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HORMONAL IMBALANCE IN BREAST CANCER

N. DESHPANDE

Department of Clinical Endocrinology, Imperial Cancer Research Fund,
Lincoln's Inn Fields, London WC2A 3PX, England

SUMMARY

The relation of the adrenal steroidogenesis in the responsiveness of patients with metastatic breast cancer to endocrine ablation was investigated using both perfusion of the gland *in situ*, and continuous peripheral infusion of pregnenolone. The results obtained by both techniques indicated that unresponsive patients converted a smaller proportion of pregnenolone to androgens relative to cortisol. Furthermore, the differences in the relative rates of synthesis were not due to abnormalities in the production or metabolic clearance rate of the precursor.

The ability of the primary breast tumours to synthesize hormones and thus become independent of the hormonal environment was investigated by both the perfusion of the human breast *in situ* and the incubation of the neoplasm *in vitro*. It was observed that the tumours were capable of converting pregnenolone and dehydroepiandrosterone to progesterone and androstenedione, respectively. Inability of these neoplasms to convert cholesterol to pregnenolone indicates that the tumours rely on a continuous supply of precursors.

INTRODUCTION

Although the role of hormones in the etiology of human breast cancer is not yet completely understood, it is generally believed that an abnormal hormonal environment may be responsible for the induction of malignancy and may also influence the clinical course of the disease. Evidence for the first of these hypotheses is inconclusive and is mainly based on epidemiological surveys [1]. Alternatively, it is possible that an abnormal endocrine environment in the pre-cancerous state enables other unknown factors to affect changes in the epithelial cell rather than itself be the cause of transformation. Endocrine function during the clinical course of the disease has been widely studied and the evidence that hormones are involved is based on the fact that surgical removal of the ovaries, adrenals and pituitary gland leads to a temporary remission in a small proportion of patients with metastatic disease. It is argued that because ablation removes the source of the secretory products of these glands, the hormones secreted by these glands were responsible for either the maintenance or growth of neoplasms.

Additional evidence for the involvement of hormones in the clinical course of human mammary cancer is based on the observation that in a small proportion of patients with metastatic disease, treatment with hormones or their synthetic analogues results in a partial or near-total remission of the known metastases for a short time. Thus, both androgens and oestrogens, together with their synthetic analogues are commonly used in the clinical management of metastatic disease. The mechanism by which additive therapy leads to a diminution of the size of metastases is not clear although it is believed that either the hormones may directly enter the neoplastic cell and alter metabolic processes or induce changes in the endocrine environment by various feed-back mechanisms.

Patients with metastatic disease fall into two broad categories, those who respond to endocrine ablation or hormonal treatment and others in whom these treatments have no effect on the tumour growth. From these observations it was postulated that in certain patients, the neoplasm is dependent on the hormonal environment and, therefore, alteration in it produces the regression. This led to vast studies in which attempts were made to detect differences in the hormonal environment of responsive and unresponsive patients by the measurement of various hormones in body fluids. The measurement of pituitary hormones in controls and early or advanced breast cancer patients have shown no differences in the plasma levels of growth hormone and prolactin [2–6]. Plasma thyroid stimulating hormone levels were raised in both early and advanced breast cancer cases and a significantly higher response to thyrotrophin-releasing hormone was reported in these patients [7]. Measurement of urinary gonadotrophins have produced inconclusive results and there is no information on plasma levels of ACTH in these patients [8]. From these studies it would appear that there are no major abnormalities associated with the pituitary in breast cancer patients as determined by the concentrations of these hormones in plasma and urine.

Measurement of steroids in plasma and urine

Between its synthesis under pituitary stimulation and excretion of its metabolites, there are a number of steps where it might be possible to detect abnormalities in the steroid hormone environment and endocrinologists have been busy exploring these for the past few decades. As in the case of pituitary hormones, the easiest way to investigate these is to measure the concentration of various steroid hormones or their metabolites in body fluids and compare them with non-cancerous controls of similar

ages, or to try to detect differences in the concentrations of these steroids between responsive and unresponsive patients.

a. Measurement of urinary and plasma oestrogens and progesterone metabolites

The measurement of urinary titres of oestrogens has produced inconclusive and contradictory results in many clinical trials [8–10]. Similarly, the measurement of oestrone and oestradiol-17β concentrations in plasmas from patients with benign breast disease, early, or advanced breast cancer and from non-cancerous controls have shown no significant differences amonst these categories (M. C. Swain, unpublished). Thus, the measurement of oestrogens in body fluids appears to be of little prognostic use in human breast cancer.

The role of progesterone, the second important secretory product of the ovary, is equally inconclusive. Using urinary pregnanediol as a unique metabolite of progesterone, it was found that the breast cancer patients excrete normal amounts of the metabolite [11, 12]. Furthermore, plasma concentrations of progesterone in benign, early, advanced and non-cancerous controls showed no significant differences (M. C. Swain, unpublished).

b. Plasma cortisol and urinary 17-hydroxycorticoids in breast cancer patients and normals

There is general agreement between various groups that plasma cortisol levels are raised in patients with metastatic disease. Thus, Schubert, Bacigalupo and Frankenberg[13] reported high resting levels which were associated with a marked response to ACTH. Deshpande, Hayward and Bulbrook[14] reported that half the advanced breast cancer patients in their series had higher total plasma 17-hydroxycorticoids and that these were not reflected in the urinary metabolites. In a further study by the same group it was reported that the high titre of plasma 17-hydroxycorticoids was due to a raised production rate of cortisol as no other factors such as plasma half-life, metabolic clearance rate or distribution of cortisol were found to affect the finding [15, 16]. Furthermore, hypophysectomy abolished the high production of cortisol indicating that the pre-operative values were dependent on pituitary function and not due to ectopic production of corticotrophin [17]. There is no conclusive evidence to show that any differences exist in the urinary titres of cortisol metabolites.

c. Androgens

Amongst the androgens, plasma testosterone measurements showed that there were no significant differences in early or advanced breast cancer patients and in non-cancerous controls [18]. Similar data on plasma androstenedione is not available. The human adrenal cortex secretes large amounts of dehydroepiandrosterone sulphate (DS) and this compound, together with androsterone sulphate (AS) is the main 17-oxosteroid in plasma. In earlier studies the measurement of these sulphate esters in plasma showed no significant differences in normal women and breast cancer patients [14, 19]. However, significant differences in these steroids were observed in early breast cancer patients between pre- and post-mastectomy samples (D. Y. Wang *et al.*, unpublished).

Use of steroid estimations in body fluids as criteria for the selection of patients for endocrine treatment

Successful attempts have been made to use groups of various steroids in the selection of patients with metastatic disease for endocrine ablation. In general, selection on the basis of measurement of urinary androgen and corticoid metabolites appear to be more successful in this respect than oestrogens [20]. The measurement of 11-deoxy-17-oxosteroids and 11-oxosteroids was first used by Allen, Hayward and Merivale[21] and expanded by Bulbrook, Greenwood and Hayward[22]. The patients who fail to respond to endocrine ablation excrete subnormal amounts of androgen metabolites relative to corticosteroid metabolites. This was further confirmed in forward studies by various workers in which the patients were selected for adrenalectomy or hypophysectomy on the basis of these measurements [20]. It is difficult to interpret the results of such studies on a simple physiological basis. The significant differences in the circulatory or excretory levels of these metabolites could be due to various factors such as,

a. Defective synthesis of precursors of these hormones;
b. Changes in the rate of conversion of precursor into the hormone;

or

c. Conversion of the hormones to metabolites not measured by the technique employed in these estimations.

The last of these hypotheses was put forward by Adams and Wong[23], who postulated conversion of dehydroepiandrosterone to the 16α-hydroxylated derivatives which were not measured in the estimation of 11-deoxy-17-oxosteroids. It appeared that the amount of dehydroepiandrosterone synthesized and secreted by the adrenal cortex was the same in both controls and cancer patients and since there is no published evidence to suggest that the human adrenal is capable of 16α-hydroxylating dehydroepiandrosterone, the site of abnormality must be elsewhere. However, the initial series is not expanded to produce conclusive evidence.

We have tested the second of these hypotheses, namely, that differences in the rate of conversion of precursors into androgens lead to the subnormal excretion of androgen metabolites by unresponsive patients, in an *in situ* perfusion of the human adrenal gland in patients with metastatic carcinoma of the

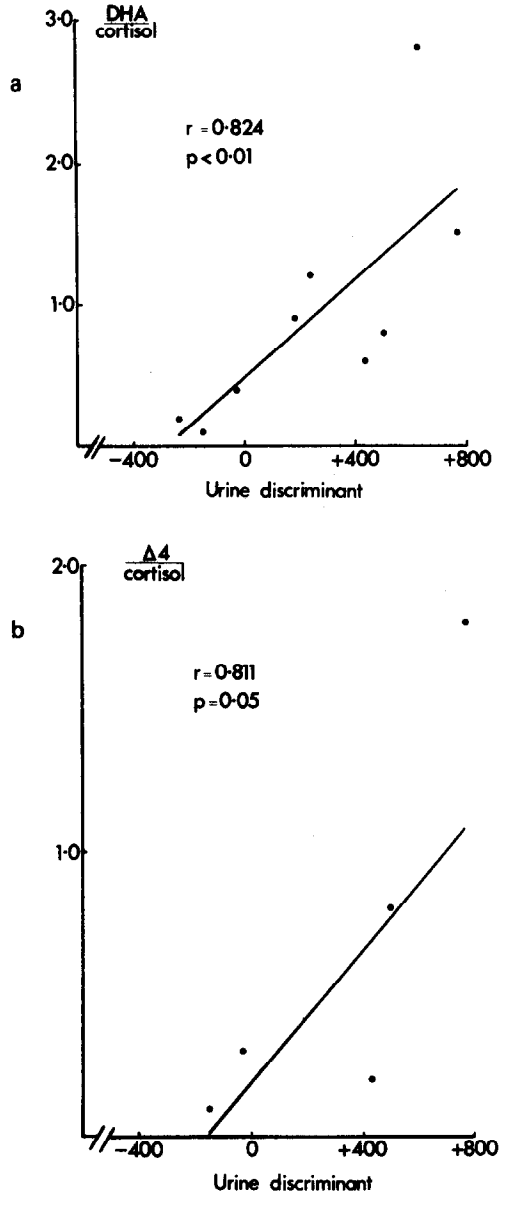

Fig. 1. The relation between the ratio of DHA/cortisol (a) and androstenedione (Δ^4)/cortisol (b) following the injection of [^{3}H]-pregnenolone into the inferior phrenic artery and urinary discriminant in patients with metastatic cancer of the breast. The urinary discriminant was estimated according to the procedure described by Bulbrook[8].

breast [24]. These patients were undergoing bilateral adrenalectomy and oophorectomy as a treatment for the disease. The anatomy of the human left adrenal gland allows one to tie most of the minor arteries, leaving either the interior phrenic or the renal artery free for injection of the precursor of androgens and cortisol. Pregnenolone acts as a common precursor of these compounds and therefore tritium-labelled pregnenolone was dissolved in a small amount of saline and injected into the artery. Following the injection of the precursor, the venous effluent was collected and from it radioactive dehydroepiandrosterone, androstenedione and cortisol were isolated. It was observed that the ratio of both dehydroepiandrosterone and androstenedione to cortisol was highly correlated with the urinary discriminant function described by Bulbrook[8].

From this series of experiments it was postulated that the patients who excrete subnormal amounts of 11-deoxy-17-oxosteroids convert a smaller amount of total precursor into androgens, relative to cortisol, and therefore the urinary abnormality was probably due to a defect in the side-chain cleavage of the C_{21} precursor of androgens.

Such studies, although useful in obtaining information regarding defective biogenesis are based on a degree of artificiality. For instance, the introduction of a radioactive precursor into the inferior phrenic artery raises the problem of incomplete mixing with the endogenous pool of the compound which is synthesized within the cell, and reliance on the isolation of radioactive metabolites for interpreting the results may be questioned. Secondly, even if one assumes that there was a complete mixing of the radioactive substrate with the endogenous pool, the study produced no evidence concerning the synthesis of pregnenolone in responsive and unresponsive patients. In order to prove that the differences observed in the synthesis of dehydroepiandrosterone and androstenedione were not related to the endogenous pool of substrate but were the result of different rates of metabolism, it would be important to obtain information regarding the production rate of pregnenolone and its interconversion to dehydroepiandrosterone and cortisol. Using the methodology devised by Horton and Tait[25] for the conversion of dehydroepiandrosterone to androstenedione and testosterone *in vivo*, the production rate of pregnenolone was estimated in early or advanced breast cancer patients and non-cancerous controls by a continuous infusion technique. The production rate was calculated as the product of the metabolic clearance rate and the plasma concentration of the compound. The results are summarised in Table 1.

There were no significant differences in the metabolic clearance rate, plasma concentration or production rate of pregnenolone in these two categories.

The percentage conversion of pregnenolone to dehydroepiandrosterone and cortisol during the continuous infusion of the compound was calculated by the isolation of these metabolites and estimation of the radioactivity associated with them. In a 3 h infusion of pregnenolone, equilibrium conditions, as judged by the constant amount of radioactivity associated with these compounds, was attained after 1 h and it was possible, therefore, to calculate the percentage conversion of pregnenolone to dehydroepiandrosterone and cortisol. These results are expressed as the ratio of radioactivity in either dehydroepiandrosterone (D/P) or cortisol (F/P) to pregnenolone. The results obtained in responsive and unresponsive patients are shown in Table 2.

It was observed that there were no significant differences between responsive and unresponsive

Table 1. The plasma concentration (ng/100 ml), metabolic clearance rate (l/day) and production rate (mg/day) of pregnenolone in early, advanced cancer patients and in non-cancerous controls

	Early	Advanced	Controls
Plasma concentration	76 ± 36 (5)	76 ± 43 (10)	112 ± 39 (3)
Metabolic clearance rate	2365 ± 290 (9)	2176 ± 369 (17)	2340 ± 187 (3)
Production rate	1·81 ± 0·83 (5)	1·70 ± 1·00 (9)	2·68 ± 1·08 (3)

Figures in brackets indicate number of patients in each category. No significant differences were observed in the three categories.

patients as far as both D/P and F/P are concerned. However, when the ratio of amounts converted to dehydroepiandrosterone and cortisol (D/F) was compared in these two categories, there was a significant difference in the ratio. The responsive patients showed a higher D/F ratio than the unresponsive, indicating that the rate of metabolism of pregnenolone to dehydroepiandrosterone relative to cortisol was higher in responsive cases. Although the differences are significant, it is to be admitted that the number of patients in each group is small at this stage.

A further hypothesis regarding the hormone responsiveness in human breast cancer concerns the role of the neoplasm itself. It is argued that the unresponsiveness of these tumours may be related to their ability to synthesize the hormones required for their growth and maintenance and therefore to be independent of the hormonal environment. This hypothesis has gained support from the publications of many groups describing the incubation of tumours with various substrates and the demonstration that the neoplasm contains enzyme systems capable of synthesizing most of the hormones normally secreted by the endocrine glands [26–30]. Furthermore, the ability of tumour tissue to sulphate androgens and oestrogens has been shown to be correlated to the recurrence after mastectomy or response to adrenalectomy [31, 32]. Since the sulphated steroids do not show any biological activity, sulphation is regarded as a de-activation mechanism by which a large pool of compound is retained for possible use.

Because the tumours can metabolize a given substrate *in vitro* does not necessarily mean that such a phenomenon takes place *in vivo*. Griffiths *et al.*[33] perfused human breast tissue during mastectomy with dehydroepiandrosterone sulphate and androstenedione and were able to detect radioactivity in dehydroepiandrosterone, androstenedione, testosterone and 5α-dihydrotestosterone. However, the anatomy of the human breast is such that it is not possible to make a complete perfusion of the breast without the injected substrate escaping into general circulation and therefore it is not certain that the metabolism occurred exclusively in the tissue. As a first step to test this hypothesis, an attempt was made to examine the biosynthetic capabilities of human breast tumours both by perfusion of the breast *in vivo* and the incubations of the neoplasm with various substrates *in vitro*.

The patients in this series were all women with primary breast cancer undergoing mastectomy. The breast was infused according to the technique described by Griffiths *et al.*[33]. The tumours removed during mastectomy were immediately frozen on cardice and remained frozen until they were incubated. The tumours were thawed, the surrounding fat and connecting tissues were removed. The tumours were weighed and homogenized in 0·25 M sucrose to a concentration of 100 mg/ml. The homogenate was centrifuged at 4°C (800 *g* for 15 min) and the supernatant was used as a source of enzymes. Instead of following the usual practice of incubating a substrate and identifying its metabolites, it was decided to look for the presence of a particular enzyme system in the neoplasm by incubating the precursor and isolating the metabolite. The same approach was adopted in perfusion studies *in vivo*. Attempts were made to identify the following enzyme systems:

1. *NADPH Dependent systems*

(a) 20–22R lyase (cholesterol to pregnenolone); (b)

Table 2. The ratios of dehydroepiandrosterone (D/P), cortisol (F/P) to pregnenolone and dehydroepiandrosterone to cortisol in responsive and unresponsive patients

D/P		F/P		D/F	
Responsive	Unresponsive	Responsive	Unresponsive	Responsive	Unresponsive
0·16 ± 0·07 (4)	0·10 ± 0·05 (6)	0·22 ± 0·17	0·25 ± 0·11	0·91 ± 0·3	0·41 ± 0·20 $P < 0·02$

No significant differences were observed in D/P and F/P in the two categories. The ratio D/F was significantly higher in responsive patients. Figures in brackets indicate the number of patients in each group.

Table 3. Isolation of progesterone after: (a) Infusion of the human breast with [^{3}H]-pregnenolone; and (b) incubation of breast tumours *in vitro*

Patient	(a) Peripheral blood (d.p.m./ml)	(a) Mammary blood (d.p.m./ml)	Tumour (d.p.m./gm)	(b) Moles x 10^{-7}/gm	(c) Moles x 10^{-7}/gm
1	—	402	302	2·48	2·38
2	—	2460	252	3·17	4·14

Column (c) represents the conversion of [^{14}C]-dehydroepiandrosterone to androstenedione by human breast tumours *in vitro*. The compounds were identified by paper chromatography and crystallization to constant specific activities.

17–20 lyase (17α-hydroxypregnenolone to DHA and 17α-hydroxyprogesterone to androstenedione); (c) aromatising enzyme (androsteronedione→oestrone and testosterone→oestradiol); and (d) 16α-hydroxylase (oestradiol→oestriol).

2. *NAD^+-Linked systems*

3β-hydroxysteroid dehydrogenase, Δ^{5-4}-isomerase (pregnenolone→progesterone and DHA→androstenedione).

Supernatant (800 *g*), (100 mg tissue) was incubated in duplicate in an 0·15 M phosphate buffer (pH 7·0 and 8·0) for 30 min with appropriate blanks in which no tissue was added. Appropriate co-factors, NADPH or NAD^+ were added at the beginning of the incubation and twice more at 10 min intervals. The reaction was stopped by the addition of alcohol and the steroids were separated by paper chromatography.

There was no conversion of cholesterol to pregnenolone, 17α-hydroxypregnenolone to dehydroepiandrosterone, 17α-hydroxyprogesterone to androstenedione, androstenedione to oestrone, testosterone to oestradiol or oestradiol to oestriol *in vitro*. The same substrates also failed to show any metabolism in perfusion of the human breast *in vivo*. Two NAD^+-linked 3β-hydroxysteroid dehydrogenase complexes (pregnenolone→progesterone and dehydroepiandrosterone to androstenedione) were detected both *in vivo* and *in vitro*. The results are summarized in Table 3.

Failure to observe the presence of 20–22R-lyase in the neoplasms indicates that, unlike endocrine glands, the breast tumours are incapable of initiating steroid hormone synthesis and are therefore dependent on a continuous supply of precursors. However, the presence of the 3β-hydroxysteroid dehydrogenase complex suggests that the neoplasm is capable of synthesizing biologically active hormones such as progesterone and androstenedione thus giving it a para-endocrine status. No evidence was found to suggest that oestrogen synthesis or metabolism takes place in the tumour.

Since a single enzyme complex is present in the mammary tumour, it was decided to test whether its presence or the amounts of progesterone or androstenedione synthesized by it give any indication of responsiveness or otherwise. In a series of 30 patients, we have studied the synthesis of both progesterone and androstenedione. The results are shown in Fig. 2. It is too early to analyse the data as the clinical evaluation of these cases is not complete.

In conclusion, human breast tumours have a limited biosynthetic capability and their dependence on a continuous supply of precursors, suggests that the tumour plays a minor role in overall steroidogenesis. Furthermore, the ability to synthesize biologically active steroids such as progesterone and androstenedione (also testosterone and dihydrostestosterone [30] indicates that these hormones may have a role in tumourgenesis which is as yet not completely understood. Studies related to effects of these hormones on both cell and organ cultures of human breast tissue have shown that testosterone and progesterone have a stimulatory effect on some of these cultures [34]. The presence of a high affinity low capacity cytoplasmic protein has been detected in human tumours for 5α-dihydrotestosterone [35], but as yet there is no conclusive evidence to suggest that a similar binding phenomenon is observed for progesterone in a majority of tumours [36]. However, it is quite possible that these endogenously synthesized hormones may affect the neoplasm without the presence of a specific binding protein.

From both, perfusion of the human adrenal gland *in situ* and continous infusion of pregnenolone in patients with metastatic disease, it is fairly clear that the abnormality associated with the subnormal excretion of androgen metabolites in unresponsive patients is related to the rate of conversion of pregnenolone to dehydroepiandrosterone. If further evidence is obtained to show that no such abnormality exists in patients with early disease prior to mastectomy or in non-cancerous controls, then it is not unreasonable to suggest that the unresponsive metasteses affect the change in steroidogenesis. Alternatively, it may be argued that these patients form a separate population and that the changes in endocrine environment in a pre-cancerous state are reflected throughout the clinical course of the disease. Attempts are now being made to show a further direct axis between the hormone unresponsive tumour and adrenal biogenesis.

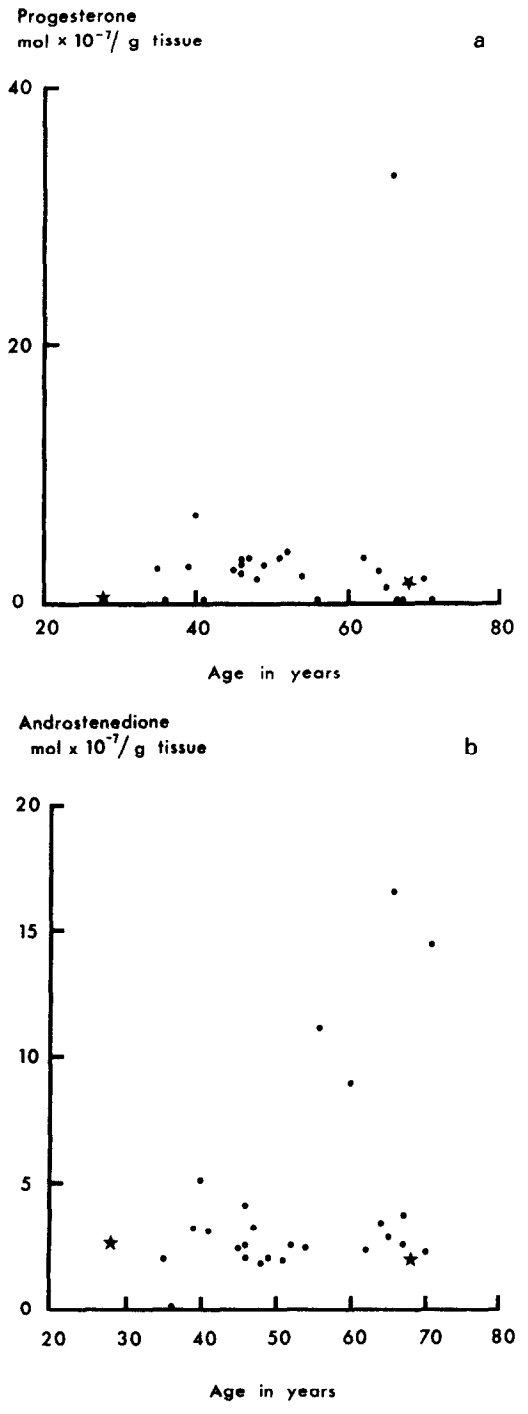

Fig. 2. Conversion of (a) pregnenolone to progesterone and (b) dehydroepiandrosterone to androstenedione by the human breast tumours *in vitro*. For incubation procedure see text. * Denotes two cases who are responding to oophorectomy.

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