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DOLICHOL-SUGAR DERIVATIVE SYNTHESIS IN HUMAN BREAST CANCER CELL LINE (T47D). EFFECTS OF ESTROGENS AND ANTIESTROGENS

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In the present paper we report evidence about the formation of polyprenyl-phosphate monosaccharides, their elongation products and the assembly of dolichyl-diphosphate-oligosaccharide to endogenous T47D clon 11 proteins upon incubation with [^{14}C]glucose. The influence of estradiol and two nonesteroidal antiestrogens -nafoxidine and tamoxifen- was examined on the dolichol pathway in T47D cell cultures. Estradiol (1 nM) does not change the rate of synthesis of dolichyl-phosphate-sugar derivatives in contrast to nafoxidine and tamoxifen both a micromolar concentration, which induce a remarkable decrease in the formation of dolichol-sugar derivatives. In addition, T47D cells were pretreated with nafoxidine or tamoxifen during one hour, fresh medium supplemented with estradiol was added to the cells simultaneously with [^{14}C]glucose. Results indicated that estradiol after nafoxidine induces a slight increase in the polyprenyl-sugar derivatives formation, however, estradiol after tamoxifen decreases the synthesis of these substances.

A considerable body of information now exists concerning the role of lipid intermediates in glycoprotein biosynthesis (1). In spite of this, little information has been provided about the regulatory mechanism of this metabolic pathway (2).

We had previously demonstrated and characterized the enzymatic conversion of UDP-Glc and GDP-Man to Dol-P-Glc and Dol-P-Man respectively in human breast neoplasia (3,4).

In the present study we have extended this investigation on the cell line T47D-c11 derived from a breast cancer patient (5). T47D-c11 contains specific intracellular receptors for several hormones including E_2 and progesterone, while clon 8, derived from the same cell line, does not (6). Indeed both clones showed different behavior in relation to polyprenyl-sugar derivatives and glycoprotein synthesis when the medium was deprived or not of endogenous steroids. Therefore, these cell clones offered an interesting model to study hormone mediated metabolic effects.

We are presenting data illustrating the participation of lipid linked sugars as intermediates in the glycoprotein synthesis in T47D-c11 cells. In addition, we have been able to demonstrate the

Abbreviations: E_2: 17β-estradiol; UDP-Glc: uridine diphosphate glucose; GDP-Man: guanosine diphosphate mannose; Dol-P: dolichyl-phosphate; T47D-c11: T47D-clon 11; T47D-c8: T47D-clon 8; FCS: fetal calf serum; Naf: nafoxidine; Tam: tamoxifen; ER: estrogen receptor; C/M/W: chloroform/methanol/water.

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inhibitory property of Naf and Tam in the various steps in the dolichol pathway, however we failed to show estrogen effects in this metabolic event.

MATERIALS AND METHODS

Chemicals. [U-^{14}C]D-Glucose (sp. activity=348.2 mCi/mmol) and [6-^{3}H(N)]D-glucosamine (sp. activity=30 Ci/mmol) were obtained from New England Nuclear Corporation. All reagents were analytical grade. 17β-estradiol was from Sigma. Nafoxidine was from Upjohn Co. Tamoxifen-citrate was kindly given by Gador Lab (Buenos Aires). All culture media and FCS were from Gibco Laboratories.
Cell culture. T47D (c11,c8) cells, provided by Dr. I. Keydar (Israel), were grown routinely as monolayer cultures in a medium consisting of RPMI 1640, 1 % non-essential aminoacids, 2 mM L-glutamine, 10^{-7}M insuline, 100 U/ml penicillin, 100 U/ml streptomycin and 10 % FCS, in a humidified atmosphere of 5 % carbon dioxide in air at 37°C. Cultures were refed with the same culture medium at intervals from 2 to 4 days; cells were subcultured at weekly intervals.
Incorporation of [^{14}C]Glc into lipid linked sugar derivatives. Four to five days before assays, growth medium was replaced for experimental medium consisting of the same one except that 10 % FCS was stripped of endogenous steroids by treatment with dextran coated charcoal (7). One hour before the assays, the medium was changed for MEM, glucose-FCS free, supplemented with BSA 1 mg/ml (8) (deprived medium). E_2 10^{-9}M, Naf 10^{-6}M or Tam o.4 x 10^{-6}M were used as agonist or antagonist. After one hour of incubation with these agents, the cultures were continued in the presence of [^{14}C]Glc for 3 additional hours. For experiments shown in Table 3, the cells were exposed to a deprived medium during 1 hour, followed by an addition of Naf or Tam for 1 hour. After that, the medium was aspirated and a fresh one plus E_2 was added and the incubation was continued for 1 additional hour; at this time, a 3 hour pulse of [^{14}C]Glc was given. Control experiments were performed in the absence of E_2 or antagonist. All assays were carried out with subconfluent cell cultures. At the end of the incubation the cells were cooled, the medium was rapidly aspirated and the reactions were stopped with 1 ml of cold 10 % TCA; the cells were washed with cold 200 mM Tris-HCl pH 7.5 buffer. The precipitated cells were harvested with the addition of 0.3 ml of 4 mM $MgCl_2$ with the aid of a rubber policeman. The samples were homogenized in a glass-glass homogenizer and partitioned by adding chloroform and methanol to bring the preparation to a C/M/W 3:2:1 ratio, according to Folch's procedure (9). The lower phase of this partition contained the lipid bound monosaccharides and oligosaccharides up to 7-8 units (10). The resultating interphase was washed with methanol and suspended in C/M/W 10:10:3 (11), to extract polyprenyl-PP-oligosaccharides containing more than 8 hexoses (12). The residue left after the above extraction was resuspended in 10 % TCA and heated a 100°C. The insoluble matter was solubilized as described (4) and the resulting radioactivity was considered as "glycoprotein" (13).
Chromatographic methods. Microanalytical DEAE-cellulose chromatography was performed as described by Quesada (14). Thin layer chromatography was carried out on silica gel G plates (Merck) and developed in chloroform/2-propanol/95 % ethanol/glacial acetic acid 2:2:3:1 (15).
Other methods. Radioactivity was measured in a liquid scintillator using a toluene-4 % Omnifluor mixture. Protein determination was performed according to Peterson (16).

RESULTS

In order to characterize the lipid-linked sugar derivatives we carried out assays where T47D-c11 control cells and cultures supplemented with E_2 10^{-9}M or Naf 10^{-6}M were exposed during 3 hours to [^{14}C]Glc (40 μCi/60 mm plastic dish/7.5 x 10^5 cells). Under these conditions the radioactivity incorporated in the lower phase (171,000, 175,000 and 121,770 cpm/dish for control, E_2 and Naf respectively) was adequate for establishing the primary biochemical characterization. Then, an aliquot of this glycosylated fraction was applied to a DEAE-cellulose column and developed as de-

scribed (4). The radiolabeled substance was eluted by a mixture of 5 mM ammonium formate in C/M/W 10:10:3 as shown in Figure 1. No other charged compounds were eluted when the salt discontinuous gradient was increased. This chromatographic behavior is characteristic of lipid-monophosphate-sugar (11) and no differences were detected among the 3 samples assayed. Further analysis of the material eluted under the afore mentioned procedure was performed by thin layer chromatography as described. Figure 2 illustrates the radiolabeled pattern obtained. T47D-c11 compound migrates with an Rf (0.42) identical to standard human breast Dol-P-[^{14}C]Glc and further ahead than standard human breast Dol-P-[^{14}C]Man (Rf=0.31)(4). These results indicate that the radioactivity extractable in the lower phase corresponds to a large extent to polyprenyl-P-[^{14}C]Glc. However, it cannot ruled out that other sugar-lipid derivatives were also formed an minor proportion. Analysis of the physicochemical nature of the material present in the C/M/W 10:10:3 and hot TCA resistant fractions are currently evaluated.

The E_2 concentration (10^{-9}M) used in the course of these experiments was based on the Kd values described in T47D cells (5). In addition, we have proved that higher levels of E_2 (10^{-8} to 10^{-6}M) decreases the rate of synthesis of polyprenyl-sugar derivatives (data not shown).

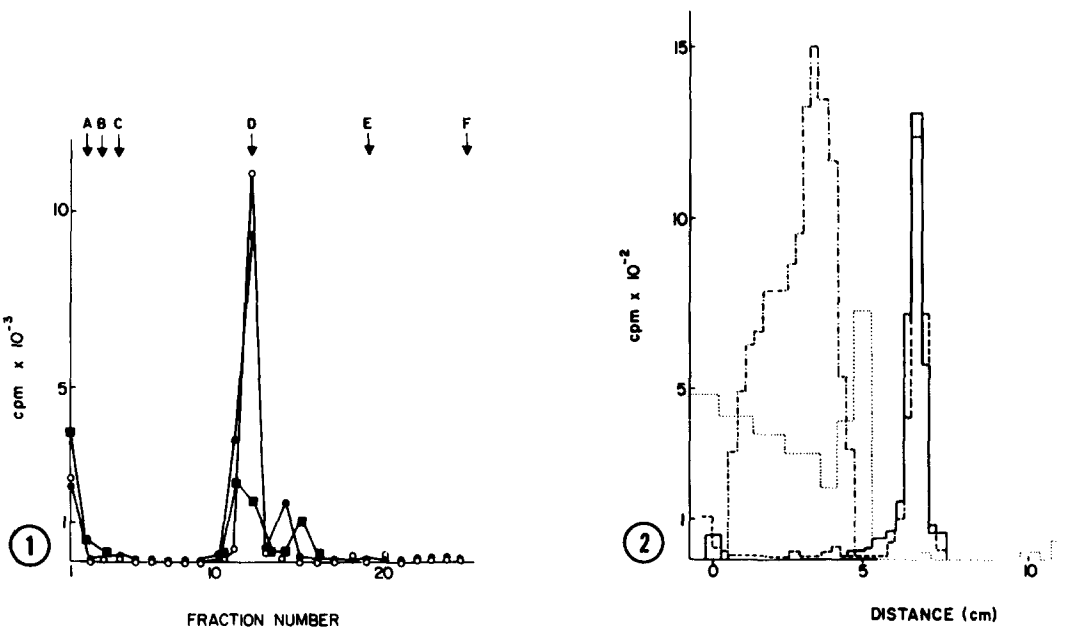

Figure 1. Ion-exchange chromatography of the T47D-c11 glycolipid. The material from the lower phase of Folch's partition from cells incubated with [^{14}C]Glc was resolved as described in Materials and Methods. Elution steps were: A) C/M 3:2; B) C/M 1:1; C) C/M/W 10:10:3, D) C/M/5 mM ammonium formate (AF) 10:10:3; E) C/M/25 mM AF 10:10:3; F) C/M/250 mM AF 10:10:3. o : control; ● : E_2; : Naf treated cells.

Figure 2. Thin layer chromatography of T47D-c11 glycolipid. The material from the control cells purified through a DEAE-cellulose column was resolved in a TLC as indicated. Radioactivity was detected by scrapping off the silica gel and counted in a toluene based scintillation fluid.
- - -: T47D-c11 [^{14}C]glycosylated lipid; ——: standard human breast Dol-P-[^{14}C]Glc; •••:standard human breast Dol-P-[^{14}C]Man;-•-•: [^{14}C]Glc.

Table 1. Effects of E_2, Naf and Tam on lipid-linked sugar derivatives in T47D-c11 cells

	RADIOACTIVITY (cpm/100 μg protein)[a]			
Fraction	Control	Estradiol	Nafoxidine	Tamoxifen
Lower phase	7,351 ± 527	7,349 ± 372	4,008 ± 598[c]	2,997 ± 439[b]
C/M/W 10:10:3	145 ± 17	155 ± 43	92 ± 23	43 ± 8[b]
Hot TCA resistant	1,432 ± 245	1,020 ± 100	373 ± 51[c]	426 ± 60[c]

Cells were plated in multiwell tissue culture dishes and cultured as described. E_2 (1 nM), Naf (1 μM) or tamoxifen-citrate (0.4 μM) were used as agonist or antagonist. The cells were pulsed with [^{14}C]Glc (2 μCi/well) in 250 μl of incubation medium, at a cell density of 78,500 ± 2,500 cells/well, and harvested after 3 hours of labeling. The reaction products were analyzed as described. [a]: Mean ± S.E., n=3; [b]: $p < 0.01$; [c]: $p < 0.02$.

These results prompted us to study further the effects of E_2 and two antiestrogens, Naf and Tam, in the formation of glycosylated lipid derivatives and glycoproteins.

Table 1 shows a comparison among the influence of E_2, Naf and Tam in the above mentioned pathway. There was no effect of E_2 on the radioactivity present in the lower phase of the Folch's partition but a significant decrease was found with Naf (45 %) and Tam (60 %). Regarding the Dol-PP-oligosaccharides, the reduction in their rate of synthesis was estimated in 37 % and 70 % for Naf and Tam respectively, while glycoprotein underwent similar decrease with both antagonist (70 %). It should be noted that E_2 slightly reduces the oligosaccharide transfer reaction to protein.

Moreover, the dependance for steroid hormones of T47D-c11 cells was confirmed when these cells were grown with serum stripped of endogenous steroids. Thus, the incorporation of radioactive Glc and glucosamine to the 3 fractions was notoriously reduced as compared with cells cultured with untreated FCS. On the other hand, similar assays using T47D-c8 cells showed no changes upon the incorporation of these precursors to lipid intermediates (See Table 2).

In agreement with this result, preliminary data from our laboratory using T47D-c8 cells cultured with a chemically defined medium (RPMI 1640 supplemented with 1 mg/ml BSA, 50 ng/ml epidermal growth factor, 10 μg/ml transferrin, 2.5 μM SO_4Fe) showed that the incorporation of [^{3}H]Man to lipid-intermediates and glycoproteins was not affected by the addition of E_2, Naf or Tam (not shown).

In order to possibly avoid the endogenous E_2 that could still remain within the cells, we designed a protocol which included a short treatment of the cells with Naf or Tam and further exposition to E_2 during 4 hours. In these experiments, besides the standard control assay we also considered two additional groups of control cultures. Thus, cells in the presence of Naf must be the reference for cells previously treated with Naf followed by E_2. Concomitantly, cells exposed to E_2 after Tam, should be compared with Tam-treated cultures. Table 3 shows again that E_2 alone did not change the synthesis of Dol-P-sugars and Dol-PP-oligosaccharides and decreases endogenous glycoprotein formation. Naf inhibited the incorporation of [^{14}C]Glc to both lower phase and glycoproteins

Table 2. Effect of steroid serum deprivation on polyprenyl-sugar derivatives and glycoproteins in T47D cll and c8 cells

	RADIOACTIVITY (cpm)[a]					
	LOWER PHASE		C/M/W 10:10:3		HOT TCA RESISTANT	
SUGAR	FCS-ch treated	FCS	FCS-ch treated	FCS	FCS-ch treated	FCS
			CLON 11			
[^{14}C]Glc	24,171 ± 287	36,118 ± 3,217	730 ± 195	1,175 ± 85	11,112 ± 1,335	14,367 ± 1,491
[^{3}H]GlcN	2,958 ± 62	17,208 ± 352[b]	53 ± 16	340 ± 1[b]	2,920 ± 381	19,671
			CLON 8			
[^{14}C]Glc	4,999	4,095 ± 685	149 ± 13	179 ± 8	284 ± 82	229 ± 13
[^{3}H]GlcN	949 ± 2	1,145 ± 98	10 ± 3	20 ± 20	176 ± 40	95 ± 21

Cells were plated as indicated in Table 1. Sixty hours before the start of an experiment the medium was changed for the same one supplemented with FCS or FCS-charcoal treatment (FCS-ch treatment). Labeled experiment was carried out with [^{14}C]Glc (4μCi/well) or [^{3}H]glucosamine ([^{3}H]GlcN)(12μCi/well) in 250μl of incubation medium during 5 hours. The reaction products were analyzed as described. [a]: Mean ± S.E., n=2; [b]: $p < 0.01$.

to an extent of 35 % and 42 % respectively, while Tam only decreased glycoprotein formation. The addition of E_2 to cells pretreated with Naf induced a slight increase in the radioactivity incorporated to the 3 fractions studied. On the other hand, E_2 after Tam-pretreatment, produced an evident decrease in the material extractable in the lower phase (36 %) and in glycoproteins (54 %).

Table 3. Influence of E_2 after Naf and Tam treatment on T47D-cll cells lipid linked sugar derivatives

	RADIOACTIVITY (cpm/100μg protein)[a]		
Conditions	Lower phase	C/M/W 10:10:3	Hot TCA resistant
Control	6,259 ± 474	186 ± 16	632 ± 31
Estradiol	5,654 ± 559	160 ± 30	409 ± 97
Nafoxidine	4,129 ± 192[b]	173 ± 29	364 ± 28[d]
Nafoxidine + E_2	4,667 ± 135	228 ± 30	442 ± 134
Tamoxifen	5,569 ± 137	154 ± 17	369 ± 32[d]
Tamoxifen + E_2	3,467 ± 242[c]	115 ± 17	169 ± 24[e]

Experimental details as given in Table 1. Assays condition were: Control: cells were exposed to the deprived medium (250μl) 3 hours before of [^{14}C]Glc (4μCi/well) labeling period (3 hs). Estradiol: cells in the same condition as control were supplemented with E_2 (1 nM) one hour before [^{14}C]Glc addition. Nafoxidine or Tamoxifen: cells as described were supplemented with Naf (1μM) or Tam (0.4μM) one hour after being exposed to the deprived medium. Incubations were continued for 1 hour, then medium was replaced. After 1 hour in fresh deprived medium the cells were labeled as above described. Nafoxidine or Tamoxifen plus Estradiol. conditions were similar to these described for Naf or Tam alone except that E_2 (1 nM) was added simultaneously with the fresh medium replacement. The reaction products were analyzed as described. [a]: Mean ± S.E., n=3; [b]: $p < 0.05$ compared to control; [c]: $p < 0.02$ compared to Tam; [d]: $p < 0.01$ compared to control; [e]: $p < 0.01$ compared to Tam.

DISCUSSION

Our results indicate that T47D cells possess an enzymatic mechanism which is able to synthetize dolichol-sugar derivatives that lead to the formation of glycoproteins (Figures 1 and 2). In addition we have proof that E_2 does not change the rate of synthesis of these lipid intermediates. However, two antiestrogens -Naf or Tam- reduce the synthesis of polyprenyl-sugar derivatives and the assembly of the oligosaccharide to proteins to a remarkable extent (See Table 1).

The importance of including antiestrogens is given by their usefulness in the treatment of breast cancer, although the mechanism by which they decrease tumor cell growth is not well known. A possible explanation for their antiestrogenic effect could be their competing property with estrogens for the ER. It has also been proposed that antiestrogens could be acting via a receptor specific for antiestrogens independently from ER (17).

The lack of response to E_2 in the lipid-sugar formation in this cell line was unexpected since hormonal induction of specific proteins or glycoproteins has already been reported (18,19,20). However, others have failed to demonstrate estrogen effects on cell growth in vitro in cell lines which nonetheless showed a striking estrogen requirement for tumor growth in vivo (7,21).

On the other hand, some steps in the dolichol-pathway have been well documented to be affected by E_2 and other steroidal hormones using different biological systems and experimental outlines (22,23).

Since we were interested in studying primary changes in dolichol pathway under E_2 influence, we designed a protocol which included a short pretreatment of the cells with Naf or Tam. This idea is based in several evidences demonstrating that cellular E_2 elimination is a slow process (24). Therefore, it is conceivable that the necessary serum-free period may be often underestimated. As a consequence, intracellular hormone levels and trophic indicators remain elevated, and no increase can be observed upon further addition of the hormone.

The smaller inhibitory effects of the antiestrogens observed in Table 3 might be explained because in this experiment Naf and Tam were withdrawn from the medium after 1 hour, while in the assay described in Table 1 the drugs remained all along the radiolabeled period. Analysis of results obtained with the addition of E_2 after Naf indicated a possible stimulatory effect of estrogen, suggesting that both agents compete for the ER. Regarding Tam pretreatment, it seems quite difficult at present to construe the increase of the inhibitory effect in lipid-sugar derivatives synthesis promoted by E_2.

A recent report should also be considered about the presence of estrogen-non competible but antiestrogen competible binding sites (25), which appear to be associated with microsomal membranes in human breast cancer cell lines. This is specially remarkable taking into account this similar subcellular locus for enzymes related to Dol-P pathway (1). Therefore, besides the classical mecha-

nism of action for antiestrogen, the effects of Tam described in this paper might be related to antiestrogen receptor.

Summarizing, the results presented in this report indicate that antiestrogens affect several steps in glycoprotein synthesis through lipid-linked sugar derivatives in T47D cells. The question is open to whether these effects are linked to ER, to antiestrogen receptor or both.

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