layer chromatography (TLC) in dichloromethane/dioxan (94:6, v/v). Ecoscint was obtained from National Diagnostics, 45 Long Plough, Aston Clinton, England. All organic solvents (AR grade) were purchased from Fisons Scientific Apparatus Ltd, Loughborough, England. All unlabelled steroids were obtained from Sigma Chemical Company, Poole, England.

Incubation and extraction

The microcysts were allowed to thaw and then sliced to ensure drainage of the cyst fluid. A 1 ml volume of 0.05 M Tris–HCl, pH 7.4, containing NADPH (1 mmol/l) was added to each microcyst in a quickfit extraction tube. Incubations were carried out at 37°C in a water bath in air with continuous shaking. After 10 min [1,2,6,7-^{3}H]testosterone (10 μCi) was added to each tube including the control tube and a 2-h incubation ensued. 200 μg of oestrone (E_1), oestradiol (E_2), testosterone (T), 5α-dihydrotestosterone (DHT) and androstenedione (A) were added as carrier steroids to each tube and mixed. The reactions were then terminated by extraction with 2 × 5 ml of diethyl ether.

The aqueous layer was frozen in a methanol/dry-ice bath and the ether layer was decanted into quickfit extraction tubes and evaporated to dryness at 40°C under nitrogen. A 5 ml volume of 90% aqueous redistilled methanol was added to the residue in each tube. Petroleum ether, 40–60°C, (5 ml) was then added to each tube and the residues partitioned between the two organic solvents. The petroleum ether layer was

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then back-partitioned with 5 ml of 90% aqueous redistilled methanol. The methanol layers for each tube were pooled together and evaporated to dryness in a vacuum desiccator.

Separation and identification of metabolites

The residues were spotted (about 0.5 cm diameter) on to precoated TLC aluminium sheets, silica gel $60F_{254}$ (Merck 5554), in 2 × 0.2 ml of diethyl ether. A set of marker steroids was spotted onto each TLC plate for the location of corresponding steroids in the sample/control. The plates were developed for 90 min in a dichloromethane dioxan (94:6, v/v) solvent system. The positions of T and A could be determined by viewing the plates under ultraviolet light. The section of each TLC plate carrying the steroid markers was cut out and the positions of the steroid markers were located by spraying with a freshly made vanillin/perchloric acid staining reagent [3] and the colour developed by heating the TLC plate on a hot plate. Bands corresponding to the positions of these steroids for the samples/control were cut out and the steroids eluted from the bands in 5 ml of diethyl ether at 4°C overnight. The subsequent steps are summarised in Fig. 1. Recoveries of the matabolites were assessed by measuring the absorbances of the samples against standard amounts of the unlabelled steroids added at 280 nm for E_1 and E_2, and 240 nm for A and T. It was not possible to assess the recovery of DHT by this method as the absorbances of TLC plate eluates are very high at the absorbance maxima (205 nm) of both DHT and its oxidised product, 5α-androstanedione (5α-dione). The average recovery obtained for A of 34.9% was, therefore, used to calculate the percentage conversion of T to DHT.

Metabolites were identified by crystallisation to constant specific activity (mean ± 5%) after the final chromatographic step in the following solvent systems:

I—redistilled methanol/distilled water;
II—iso-octane/ethyl acetate;
III—cyclohexane/acetone.

The corresponding unlabelled steroid (100 mg) was added prior to crystallisation of the metabolites of interest.

Measurement of radioactivity

Crystals were weighed on a Unimatic CL43 (Stanton Instruments, England). About 10 mg of crystals were dissolved in 0.5 ml of dioxan. Scintillation fluid (10 ml ecoscint) was then added. The vials were shaken and counted for 10 min each in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Oxidation of DHT to 5α-dione

Chromium trioxide (26.72 g) was added to 23 ml of concentrated sulphuric acid and the volume made up to 100 ml with distilled water (stock reagent). Acetone (10 ml) was added to 0.1 ml of the reagent. The sample residue was dissolved in 0.1 ml of the acetone. A 0.1 ml aliquot of the diluted reagent was then added. After 10 min at room temperature 1 ml of a freshly made solution of 5% sodium metabisulphite in 2 N sodium hydroxide was added. After a further 10 min incubation at room temperature the oxidised steroid was extracted with 5 ml of diethyl ether.

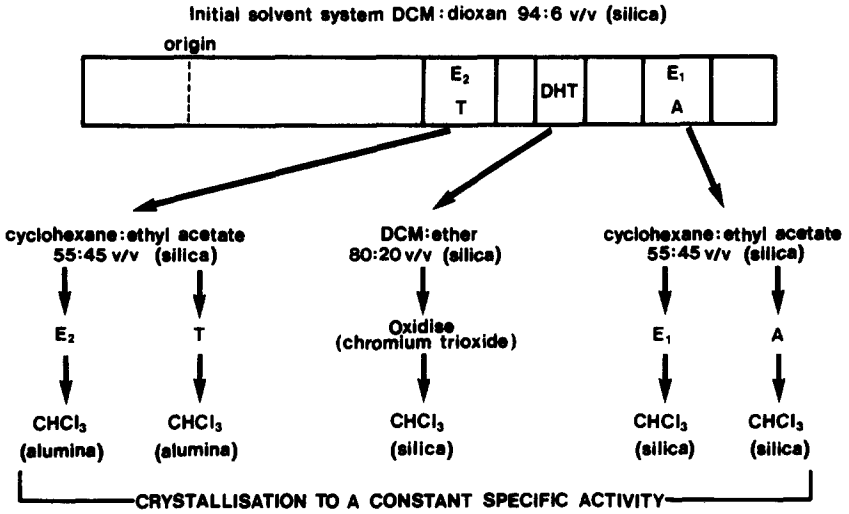

Fig. 1. Procedure for the identification of metabolites formed during incubation of radiolabelled testosterone with breast microcysts. Chloroform ($CHCl_3$), dichloromethane (DCM).

Table 1. R_f values of steroids in various solvent systems

Steroid	Solvent system: DCM:dioxan 94:6, v/v (silica)	DCM:ether 80:20, v/v (silica)	$CHCl_3$ (silica)	$CHCl_3$ (alumina)	Cyclohexane:ethyl acetate 55:45, v/v (silica)
E_1	0.82	0.87	0.31	0.48	0.58
E_2	0.45	0.58	0.14	0.22	0.42
T	0.46	0.43	0.22	0.73	0.27
DHT	0.63	0.62	0.30	0.78	0.41
A	0.83	0.77	0.42	0.92	0.36

Table 2. Percentage conversion of [1,2,6,7-^{3}H]testosterone to metabolites identified

Sample	% Conversion to A	% Conversion to DHT
1	1.34	2.98
2	1.25	0.39
3	0.55	3.82
4	0.83	7.58
5	0.44	8.62

RESULTS

The R_f values obtained for the steroids in the various solvent systems are shown in Table 1. A and DHT were identified on incubation of [1,2,6,7-^{3}H]testosterone with all five breast microcysts. The percentage conversion of T to A and of T to DHT are shown in Table 2. No oestrogens were identified in any of the incubations. Negligible conversion of T occurred in the control tube.

DISCUSSION

In this study we have demonstrated the presence of the 5α-reductase (T → DHT) and 17-oxidoreductase (T → A) enzyme systems in breast microcysts. Conversion of T to DHT and other metabolites have been shown to occur in breast cancers, benign breast disease, e.g. fibroadenomata, and in normal breast tissue *in vitro* [4–6]. It is, therefore, not surprising that microcysts have 5α-reductase activity. We have examined cyst walls from 2 breast macrocysts and found 5α-reductase activity to be present (unpublished observation). Breast cyst fluid aspirates from palpable breast cysts have been shown to contain significantly higher levels of DHT, which may be synthesised *in situ*, than in plasma [7]. The significance of the presence of high levels of this potent androgen in cyst fluid is unclear.

All 111 human breast cancers in a study by Miller *et al.* [6] were able to metabolise T to A, in addition to DHT. The proportion of T metabolised, however, varied from tumour to tumour and was found to be significantly higher in tumours with marked apocrine features than in tumours with minimal or moderate apocrine features. Microcysts, all of which are lined by apocrine epithelium [1], have been shown in this study to possess the ability to convert T to A. The enzyme responsible for this conversion, 17-oxidoreductase, was also found to be present in the two macrocysts studied, both of which were lined by apocrine epithelium (unpublished observation). It would be interesting to see if macrocysts which are lined by attenuated epithelium have much lower 17-oxidoreductase activities than macrocysts lined by apocrine epithelium.

This study suggests that some of the steroids found in breast cyst fluid may be synthesised *in situ*. No evidence for the presence of the aromatase–enzyme complex was found in this study. This does not, however, exclude the possibility that breast microcysts may possess the ability to aromatise androgens to the corresponding oestrogens.

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