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In vitro responsiveness of ovarian epithelial carcinomas to endocrine therapy

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Summary. As previously reported, ovarian epithelial carcinomas may respond to endocrine therapy. We examined the direct effect of progesterone, medroxyprogesteroneacetate, gestoneron, 17-β-estradiol, tamoxifen, 4-OH-tamoxifen, or *N*-desmethyltamoxifen on the proliferative capacity of ovarian carcinoma cells by means of the colony assay described by Hamburger and Salmon. The growth rate of 25 tested tumors (ascitic fluid, primary tumor, metastases) was 68%. The plating efficiency was 0.078%. Beside the drug testing estrogen and progesterone receptor levels were determined.

The inhibition of colony survival was slightest with 17-β-estradiol, more pronounced with medroxyprogesteroneacetate, gestoneron, *N*-desmethyltamoxifen, and progesterone, and greatest with 4-OH-tamoxifen and tamoxifen. Significant and dose-dependent inhibition of $>70\%$ was observed with tamoxifen and 4-OH-tamoxifen in 80% of the tested tumors. There was no significant correlation between the in vitro responsiveness and the level of hormonal receptors. It is presumed that tamoxifen and its metabolites act not only via an estrogen receptor but also via an antiestrogen-binding site.

Introduction

Patients with advanced breast cancer whose tumors contain estrogen receptors are likely to respond to endocrine therapy [6, 17]. If the tumors are positive for both estrogen and progestin receptors the likelihood of response to endocrine therapy with antiestrogens or high-dose medroxyprogesteroneacetate is even higher [17, 20]. Patients with negative receptor levels rarely respond to endocrine therapy [17]. The prognostic and therapeutic value of steroid receptor levels in ovarian epithelial carcinomas have been discussed in few communications [3, 13, 28, 32, 33]. The estrogen and progestin receptor level seems to be independent of patient age, histological grade of tumor, clinical stage, and residual tumor after surgery [28]. As the receptor level is the prognostic factor that correlates with the survival time in patients with breast cancer, the prognostic significance of steroid receptors in ovarian epithelial carcinomas is unknown.

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In a retrospective study, 36% of 159 advanced ovarian carcinomas were found to respond to therapy with various progestins [18, 19]. Trials with medroxyprogesteroneacetate in the treatment of advanced ovarian cancer did not demonstrate a convincing therapeutic effect such as is known to result [10, 12, 25] from endocrine therapy of advanced breast cancer.

As previously reported [13, 23–25, 29], the antiestrogen tamoxifen seems to be of use in the treatment of advanced ovarian cancer that has been pretreated with chemotherapeutic agents.

This report describes the in vitro responsiveness of ovarian epithelial carcinomas to endocrine therapy with antiestrogens, estrogen, and various progestins according to the human tumor stem cell assay described by Hamburger and Salmon [1, 7, 8].

Materials and methods

Tumor material. Specimens from untreated human ovarian carcinomas were obtained from patients ($n=25$) undergoing gynecological operations. The tumor tissue was prepared under sterile conditions in a laminar flow system. Malignant effusions were collected in heparinized bottles (100 units/ml). After centrifugation at 150 *g* for 10 min the cells were collected and washed twice in Hank's balanced salt solution (HBSS, Gibco) with 10% heat-inactivated fetal calf serum (FCS, Gibco). Tumor nodules were mechanically dissociated with a scalpel as described elsewhere [8]. The cell suspension was filtered through a nylon gauze sieve (200, 100, 50, 20 μm) to remove cell clumps. After washing the viable cell count was determined by the trypan blue exclusion test. Viability was routinely more than 60% if samples were obtained with 1 h of surgery.

Cells (2×10^5/dish) were cultured as described by Hamburger and Salmon [1, 7, 8]. Briefly, an underlayer was prepared containing 0.5% Bacto-Agar (Gibco) in enriched McCoy's medium (Gibco) with 10% heat-inactivated FCS and 5% horse serum (Gibco). The tumor cells to be plated were suspended in the upper layer of enriched CMRL-1066 (Gibco) medium with 15% heat-inactivated horse serum containing 0.3% Bacto-Agar (Gibco), and pipetted onto the top of the feeder layer. Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

Examination of cells and colonies. Freshly plated cultures were examined by an inverted light microscope to ascer-

tain that cell clumps were not present. Colonies were considered as aggregates of more than 30–40 tumor cells [7, 8] with a diameter of 80–100 μm or more. Cells from cell suspension prior to plating and cells from colonies plucked by a fine capillary pipette were subjected to further cytological and cytochemical analysis. Cells were stained by the Papanicolaou and H & E methods [30]. Cytochemical tests were performed with peroxidase stain [10], nonspecific esterase [14], and periodic acid-Schiff (PAS) reactions [21] to exclude a hematopoetic origin.

Individual colonies were assessed for their ability to form E-rosettes with aminoethylisothiouronium bromide hydrobromide (AET)-treated sheep red blood cells [4, 5] to rule out T cell colonies.

Drug testing. 17-β-Estradiol (Sigma), medroxyprogesteroneacetate (Sigma), progesterone (Sigma), gestoneron (Schering), tamoxifen, and its major metabolites 4-OH-tamoxifen and *N*-desmethyltamoxifen (Imperial Chemical Industries) were dissolved in absolute ethanol. The drugs were prepared freshly every 4 weeks and kept in light-protected glass vials at −80 °C to avoid releasing uncontrolled hormonal activity. The final ethanol concentration in the upper layer was 1%, as we found that this concentration did not influence colony growth in the control dishes.

Cultures were continuously exposed to the hormones at 10^{-9} to 10^{-6} *M* as other authors [13] did not find any inhibition of colony formation with short 1 h drug exposure.

Steroid receptor assay. The tumor specimens were placed on ice in the operating room as soon as they had been excised, frozen within a few minutes to −80 °C and stored at this temperature until the receptor assay.

The samples were analyzed for estrogen and progesterone receptors by the dextran-coated charcoal method (DCC) as described elsewhere [32], with or without un-

Table 1. Patient information, histological diagnosis and plating efficiency of 17 untreated human epithelial ovarian carcinomas

Patient	Age (years)	Stage[a]	Tumor site	Histology	Grade of differentiation	Plating % efficiency
1	72	III	Primary	Endometrioid adenocarcinoma	Poor	0.094
2	50	III	Metastasis	Serous adenocarcinoma	Poor	0.115
3	51	III	Primary	Papillary serous adenocarcinoma	Mixed well and poor	0.068
4	59	III	Ascitic fluid	Clear cell adenocarcinoma	Well	0.029
5	72	III	Metastasis	Endometrioid adenocarcinoma	Poor	0.108
6	46	III	Primary	Serous adenocarcinoma	Well	0.138
7	48	III	Ascitic fluid	Papillary serous adenocarcinoma	Poor	0.046
8	49	III	Metastasis	Papillary serous adenocarcinoma	Poor	0.091
9	46	III	Metastasis	Serous adenocarcinoma	Well	0.139
10	50	III	Metastasis	Endometrioid adenocarcinoma	Poor	0.070
11	55	III	Primary	Papillary serous adenocarcinoma	Well	0.019
12	51	III	Metastasis	Papillary serous adenocarcinoma	Mixed well and poor	0.065
13	71	III	Primary	Mucinous adenocarcinoma	Well	0.060
14	67	III	Ascitic fluid	Serous adenocarcinoma	Moderate	0.049
15	60	IV	Ascitic fluid	Serous adenocarcinoma	Well and moderate	0.059
16	50	III	Primary	Endometrioid adenocarcinoma	Poor	0.089
17	55	III	Primary	Mucinous adenocarcinoma	Poor	0.086

[a] International Federation of Gynecologists and Obstetricians staging system

labeled diethylstillbestrol and R5020, respectively. Tissue protein was determined according to the Lowry method [15]. All data were analyzed according to Scatchard analysis [11, 32]. A tissue was receptor-positive if the receptor concentration exceeded 10 fmol/mg tissue protein [32].

Results

Tumor cells from 25 untreated patients with ovarian carcinoma (ascitic fluid, solid tissue from primary tumors and metastases) were cultured. In 17 samples a sufficient number of colonies (>30 colonies/dish) for drug testing was found. Thus, the growth rate was 68%.

The relevant patient information for the 17 tumor samples that grew in soft agar is listed in Table 1.

The plating efficiency ranged between 0.019% and 0.139%. The median plating efficiency was 0.078%.

There was a linear relationship between the number of nucleated cells plated and the number of colony-forming units (Fig. 1), suggesting that the colonies were derived from single tumor cells [1, 7]. This important parameter was checked five times within the 17 tumor samples. If the tumor cell suspension was not filtered through a cytosieve as mentioned above no linearity could be shown. The cytological analysis of cells prior to plating and of cells taken from the colonies in the agar revealed identical cell morphology. Cytochemical analysis demonstrated that the cells were negative for peroxidase, nonspecific esterase and the PAS reaction.

As the hormones were dissolved in pure ethanol, we studied the influence of increasing ethanol concentrations on colony formation. The presence of 10 µl pure ethanol in the upper layer (1 ml) with the tumor cells did not significantly influence the colony growth. Higher ethanol concentrations yielded an increasing inhibition of colony formation (Fig. 2). Thus, all hormones (10^{-9} to 10^{-6} mol/l) were dissolved in 10 µl pure ethanol as the control dishes also contained 10 µl ethanol. Tamoxifen concentrations of more than 2 µmol/l were followed by crystallization of the drug in the upper layer.

All tumor samples were simultaneously assayed for estrogen (ER) and progesterone (PR) receptors. The ranges of hormonal receptor levels were 10–1580 fmol/mg tissue protein for ERs and 10–4414 fmol/mg tissue protein for PRs. Of the receptor-positive carcinomas more than 90% showed ER and PR levels between 10 and 150 fmol/mg. There was no obvious difference in receptor levels between primary tumors and their metastatic tissues in the abdomen.

Our data show that there was no significant correlation between the ER or PR levels and the responsiveness to any of the antiestrogens we used. Even receptor-negative tumors showed a good in vitro responsiveness to antiestrogens (Table 2).

Figure 3 and Table 3 provide a representative view of the data we obtained and show the influence of various hormones and antiestrogens on the colony formation of human ovarian carcinomas.

17-β-Estradiol stimulates the colony formation in concentrations from 10^{-9} to 10^{-8} mol/l. Estrogen concentrations from 10^{-7} to 10^{-6} mol/l showed no significant effect.

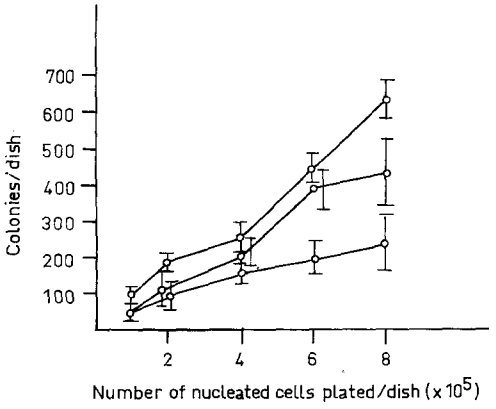


Fig. 1. Linear relationship between colony formation and the number of nucleated cells plated in three human ovarian carcinomas. *Points*, means of 4 dishes; *bars*, ±SEM

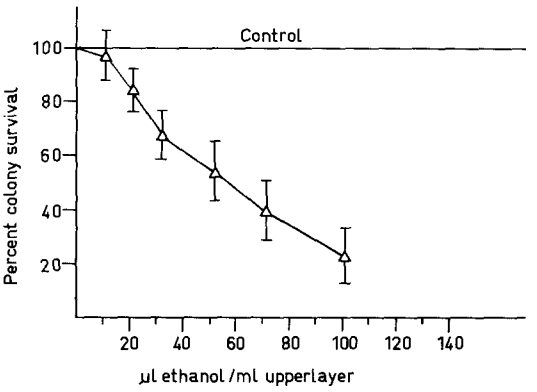


Fig. 2. Inhibition of colony growth of human ovarian carcinoma cells by increasing ethanol concentrations in the upper layer. *Points*, means of quadruplicate cultures; *bars*, ±SEM

Table 2. Inhibition of colony formation (%) after tamoxifen treatment (continuous exposure to 1 µM tamoxifen) and steroid receptor content[a]

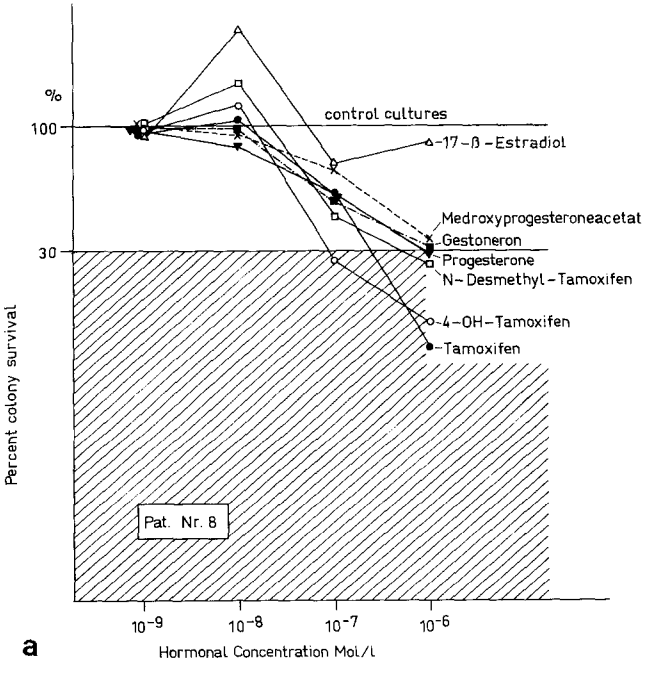
Patient	(fmol/mg tissue protein)		Inhibition of colony formation % of control
	ER	PR	
1	Negative	15	59
2	23	44	88
3	49	100	86
4	Negative	Negative	76
5	Negative	37	73
6	56	109	80
7	Negative	Negative	51
8	57	18	86
9	55	130	81
10	48	112	87
11	50	Negative	86
12	36	40	85
13	Negative	32	79
14	141	109	62
15	1580	4414	80
16	33	139	89
17	Negative	Negative	91

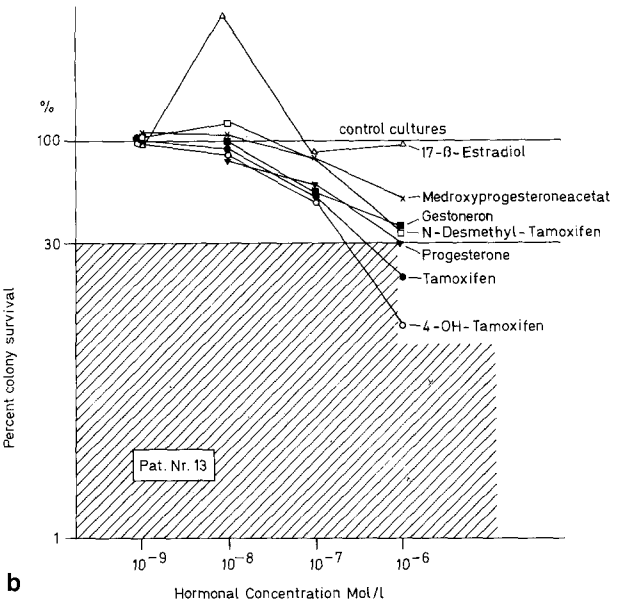
ER, estrogen receptor; PR, progesterone receptor

[a] ER-negative and PR-negative ≙ <10 fmol/mg tissue protein

Table 3. Dose-dependent influence of various hormones and antiestrogens on the colony formation of human ovarian carcinoma cells. Inhibition of colony growth was measured at hormonal concentrations from 10^{-8} Mol/l (10 nM), 10^{-7} Mol/l (100 nM) and 10^{-6} Mol/l (1 μM), (n) = number of assayed tumor samples from different patients

Drug concentration		Inhibition of colony survival > 50%		Inhibition of colony survival > 70%	
Tamoxifen	10 nM	0 (6)		0 (5)	
	100 nM	2 (14)		0 (14)	
	1 μM	17 (17)	100%	14 (17)	82%
	2 μM	10 (10)	100%	8 (10)	80%
4-OH-Tamoxifen	10 nM	0 (7)		0 (7)	
	100 nM	11 (16)	69%	7 (16)	44%
	1 μM	15 (16)	94%	13 (16)	81%
N-Desmethyl-tamoxifen	10 nM	0 (5)		0 (5)	
	100 nM	8 (12)	67%	3 (12)	25%
	1 μM	12 (12)	100%	6 (12)	50%
Progesterone	10 nM	0 (8)		0 (8)	
	100 nM	4 (8)	50%	4 (8)	50%
	1 μM	4 (8)	50%	5 (8)	63%
Gestonexone	10 nM	0 (8)		0 (8)	
	100 nM	3 (8)	38%	0 (8)	
	1 μM	6 (8)	75%	3 (8)	38%
Medroxy-progesterone-acetate	10 nM	0 (10)		0 (10)	
	100 nM	1 (10)	10%	0 (10)	
	1 μM	0 (10)	60%	3 (10)	30%
17-β-Estradiol	10 nM	0 (5)		0 (5)	
	100 nM	0 (5)		0 (5)	
	1 μM	1 (5)	20%	0 (5)	





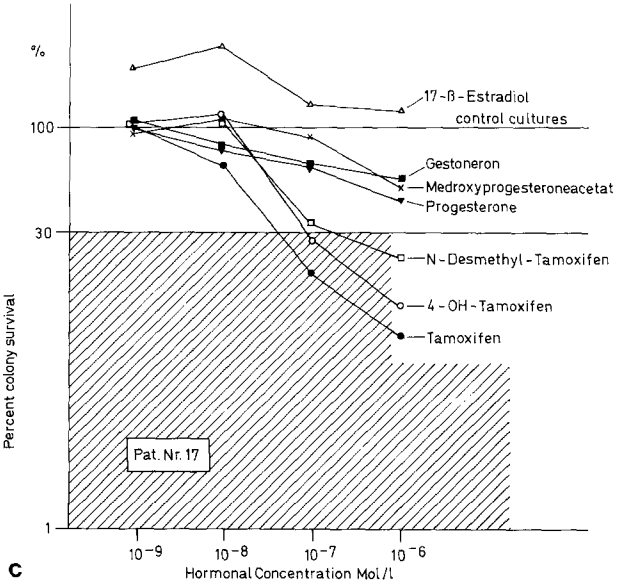


Fig. 3a–c Inhibitory effect of various hormones and antiestrogens on the colony survival of three human ovarian carcinomas. *Points*, means of triplicate cultures. The level of significance between the control and treated groups (at 10^{-7} and 10^{-8} M was found in the t-test ($P < 0.005$). ▨, inhibition of colony growth by over 70%

Tamoxifen and its major metabolites 4-OH-tamoxifen and *N*-desmethyltamoxifen showed no significant stimulatory or inhibitory effect on the colony growth at a concentration of 10^{-9} to 10^{-8} mol/l, whereas higher concentrations (10^{-7}–10^{-6} mol/l) yielded a progressive, strictly dose-dependent inhibition of colony formation. Tamoxifen and 4-OH-tamoxifen inhibited colony growth by over 70% in 80% of the tested tumors (Fig. 3, Table 3). Inhibition of colony growth by over 70% indicates drug sensitivity of the colony-forming tumor cells to anticancer drugs [27]. *N*-Desmethyltamoxifen, gestoneron, medroxyprogesteroneacetate, and native progesterone were significantly less effective. Among the progestins, the native progesterone was even more effective than gestoneron or the clinically used medroxyprogesteroneacetate (Table 3).

Discussion

The influence of various hormones and antiestrogens on the colony formation of ovarian carcinoma cells from 25 untreated patients was studied in the human tumor stem cell assay [1, 7, 8]. The growth rate (>30 colonies/dish) was 68% and thus comparable to the results of other authors [1, 8]. The median plating efficiency was 0.078%.

As other authors [13] did not observe a decrease in colony formation with brief exposure (1 h) to any of several hormones and antiestrogens, it is suggested that brief exposure to tamoxifen at a concentration of 1 μmol was not cytostatic or cytotoxic. As some cytostatics need a long-term exposure if the drug action depends on the cell cycle [16], hormones probably need a long incubation too before they can interact with the receptor proteins or the cell metabolism. Thus, we decided to use a long-term incubation with hormones, which is supported by other results [26].

Hormones are usually dissolved in an oily solution. As the agar double layer is a hydrophilic medium it necessitates a different mode of hormonal application. If the hormones were dissolved in pure ethanol and brought into the assay in a final concentration of 1% ethanol/ml upper layer the ethanol did not influence the colony growth (Fig. 2).

The prognostic significance of steroid receptors in ovarian epthelial carcinoma has been handled in few communications [28, 32, 33]. Retrospective and prospective therapeutic trials with progestins could not provide a convincing therapeutic effect in advanced ovarian cancer, as is known from endocrine therapy of breast cancer [9, 12, 18]. As previously reported, antiestrogens may be of use in the treatment of advanced ovarian cancer [23, 24, 29].

Besides the hormones and antiestrogens in clinical use we studied the influence of the naturally occurring 17-β-estradiol and native progesterone on the colony formation.

The stimulating effect of 17-β-estradiol in physiological concentrations (10^{-9} to 10^{-8} mol/l) can be explained by the enhanced synthesis of proteins in the cell following the binding of estradiol to the intracellular ERs. At pharmacological concentrations (10^{-7} to 10^{-6} mol/l) no significant effect was seen.

In our experiments we assume sensitivity of colony formation to hormonal treatment if the colony formation is inhibited by at least 70% (Fig. 3a–c) [1, 27]. Of the progestins we used, the clinically used medroxyprogesteroneacetate revealed a sufficient inhibition of colony growth only in 30% of the tested tumors. These results correspond to the clinical results of other authors [9, 12, 25], who have reported that the therapy of advanced ovarian cancer with medroxyprogesteroneacetate cannot improve the prognosis of the disease. Native progesterone and gestoneron brought about a colony inhibition of 70% in about 50% and 38% of the tested tumors (Fig. 3, Table 3).

The best in vitro results were obtained with tamoxifen and its metabolite 4-OH-tamoxifen, with inhibition of colony survival by over 70% in 80% of the tested tumors at pharmacological concentrations (10^{7} to 10^{6} mol/l). This inhibition of colony growth was strictly dose-dependent and significant. Nonsteroidal antiestrogens antagonize the effect of estrogens on the target tissues, and this activity has led to the use of tamoxifen for the treatment of advanced breast cancer [31], suggesting that antiestrogens bind to the ER. Furthermore, estrogens and antiestrogens are mutually competitive for binding to these sites [31]. Thus, it has been generally accepted that antiestrogens exert most of the effects through the specific ERs. Other authors [13] conclude from their in vitro experiments that the responsiveness of ovarian carcinoma to antiestrogens depends on the level of hormonal receptor. Our data cannot prove a significant correlation between the ER or PR levels and the responsiveness to our hormonal treatment. Even receptor-negative tumors showed a good in vitro response to antiestrogens (Table 2).

The absence of any obvious difference in the receptor levels between primary tumors and their metastatic tissues in the abdomen is supported by the results of Teufel [32, 33].

These results support the idea that tamoxifen and its metabolites do not act only via the ER. In breast cancer cells Sutherland et al. found receptor complexes for tamoxifen and 4-OH-tamoxifen that are different from the estradiol receptor complexes [2, 22, 31]. These antiestrogenic binding sites with a high affinity for tamoxifen may have a role in regulating the effects of nonsteroidal antiestrogens [31].

If we compare the antiestrogenic activity of tamoxifen and 4-OH-tamoxifen with *N*-desmethyltamoxifen, we find that there is a marked difference in their ability to inhibit colony growth. *N*-Desmethyltamoxifen is significantly less effective (Fig. 3, Table 3).

In vitro experiments of Murphy et al. [22] showed that demethylation in the aminomethoxy side chain of the tamoxifen molecule had little effect on ER binding but markedly reduced the affinity for the antiestrogen-binding site.

This in vitro inhibition of tumor colony formation of ovarian epithelial carcinomas by tamoxifen and its metabolite 4-OH-tamoxifen supports the clinical finding that patients with advanced ovarian cancer can benefit from therapy with antiestrogens. The supposed antistrogenic effect via an antiestrogenic binding site in the tumor cell should encourage further in vitro investigations.

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