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Steroid Sulphotransferase Activity in Human Hormone-independent MDA-MB-468 Mammary Cancer Cells

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The metabolism of various unconjugated oestrogens [oestrone (E_1), oestradiol (E_2) and oestriol (E_3)], in MDA-MB-468 human mammary cancer cells, a cell line characterised by the absence of oestrogen receptor has been investigated. 24 h after incubation of these cells with 5×10^{-9} mol/l of tritiated E_1, E_2, or E_3, 62–90% of the total radioactivity was localised in the sulphate fraction. Analysis showed that 73–90% of the sulphate fraction corresponds with the oestrogen incubated. The formation of the oestrogen sulphates is rapid and maximum values are found after 3 h incubation. Intense sulphotransferase activity was also found for testosterone, pregnenolone and dehydroepiandrosterone. Thus these cells contained high oestrogen sulphotransferase activity, suggesting that the presence of high levels of oestrogen sulphates in breast cancer can be synthesised in the tumour itself. In addition, the control of this enzymatic activity could open new possibilities in the knowledge of oestrogen responses and of therapeutic applications in breast cancer.
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INTRODUCTION

ACCUMULATION OF oestrogen sulphates in breast cancer tissues is of importance because a great proportion of these conjugates (e.g. oestrone sulphate) in hormone-dependent mammary cancer cell lines is converted to oestradiol [1, 2] and quantitative data show that in breast tumours oestrone sulphate 'via sulphatase' is a much more likely precursor for oestrone than is androstenedione 'via aromatase' [3].

The presence of sulphotransferases for various steroids was demonstrated in breast tumours [4, 5] and it was suggested that there is a correlation between the oestrogen sulphotransferase activity and the presence of oestrogen receptors [6]. Different authors have reported high concentrations of oestrogen sulphates in the breast cyst fluid [7] as well as in the breast cancer tissue [8] or in plasma [9]. However, at present it is not clear whether the accumulation of these conjugates could originate from the circulating oestrogen sulphates or through biosynthesis in the breast tissue itself. This paper concerns the formation of various oestrogen sulphates in the hormone-independent MDA-MB-468 mammary cancer cells [10], a cell line characterised by the absence of oestrogen receptors and whose growth is inhibited by epidermal growth factor [11].

MATERIALS AND METHODS

Cell culture and chemicals

The human mammary cancer cell line MDA-MB-468 (kindly provided by Drs M.E. Lippman and R. Dickson, Georgetown University, Washington, USA) was routinely grown in Eagle's minimal essential medium (MEM) containing 10 mmol/l HEPES supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin– streptomycin (Gibco) and 5% fetal calf serum (FCS) (Flow). The cells were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37°C, passed every 10 days and replated in 75 cm^2 flasks (C.M.L., Nemours) at 10^6 cells per flask - 3'-phosphoadenosine-5-phosphosulphate (PAPS) (Sigma).

Metabolism of different labelled oestrogens after incubation with the MDA-MB-468 cells

After 7 days of culture in 5% charcoal-treated FCS (DCC–FCS) preconfluent cells were incubated for 24 h at 37°C in MEM containing 5×10^{-9} mol/l of: [^{3}H]oestrone sulphate (E_1-S) (SA: 2.22 TBq/mmol), [^{3}H]oestrone (E_1) (SA: 2.22 TBq/mmol), [^{3}H]oestradiol (E_2) (SA: 1.57 Tbq/mmol), [^{3}H]oestriol (E_3) (SA: 3.88 TBq/mmol), [^{3}H]-dehydroepiandrosterone (SA: 740 GBq/mmol), [^{3}H]testosterone (SA: 1.92 TBq/mmol), or [^{3}H]pregnenolone (SA: 1.29 TBq/mmol); all purchased from NEN (Paris). At the end of incubation the medium was removed, the cells washed twice with ice-cold HBSS (Hank's balanced salt solution) and harvested by scraping. After centrifugation the pellet, or the culture medium, was precipitated by 80% ethanol and the radioactive material extracted for 24 h at −20°C. The cellular radioactivity uptake was determined in the ethanolic supernatant and the DNA content in the remaining pellet was evaluated according to Burton [12]. The qualitative analysis and the quantitative evaluation of the unconjugated and conjugated oestrogens were carried out after isolation by thin layer chromatography on silica gel (Merck F254) which was developed either with chloroform/ethyl acetate (4:1 v/v) for the unconjugated oestrogens, or with ethyl acetate/methanol/ammonium (75:25:2 by vol) for the separation of oestrogen sulphates. [^{3}H]oestradiol or [^{3}H]oestrone obtained from respective incubations of [^{3}H]oestrone or oestradiol or by solvolysis of the sulphate fraction were identified by crystallisation to a constant specific activity in different solvent systems using synthetic oestradiol or oestrone as carriers. [^{14}C]E_1 or [^{14}C]E_2 were used to evaluate the losses.

Analysis of the sulphate fraction

The sulphates obtained in the cells and medium after incubation with the different oestrogens were separated by TLC and submitted to solvolysis. The fraction containing the sulphates

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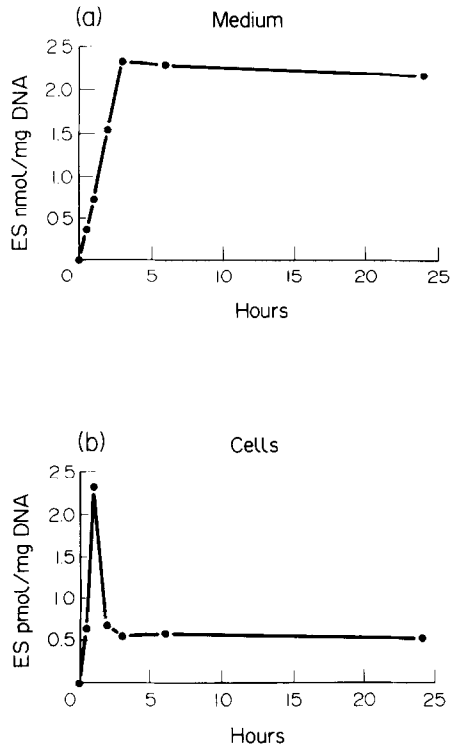

Fig. 1. Conversion of oestrone to the oestrogen sulphates (ES) in function of time by the MDA-MB-468 mammary cancer cells in the culture medium (a) and in the cells (b). Preconfluent cells were incubated with [^{3}H]oestrone (5×10^{-9} mol/l) for 0.5, 1, 2, 3, 6, and 24 h and the sulphate fractions were suspended and quantified as indicated in 'Materials and Methods'. The data represent the mean of three determinations for each time.

was dissolved in 1.8 ml 0.9% NaCl × 0.2 ml of 2 mol/l H_2SO_4, extracted with 4 ml of ethyl acetate and incubated overnight at 37°C. After neutralisation with a concentrated ammonium solution, the dry residue was dissolved in 1 ml water and extracted with 10 vol ethyl acetate:hexane (3:2, v/v) and the freed oestrogens were then analysed and quantified as indicated above. These conditions of solvolysis resulted in 90% cleavage of authentic [^{3}H]E_1S.

Estimation of sulfotransferase activity in subcellular fractionation

The MDA-MB-468 cells were resuspended in 1 ml of 0.02 mol/l Tris–HCl, 1.5 mmol/l $CaCl_2$ (pH: 7.4) solution and homogenised with a Teflon–glass Potter-Elvejhem homogeniser and centrifuged at 900 *g*. The supernatants were centrifuged at 200000 *g* for 30 min to obtain the cytosol and the mitochondrial–microsomal fractions. The pellets of the 900 *g* (the nuclear myofibrillar fraction) and that of 200000 *g* (mitochondrial–microsomal fraction) were resuspended in 0.02 mol/l Tris–HCl buffer, pH: 6.5, and sonicated. Sulphotransferase assay consisted of 100 µl enzyme sample (0.1–0.15 mg protein) and 200 µl of 0.02 mol/l Tris–HCl buffer containing [^{3}H]E_1 (5×10^{-9} mol/l). After incubation for 1 h at 37°C, the reaction was stopped by addition of 0.3 ml cold 0.1 mol/l Na_2CO_3 and the formation of E_1S was analysed in each subcellular fraction as indicated above.

Oestrogen sulphates biosynthesis in function of time and dose

The MDA-MB-468 cells were grown to confluence and incubated with 5×10^{-9} mol/l of [^{3}H]oestradiol or [^{3}H]oestrone for 0.5, 1, 2, 3, 6, and 24 h, following which the radioactive material in the cells and in the medium was analysed and quantified as indicated above. In another series of experiments, the oestrogen sulphotransferase activity was tested using a concentration range of 5×10^{-9} mol/l to 5×10^{-7} mol/l of [^{3}H]oestrone.

RESULTS

Formation of oestrogen sulphates

The biosynthesis of oestrogen sulphates after incubation of [^{3}H]oestrone (5×10^{-9} mol/l) with these cells is indicated in Fig. 1. The oestrone concentration of 5×10^{-9} mol/l was chosen because it corresponds with the levels of this oestrogen in the breast cancer tissues [8]. Figure 1(a) gives the formation of the oestrogen sulphates (in nmol/mg DNA) in the culture medium in function of time; maximal values are obtained after 3 h incubation. Figure 1(b) gives (in pmol/mg DNA) the formation of these conjugates in the cells. It can be observed that the values at 3 h of incubation (maximum formation) are 800 times less than in the culture medium and 2500 times less after 24 h incubation. Similar results are found after incubation of [^{3}H] oestradiol (Fig. 2a and b), where the concentration values of oestrogen sulphates are also very high in the culture medium, 1000–4000 times those in the cells. This very intense oestrogen sulphotransferase activity was confirmed using a concentration of E_1 100 times higher than those used previously, where after 24 h of incubation the concentration of oestrogen sulphates was 25.2 (2) nmol/mg DNA in the culture medium and 10.5 (2) pmol/mg DNA in the cells [mean (S.E.) of three experiments]. The data with 100-fold excess oestrone yielded only a 10-fold increase in oestrone sulphate in the media and a 20-fold increase in the cells, suggesting a saturation of the enzyme at that concentration.

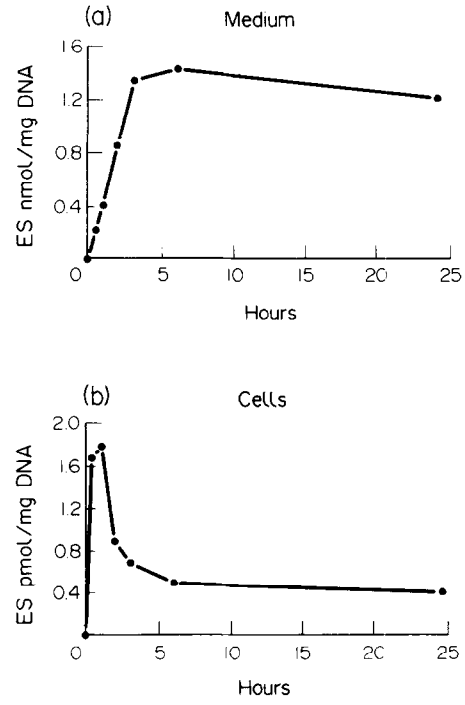

Fig. 2. Conversion of estradiol to the oestrogen sulphates (ES) in function of time by the MDA-MB-468 mammary cancer cells in the culture medium (a) and in the cells (b). Preconfluent cells were incubated with [^{3}H]oestradiol (5×10^{-9} mol/l) for 0.5, 1, 2, 3, 6, and 24 h and the sulphate fractions were separated and quantified as indicated in 'Materials and Methods'. The data represent the mean of three determinations for each time.

Table 1. Metabolism of oestrone (E_1), oestradiol (E_2), and oestrone (E_3) after incubation with MDA-MB-468 cells

Oestrogen incubated	Medium (nmol/mg DNA)				Cells (pmol/mg DNA)			
	Sulphate fraction	Unconjugated			Sulphate fraction	Unconjugated		
		E_1	E_2	E_3		E_1	E_2	E_3
[^{3}H]oestrone	1.2 (0.2)	0.25 (0.07)	ND	ND	0.55 (0.16)	1.40 (0.22)	ND	ND
[^{3}H]oestradiol	2.2 (0.3)	ND	0.4 (0.2)	ND	0.54 (0.30)	1.28 (0.34)	0.77 (0.20)	ND
[^{3}H]oestriol	1.7 (0.1)	ND	ND	0.07 (0.03)	0.10 (0.01)	ND	ND	0.32 (0.03)

Preconfluent cells were incubated for 24 h at 37°C in MEM with 5×10^{-9} mol/l [^{3}H]E_1, [^{3}H]E_2, or [^{3}H]E_3. The radioactive material was analysed and quantified as described in 'Materials and Methods'.
The data represent the mean (S.E.) of five determinations.
ND, not detectable.

Analysis of the radioactive material after incubation of MDA-MB-468 cells with unconjugated oestrone, oestradiol or oestriol

Table 1 gives the main products obtained in the culture medium and in the cells after incubation of the different labelled oestrogens (E_1, E_2, E_3). It is observed that in the medium a great percentage (72–95%) of the radioactive material corresponds with the sulphate fraction. The formation of sulphates is particularly intense with oestriol, this conjugate in the culture medium represents more than 90% of the total radioactivity and the concentration values are 14 000–18 000 times those found in the cells. Another interesting aspect is the interconversion oestrone $\rightleftharpoons$ oestradiol; in the incubation with oestrone no conversion to oestradiol in either the medium or the cells was observed, whereas in the incubation with oestradiol a significant proportion in the cells, 50–75% of the unconjugated fraction is represented by oestrone. These data agree with those obtained with other hormone-independent cell lines (MDA-MB-231, MDA-MB-436) in which the enzymatic reaction is also orientated to the formation of oestrone (unpublished data).

Analysis of the sulphate fraction after incubation with the different [^{3}H]oestrogens

Table 2 gives the percentage distribution in the culture medium of the different oestrogen sulphates after incubation of the MDA-MB-468 cells with [^{3}H]oestrone, [^{3}H]oestradiol or [^{3}H]oestriol. The data show that most (73–90%) of the oestrogen liberated after solvolysis of the sulphate fraction corresponds with the oestrogen incubated. It is to be remarked that, as is the situation for the unconjugated fraction, after incubation with E_1, in the sulphate fraction most are represented by E_1-S, but after incubation with E_2 a significant proportion (15–20%) are oestrone sulphate.

Table 2. Analysis of the sulphate fraction in the culture medium after incubation of the MDA-MB-468 cell with [^{3}H]oestrone ([^{3}H]E_1), [^{3}H]oestradiol ([^{3}H]E_2), or [^{3}H]oestriol ([^{3}H]E_3)

Oestrogen incubated	Sulphate fraction %		
	E_1	E_2	E_3
[^{3}H]E_1	75 (5)	2 (1)	ND
[^{3}H]E_2	20 (5)	73 (6)	ND
[^{3}H]E_3	ND	ND	90 (4)

The sulphate fractions were separated as indicated in 'Materials and Methods' and after solvolysis the different liberated oestrogens were analysed by TLC.
Results are expressed as the mean (S.E.) of five determinations.
ND, not detectable.

Sulphotransferase activity using other steroids

In order to explore the possible sulphotransferase activity for other steroids, a series of neutral steroids: dehydroepiandrosterone, pregnenolone, testosterone, were incubated for 24 h at a similar concentration (5×10^{-9} mol/l) to that for oestrogens. Table 3 indicates that with these neutral steroids a significant conversion into the sulphate is obtained. In the culture medium, the values in percentage account for 42–67% and the analysis (after solvolysis) of the sulphate fraction shows that more than 90% of the liberated radioactive material of this fraction corresponds with the steroid incubated.

Studies on the possible mechanism of the sulphotransferase activity and subcellular localisation

As the concentrations of the oestrogen sulphates are very high in the culture medium, a sample of medium which had been conditioned by exposure to cells (2–4 days), in the absence of radioactive oestrogen, was separated from the cells and used in

Table 3. Conversion of various steroids into their respective sulphates by MDA-MB-468 cells

	Medium (nmol/mg DNA)		Cells (pmol/mg DNA)	
	Sulphate fraction	Unconjugated	Sulphate fraction	Unconjugated
[^{3}H]dehydroepiandrosterone	1.20 (0.20)	1.10 (0.30)	0.39 (0.05)	1.55 (0.30)
[^{3}H]testosterone	0.30 (0.05)	0.22 (0.07)	18 (1)	7.00 (0.60)
[^{3}H]pregnenolone	0.47 (0.03)	0.09 (0.01)	55 (3)	38.00 (2.50)

Preconfluent cells were incubated for 24 h at 37°C in MEM with 5×10^{-9} mol/l [^{3}H]dehydroepiandrosterone, [^{3}H]testosterone, or [^{3}H]pregnenolone. The radioactive material was analysed and quantified as described in 'Materials and Methods'.
The data represent the mean (S.E.) of four determinations.

a 'non-cells' control incubation. This medium was incubated with 5×10^{-9} mol/l of [^{3}H]E_1 or [^{3}H]E_2 for various periods of time (1–5 h) and no formation of oestrogen sulphates was detected. Also, after addition of the cofactor necessary for the sulphate biosynthesis: 3'-phospho-adenosine-5'-phospho-sulphate no presence of oestrogen sulphates was observed, suggesting that the sulphotransferase is not secreted to the medium.

In another series of experiments, and in order to investigate the subcellular localisation of the enzyme, the MDA-MB-468 cells were homogenised and the different subcellular fractions, cytosol, mitochondria–microsomes, and nuclear pellet, were obtained as indicated in 'Materials and Methods'. After incubation of [^{3}H]E_1 (5×10^{-9} mol/l) with the different isolated subcellular fractions, the presence of oestrogen sulphates was observed in the mitochondrial–microsomal and cytosol fractions, but not in the nuclear-myofibrillar fraction. In the cytosol fraction E_1S represents 50% (12) of the total radioactive material of this fraction and in the mitochondrial–microsomal fraction E_1S accounts for 20% (5) [mean (S.E.) of four experiments].

DISCUSSION

The most striking aspect of the present data is the very high oestrogen sulphotransferase activity in the MDA-MB-468 mammary cancer cell line. The formation of the oestrogen sulphates is very rapid and in less than 3 h, 70–90% of the total radioactivity in the culture medium corresponds with the sulphate fraction. The data suggested that the high concentration of oestrogen sulphates in breast cancer [8, 9] could be formed in the tissue itself and these cells are an important source for the formation of the sulphate. Using a homogenate of human breast tumour, Raju *et al.* [4] found less than 0.6% of the conversion of oestriol to oestriol sulphate, but this can be explained by the presence in this homogenate of other cells which contain high sulphatase activity as was extensively demonstrated by various investigators [1, 2, 13–15]. Adams *et al.* [6] suggested the possibility of a correlation between the oestrogen sulphotransferase levels and the oestrogen receptor status. However, in the present study the breast cancer cell line studied contained high sulphotransferase activity and the oestrogen receptor is negative. In addition, Tseng *et al.* [5] concluded that the oestrogen sulphotransferase activity cannot be used to differentiate between hormone-dependent and -independent tumours. As it is well known that the oestrogen sulphates 'per se' are biologically inactive, the exploration of the control of this enzyme could be of importance as a possibility to block the effect of the unconjugated oestrogens.

Further interesting information provided by the present study is the very high concentration ratio: culture medium/cells of the various oestrogen sulphates, which suggests that the biosynthesised sulphates are secreted to the medium. This is supported by the fact that, when in our experimental conditions, the volume of the culture medium (10 ml) is compared with that of the total cells of one flask (0.4 ml) the ratio value is 250. As we obtained an oestrogen sulphate concentration ratio medium/cell between 2000 and 17000, we think that the cell secretes the sulphates to the medium. The present data open the question of the mechanism of the formation of sulphates, as the culture medium which was in contact with the cells is unable to produce any sulphate, suggesting that the enzyme is not secreted by the cells.

Consequently, we can assume that the unconjugated oestrogen penetrated the cells, the formation of the sulphate is very rapid and secreted to the medium. In previous studies we observed that when we incubated different mammary cancer cell lines (MCF-7, T-47D, R-27, MDA-MB-231, MDA-MB-436) with tritiated E_1S, inside the cells more than 95% of the radioactive material is represented by unconjugated oestrogens [1, 8, 16]. However, more information is necessary to elucidate the steps in the mechanism and in the subcellular area that the sulphates are formed. It is to be remarked that in the different cell lines MCF-7, T-47D, R-27, MDA-MB-231, MDA-MB-436 we explored the conversion of the unconjugated oestrogens (E_1, E_2, E_3) to the sulphates, but the formation of these conjugates was very low or negative (data not shown). Consequently, we can consider that the MDA-MB-468 cell line is very specific for the production of oestrogen sulphates in breast cancer tissue. In addition, the data showing that testosterone, pregnenolone, or dehydroepiandrosterone can be converted to the sulphates by these cells suggested that this sulphotransferase activity can be extended to other steroids.

It is concluded that these hormone-independent cells contained very high oestrogen sulphotransferase activity, suggesting that the presence of high levels of oestrogen sulphates in breast cancer can be synthesised in the tissue itself; the control of this enzymatic activity can open new possibilities in the knowledge of the oestrogen responses and in therapeutic applications in human breast cancer.

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In vitro Karyotypic and Immunophenotypic Characterisation of Primitive Neuroectodermal Tumours: Similarities to Malignant Gliomas

Mark T. Jennings, DeVeta L. Jennings, Salah A.D. Ebrahim, Mahlon D. Johnson, Claude Turc-Carel, Thierry Philip, Irene Philip, Claude Lapras and Joan R. Shapiro

Monoclonal antibody (Mab) mediated immunotherapy of brain tumours requires the identification of tumour-restricted cell surface antigens. We have characterised four primitive neuroectodermal tumours, which included pineoblastoma, medulloblastoma and ependymoblastoma cultures, that demonstrated *in vitro* evidence of malignant behaviour (anchorage-independent growth and nu/nu xenograft tumour formation). The cytogenetic findings ranged from normal G-banded and Q-banded karyotypes through mixed near-diploid/hyperdiploid. These cultures resembled the cell surface immunophenotypic spectrum of malignant gliomas. They were distinguished from normal glia *in vitro* by the expression of restricted fetal mesenchymal, neuronal, myoblastic, melanocytic, epidermal, chondrocytic, lymphoid and epithelial antigens. Certain antigens appeared sufficiently represented among central nervous system (CNS) neoplasms to afford potential targets for Mab-mediated immunotherapy.
Eur J Cancer, Vol. 28A, No. 4/5, pp. 762–766, 1992.

INTRODUCTION

LITTLE is understood of the biological determinants of central nervous system (CNS) neoplasms which are responsible for their poor prognosis. However, tumour-specific traits may be exploited for the development of novel therapies. This preliminary report seeks to correlate the cytogenetic findings, experimental growth characteristics (anchorage-independent growth, nu/nu xenograft tumour formation) and the surface immunophenotype of early passage CNS primitive neuroectodermal tumours (PNET), which include pineoblastoma (PBL), medulloblastoma (MBL) and ependymoblastoma (EPD) cultures. The purpose was to identify qualitative and quantitative differences in cell surface immunophenotype between normal glia with malignant gliomas and PNET, which may be targeted for passive immunotherapy with monoclonal antibodies (Mabs) [1, 2]. The process of inquiry has emphasised the demonstration of tumour-restricted cell surface antigenic expression with correlation to other attributes of the transformed phenotype. The tumour cell surface immunophenotype is relevant for therapeutic purposes as systemically administered Mab will probably be afforded access principally to cell surface and interstitial antigens. Previous studies have demonstrated general correspondence between *in vivo* and *in vitro* expression for many of these antigens [3, 4].

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PATIENTS AND METHODS

Derivation of cell cultures

Solid tumour cell cultures of intrinsic CNS neoplasms were generated and characterised, as previously described [1]. The specimens were obtained through the tumour procurement