METABOLISM AND EFFECTS OF PROGESTERONE IN THE HUMAN ENDOMETRIAL ADENOCARCINOMA CELL LINE HEC-1

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ABSTRACT

Human endometrial adenocarcinoma cells (HEC-1 line) were incubated with ^{14}C-progesterone. Four major labeled metabolites, 3β-hydroxy 5α-pregnan-20-one, 5α-pregnane-3β,20α-diol, 20α-hydroxy-4-pregnen-3-one and 5α-pregnane-3,20-dione were separated by thin layer chromatography, further purified by high pressure liquid chromatography, and finally identified by addition of carriers and crystallization to constant specific activity. Among these metabolites, 5α-pregnane-3β, 20α-diol seems characteristic of this cell line since its formation from labeled progesterone was not detected in normal endometrium or in 2 specimens of endometrial adenocarcinoma.

The growth of HEC cells was unaffected by either progesterone or medroxyprogesterone acetate, a slowly metabolized progestin, at about 10^{-6} M levels but was inhibited by about 10^{-5} M concentrations of these compounds.

INTRODUCTION

Since progestins were found effective in the treatment of some patients with endometrial adenocarcinoma (1) efforts were made to develop culture systems in which the effects of these compounds on endometrial cancer could be investigated (2-5).

In the human endometrial carcinoma cell line HEC-1, established by Kuramoto et al (5,6), growth inhibition effects of a synthetic progestin were noted. In contrast, Shapiro et al (7) were unable to show any effect of estradiol (E_2) or progesterone (P) on amino acid incorporation into proteins in this cell line. We have observed a rapid

metabolism of P by HEC cells (8) and therefore considered the possibility that degradation of the hormone added to the culture medium may reduce its effectiveness on these cells.

In this report, we describe the pattern of metabolism of P in the HEC cell line and present results on the effects of P and medroxyprogesterone acetate (MPA) on rates of growth of HEC cells in culture.

MATERIALS AND METHODS

HEC-1 cells for starting cultures were kindly provided by Dr. J. Fogh, Sloan-Kettering Institute for Cancer Research. The original cell line was established in 1968 by Kuramoto et al (5,6) from explants of moderately differentiated papillary endometrial adenocarcinoma obtained from a 71 year old woman. These cells have been maintained in continuous culture in Ham F-10 medium (Flow Labs) containing 10% fetal calf serum (Flow Labs), 10 µg/ml insulin (Eli Lilly), 2.5 ng/ml estradiol and 1% antibiotic-antimycotic mixture (GIBCO), with glucose (final concentration: 5 mg/ml).

Progesterone 4-^{14}C (55.7 mCi/mmol), [1,2-^{3}H]-P (50 Ci/mmol), and [methyl-^{3}H]-thymidine (20 Ci/mmol) were obtained from New England Nuclear. Steroid standards were purchased from Steraloids and from Sigma Chemical Co. Medroxyprogesterone acetate was a gift from Upjohn Co., Kalamazoo, Michigan.

Incubations

HEC cells or glandular epithelial cells isolated according to a previously reported procedure (9) from a specimen of well-differentiated endometrial adenocarcinoma of a 75 year old patient, were cultured in T-75 flasks (Falcon) using the medium described above. Upon reaching confluence, the cells were collected by treatment with a solution of 0.05% trypsin and 0.02% EDTA in buffer (Flow Labs) for 5 min. The washed cells were incubated for 2 h in a shaking bath at 37°C with 2 ml Tris buffer (0.05 M, pH 7.4) containing about 200,000 cpm/ml ^{14}C-P. At the end of the incubation period, carriers of P and 20α dihydroprogesterone (20αDHP) were added (200 µg of each) and the cells were homogenized without separation of the medium. The homogenate

was extracted twice with 2 ml ethyl acetate. The aqueous phase was further extracted with benzene and an aliquot was counted in a liquid scintillation spectrometer (Isocap, Nuclear Chicago Corp.) using Scintiverse counting fluid, in order to determine whether labeled highly polar or conjugated steroids were formed during the incubation.

Metabolism of progesterone by HEC cells and confluent primary cultures of epithelial cells prepared from an endometrial adenocarcinoma was also studied by adding ^{3}H-P (10^{6} cpm/ml) to cultures in T-75 flasks. Aliquots of the culture medium (0.5 ml) were removed at various intervals between 0 and 24 h of incubation and the labeled metabolites were extracted with ethyl acetate.

A specimen of moderately to poorly differentiated endometrial carcinoma obtained from a 60 year old patient was cut in small pieces and 200 mg of the minced tissue incubated with ^{14}C-P in Tris buffer as indicated above. At the end of the incubation, the tissue was separated from the medium by centrifugation and homogenized in 3 ml methanol after addition of P and 20αDHP carriers. An ethyl acetate extract of the medium was combined with the methanolic extract of the tissue.

Chromatographic separation of metabolites

(a) Thin layer chromatography

The extract containing the labeled steroids were chromatographed on silica gel GF thin layer plates (Analtech) using the solvent system chloroform:acetone:hexane, 4:1:3. The plates were scanned for radioactivity with a Packard Radioscanner, Model 7201, and the carriers added were localized under U.V. light. The radioactive zones were scraped off the plate, eluted with ethyl acetate and an aliquot was used for measurement of radioactivity.

(b) High pressure liquid chromatography

Further purification and identification of the radioactive metabolites eluted from TLC was accomplished by high pressure liquid chromatography (HPLC) after addition of various steroids as carriers. Elution patterns of labeled and unlabeled compounds were determined by U.V. and refractometric monitoring of the effluent and by measurement of radioactivity in collected fractions.

The HPLC apparatus used was a Waters Associates Model 6000, equipped with a U6K injector, a Model 440 high sensitivity absorbance detector (8 μ l cell volume), a differential refractometer R 401 and a dual Omniscribe recorder (Houston Instruments). Progesterone metabolites were

chromatographed on a stainless steel 0.4 x 30 cm microporasil column (Waters Associates, Milford, Mass.), using the solvent system chloroform: isooctane, 4:1, at a flow rate of 3 ml/min. Purification of polar metabolites (diols) was carried out on a reverse phase C_{18} micro Bondapak 0.4 x 30 cm column (Waters Associates), using the solvent system acetonitrile: water, 1:1, at a flow rate of 3 ml/min.

Identity of the metabolites was finally established by crystallization to constant specific activity after the addition of 10 mg of reference standards.

Measurement of thymidine incorporationn into DNA and cell growth

In order to study the incorporation of ^{3}H-thymidine into DNA, 5 x 10^{4} HEC cells were plated on 60 mm dishes (Falcon) with 5 ml Ham's F-10 medium per dish. Twenty-four hours later, P or MPA in 50 µl ethanol was added to each dish to obtain concentrations of 0.5 or 1 µg/ml. The same amount of ethanol was added to the culture medium in control dishes. After a period of 18 h, 50 µl of medium containing 5 µCi ^{3}H-thymidine and 6 µg unlabaled thymidine were added to each dish and the incubations were continued for 28 h. At the end of this period, the medium was removed and the cells were collected by trypsinization. DNA was precipitated by adding TCA to a final concentration of 30%. The precipitates were collected on Whatman GF/A glass fiber filters, washed 3 times with 15% TCA and counted in 10 ml Econofluor (New England Nuclear Corp.) to determine the amount of ^{3}H incorporated into DNA. Each assay was conducted in triplicate.

For growth determinations, 3 x 10^{4} cells/dish were plated. Progesterone or MPA were added 24 h later, as described above to a final concentration of 1 or 10 µg/ml. The cells were counted in triplicate, after trypsinization, every day for 7 days, using a hemacytometer. The medium was renewed every alternate day during this study.

RESULTS

Figure 1 shows a radiochromatogram obtained from a TLC separation of metabolites formed during a 2 h incubation of HEC cells with ^{14}C-P. The bands corresponding to P and 20αDHP carriers, located under the U.V. light, are indicated in the figure. It is apparent from this chromatogram that

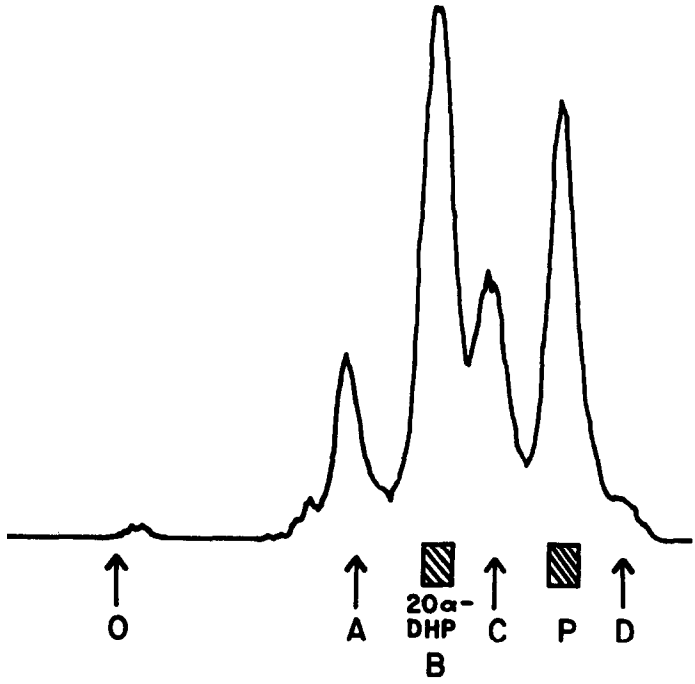

Fig. 1. A TLC radioscan of progesterone metabolites formed by HEC-1 cells.

more than half of the ^{14}C-P was converted to other products. The major metabolites formed have chromatographic mobility corresponding to pregnane diols (zone A), to 20αDHP (zone B), and to 3β-hydroxy-5α-pregnan-20-one (zone C). Formation of 5α or 5β dihydroprogesterone (zone D) is also suggested by this chromatogram.

Figure 2 shows the pattern of metabolites of ^{3}H-P released to the medium at 2 and 24 h after the addition of the substrate to HEC cell cultures in T-75 flasks. Chromatograms of samples taken between these time periods showed all the radioactive metabolites already present at 2 h.

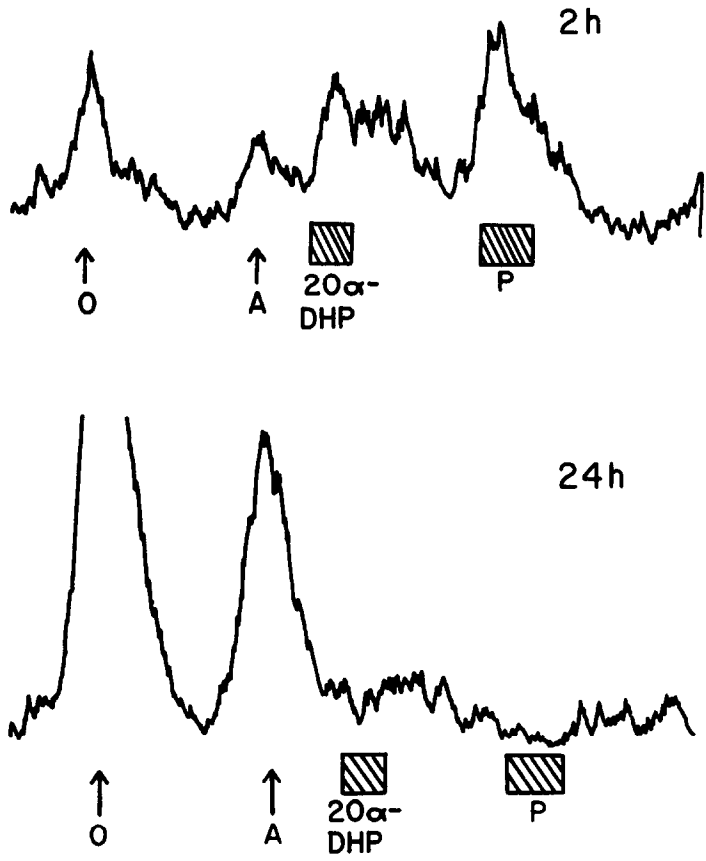

Fig. 2. Radioscan of progestersone metabolites formed at 2 h and 24 h after the addition of ^{3}H-P to the monolayer culture of HEC cells.

However, the radioactivity associated with 5α dihydroprogesterone (5αDHP) had completely disappeared by 4 h and that associated with 20αDHP, by 6 h. The 3β-hydroxy-5α-pregnan-20-one constitutes about 15% of the total radioactivity between 2 and 6 h, but by 24 h, it had been completely metabolized. The disappearance of radioactivity in

these metabolites is reflected in the concomitant accumulation of radioactivity in diol (zone A) and the highly polar compound (origin) between 2 and 24 h. At 24 h only these 2 metabolites are present and practically no P remains unmetabolized. This pattern suggests the following metabolic pathway of P metabolism by HEC cells:

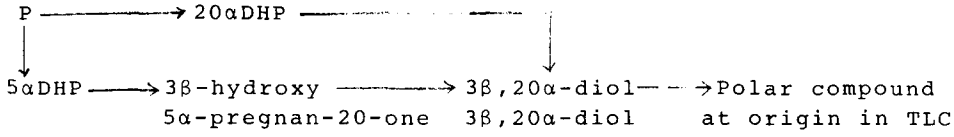

Figure 3 shows the HPLC profile of progesterone and its metabolites separated on a microporasil column. Baseline separation of P and its metabolites are achieved with this system. The polar metabolite 5α-pregnane-3,20 diol, however, is retained in this column, but can be conveniently isolated using a reverse phase separation system with the μ C_{18} Bondapak column (Fig. 4). Using the microporasil column, almost all the radioactivity in zone B from TLC coincided with the 20αDHP peak, that of zone C with 3β-hydroxy-5α-pregnan-20-one and that of zone D with 5αDHP. There was no radioactivity associated with any other steroids shown in Fig. 3. Only 55% of the radioactivity in zone A, however, had the mobility of 5α-pregnane-3β,20α-diol. The remaining radioactivity was eluted after this peak and did not correspond to 5β-pregnane-3β,20α-diol.

Thus, 4 major metabolites of ^{14}C-P formed during incubations with HEC cells were identified by HPLC to be 20αDHP, 5αDHP, 3β-hydroxy-5α-pregnan-20-one and 5α-pregnane-3β,20-diol.

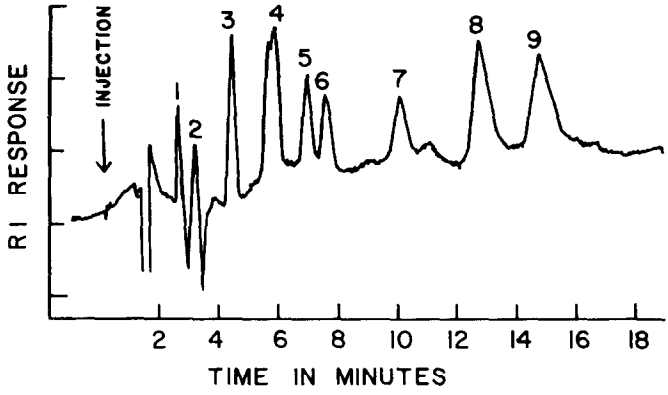

Fig 3. HPLC separation of progesterone and its metabolites on a microporasil column using the solvent system chloroform: isooctane (4:1). Flow rate - 3 ml/min; chart speed - 1 cm/min, RI-4X. The compounds separated are 1. 5α-pregnane-3,20-dione, 2. 5β-pregnane-3,20-dione, 3. progesterone, 4. 3α-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one, 5. 4-pregnen-3,6,20-trione, 6. 3β-hydroxy-5α-pregnan-20-one, 7. 3β-hydroxy-5β-pregnan-20-one, 8. 20α-hydroxy-4-pregnen-3-one, and 9. 20β-hydroxy-4-pregnen-3-one.

The identity of these radioactive metabolites was confirmed by cocrystallization with standard steroids to constant specific activity. The crystalization data are presented in Table 1.

The identity of the labeled material at the origin during TLC was not established but it appears not to be a steroid conjugate since practically all the radioactivity eluted with methanol from the origin remained in the organic phase when subjected to benzene-water partition.

Metabolism of ^{14}C-P in minces of a specimen of

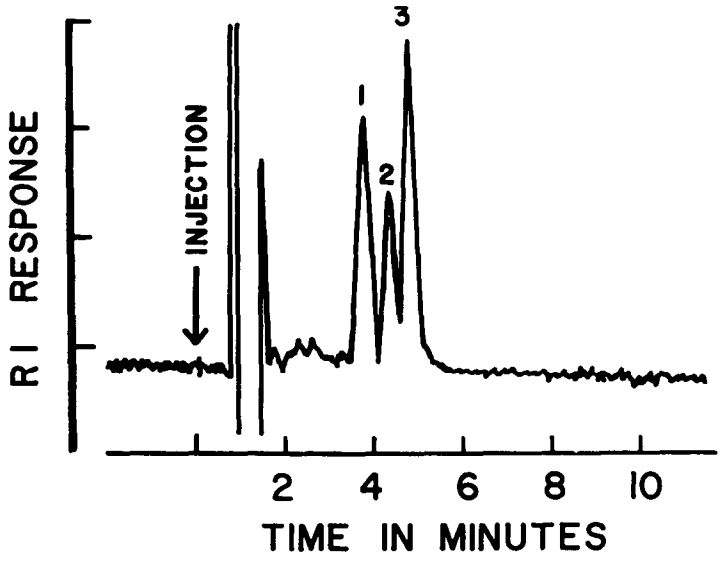

Fig. 4 HPLC separation of (1) 20α-hydroxy-4-pregnene-3-one, (2) 5α-pregnane-3β-20α-diol, and (3) 20β-hydroxy-4-pregnene-3-one on a microBondapak reverse phase column using the solvent system acetonitrile:water (1:1). Flow rate - 3 ml/min chart speed - 1 cm/min, RI-4X.

endometrial adenocarcinoma during a 2 h incubation was slow. As shown in the TLC chromatogram, in Fig. 5a, the major metabolite formed was 20αDHP. Small amounts of 5αDHP and 3β-hydroxy-5α-pregnan-20-one were also present. No 5α-pregnane-3β,20α-diol could be detected by scanning the TLC plates or by eluting the zone corresponding to this compound and counting the eluate in a liquid scintillation spectrometer.

Table 1.

Crystallization of Metabolites of Progesterone Produced by HEC-1 Cells

Metabolites	Specific Activity - cpm/mg			
	Initial	C_1	C_2	C_3
3β-hydroxy-5α pregnan-20-one	2200	2200(a)	2200(b)	2100(a)
20αDHP	2000	1900(a)	1900(b)	1900(a)
5α-pregnane-3, 20-dione	1200	1100(a)	1200(c)	1200(d)
5α-pregnane-3β-20α-diol	1100	550(a)	520(b)	550(e)

Metabolites 3β-hydroxy-5α-pregnan-20-one, 20αDHP and 5α-pregnane-3,20-dione were crystallized after purification through TLC and HPLC. Metabolite 5α-pregnane-3β,20α-diol was crystallized after purification by TLC. C - crystallization (a) methanol/water; (b) acetone/hexane; (c) methanol; (d) heptane; (e) acetone.

Similar results, shown in Fig 5b were obtained with confluent epithelial cells derived from another specimen of endometrial carcinoma. The cells were grown in monolayer for 20 days before trypsinization and incubation of these cells with ^{14}C-P for 2 h. There was also no indication of formation of 5α-pregnane-3β,20α-diol when the pattern of P metabolites released into the medium was followed up to 24 h after the addition of radioactive substrate to the monolayer of these cells growing in T-75 flasks. A radioscan of the chromatographed extract corresponding to 24 h incubations is shown in Fig 6. No radioactivity was detected in the zone where 5α-pregnane-3β,20α-diol was expected, even though ^{14}C-P was extensively metabolized and accounted for

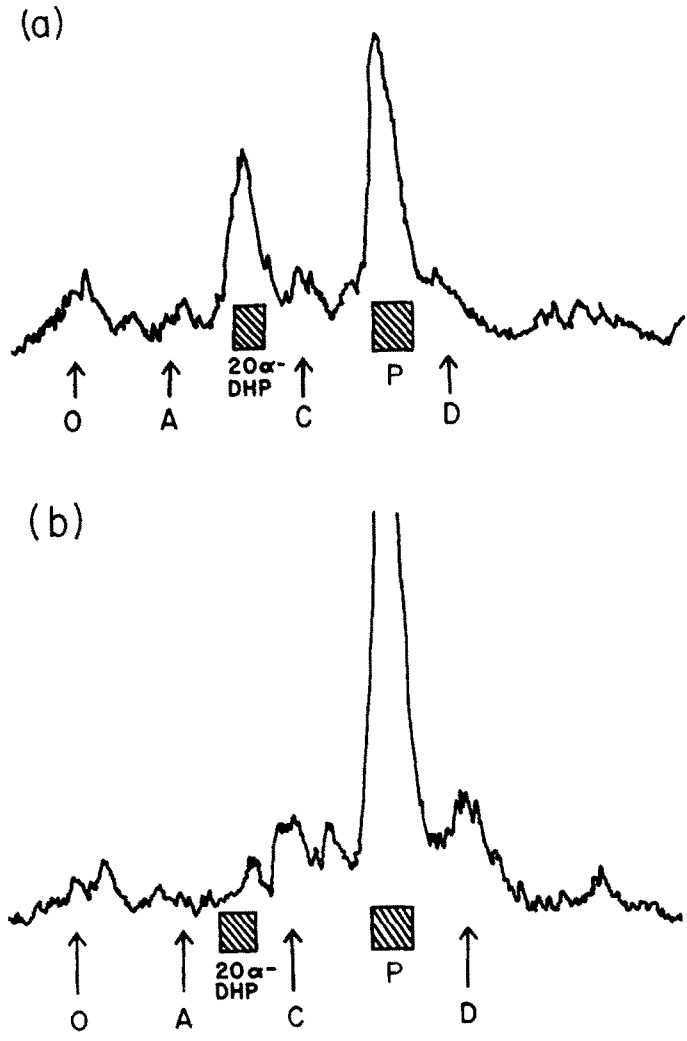

Fig. 5. Radioscans of progesterone metabolites (a) by minced endometrial adenocarcinoma incubated with ^{14}C-P for 2 h and (b) by a 20 d monolayer culture of an endometrial adenocarcinoma 2 h after the addition of ^{14}C-P to the growth medium.

less than 50% of the total radioactivity in the TLC plate.

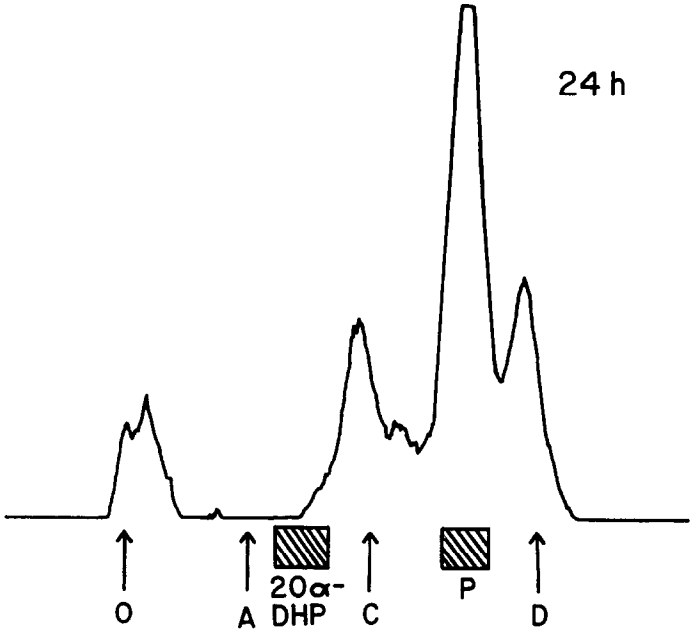

Fig. 6 A TLC radioscan of progesterone metabolites formed 24 h after the addition of radioactive substrate to an epithelial monolayer preparation of an endometrial adenocarcinoma.

The growth of HEC cells followed each day during a 7 day period were identical in the absence or in the presence of P or MPA at concentrations of 1 µg/ml (Fig. 7). At 10 µg/ml, however, both compounds were inhibitory. Tritiated thymidine incorporation into DNA was also unaffected by the presence of these steroids (1 µg/ml) in the growth medium (Table 2).

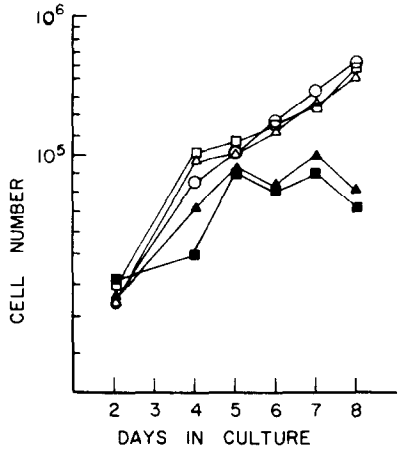

Fig. 7. The effect of progesterone and medroxyprogesterone acetate on the growth of HEC-1 cells. o—o control; Δ—Δ 1 μg/ml P; □—□ 1 μg/ml MPA; □—□ 10 μg/ml MPA; Δ—Δ 10 μg/ml P.

Table 2.

Effect of Progesterone and MPA on DNA Synthesis in HEC-1 Cells

Additions	Concentration (μg/ml)	^{3}H-thymidine incorporation dpm ± S.D.
None	-	54000 ± 5500
Progesterone	0.5	56000 ± 5200
MPA	0.5	61000 ± 4000
None	-	104000 ± 10700
Progesterone	1.0	76000 ± 3900
MPA	1.0	84000 ± 3500

Discussion

The progesterone metabolic profiles of HEC-1 cells and the endometrial adenocarcinomas appear to be different. While a major metabolite in HEC cells is 5α-pregnane-3β,20α-diol, there is no detectable formation of this product by the 2 endometrial adenocarcinomas or by the normal endometrium studied (unpublished results). Small amounts of 3β-hydroxy-5α-pregnan-20-one were formed, however, by the endometrial adenocarcinomas. The origin material that accumulates over a 24 h period is not a steroid conjugate as ascertained by partition between benzene and water but its identity remains to be established. The lack of formation of 5α-pregnane-3β,20α-diol by the endometrial adenocarcinomas does not appear to be due to low P metabolizing activity. When epithelial cells of one of these specimens were grown as confluent primary monolayers and incubated with radioactive progesterone for up to 24 h, no diol was formed even though half the added substrate had been metabolized. Incubations of pieces of secretory endometrium (300 mg) with ^{14}C-P for 24 h also failed to form this metabolite (data not presented).

The rapid metabolism of P by HEC cells ($t_{1/2} \sim 2$ h) has previously been reported by us (8). We have now identified 5α-pregnane-3β,20α-diol and 3β-hydroxy-5α-pregnan-20-one as 2 major metabolites of P in HEC cells. We are not aware of any report on the formation of these compounds

from P by either normal human endometrium during the menstrual cycle (9-12) or by endometrial adenocarcinomas of differing degrees of differentiation (13, 15). Bryson and Sweat (10) reported on the formation of an unidentified metabolite of P by normal proliferative endometrium and inferred that it could be a dihydroxy metabolite. Pollow et al (13), however, were unable to detect any dihydroxy compound in their studies.

Collins and Jewkes (15) inferred from their studies on P metabolism by human endometrial adenocarcinoma that the enzyme 5α reductase may be predominantly localized in the glandular epithelium while stromal components may be enriched in 20α-hydroxysteroid oxidoreductase activity (20α-HSD). Maeyema et al (16), however, histochemically localized the 20αHSD in the glandular epithelium. Our studies on the metabolism of P by homogeneous cultures of HEC cells serve to indicate the presence of 5α reductase, 3β-hydroxysteroid oxidoreductase and 20αHSD activities in endometrial epithelial cells. Pollow et al (13) showed that the specific activity of the enzyme 5α reductase increased with decreasing differentiation of the tumor while the specific activity of the 20α hydroxysteroid oxidoreductase decreased.

We considered the possibility that the extensive metabolism of P by HEC cells might explain the lack of effect of P on the incorporation of amino acids into protein reported by Shapiro et al (7) in these cells.

However, we were also unable to induce the activity of the enzyme estradiol -17β-hydroxysteroid oxidoreductase in this cell line with MPA, a steroid which is very slowly metabolized by HEC cells. Furthermore, the failure of both P and the synthetic progestin to affect growth of these cells or the incorporation of thymidine into DNA up to a concentration of 1 μg/ml, points strongly to other factors for the insensitivity of HEC cells to progestins, e.g. absence of P receptors. Cytoplasmic P receptors could not be detected in this cell line, even when estradiol was added to the culture medium for several days (Holinka and Gurpide, unpublished observations). The non-specific growth inhibitory effect of high steroid concentrations (above 10^{-5} M) has previously been reported by Ishiwata et al (4). They showed that the growth of both the endometrial adenocarcinoma cell line SNG-M, as well as the fetal lung cell line WI-38 were inhibited by P at these concentrations.

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FOOTNOTE

The following abbreviations and trivial names are used in the text:

Estradiol; 1,3,5(10)-estratriene,3,17β-diol (E_2)
Progesterone; 4-pregnene-3,20-dione (P)
Medroxyprogesterone acetate;17-acetoxy-6α-methylpregn-4-ene-3,20-dione(MPA)
20α dihydroprogesterone; 20α-hydroxy-4-pregnene-3-one (20αDHP)
5α-dihydroprogesterone; 5α-pregnane-3,20-dione (5αDHP)
5β-dihydroprogesterone; 5β-pregnane-3,20-dione (5βDHP)
20α-hydroxysteroid oxidoreductase (20αHSD)
3β-hydroxysteroid oxidoreductase (3βHSD)
Trichloracetic acid (TCA)