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Growth patterns and Hormonal Sensitivity of Primary Tumor, Abdominal Metastasis and Ascitic Fluid from Human Epithelial Ovarian Carcinomas in the Tumor Colony-forming Assay

H.M. RUNGE,*‡ H.A. NEUMANN,† T. BAUKNECHT,* and A. PFLEIDERER*

*Lehrstuhl f. Geburtshilfe und Gynäkologie II der Univ.-Frauenklinik der Albert-Ludwigs-Universität D-7800 Freiburg, Hugstetter Str. 55, FRG and †Med.-Univ.-Klinik, Hugstetter Straße 55, D-7800 Freiburg, FRG

Abstract—*The human tumor colony forming assay was used to evaluate the response of ovarian carcinoma cells from primary tumors, ascitic fluids and metastasis to hormonal treatment. In* 12/35 *patients a sufficient colony formation* (> 30 colonies/dish) *was obtained in order to perform a simultaneous drug testing. The plating efficiency of the metastatic samples* (0.12%) *was significantly higher* ($P < 0.053$) *than those from the primary tumor* (0.076%) *or those that were derived from the ascitic fluid* (0.082%). *Colonies from the metastatic tissues could be evaluated* 2–4 days *earlier than those from primary tumors. These discrepancies may be due to a heterogeneity in the clonable tumor cell compartment of primary tumor and metastasis. The antiproliferative properties of the antiestrogen tamoxifen and the progestin gestoneron were studied. In* 9/12 *cases a significant, dose-dependent reduction of colony formation* (> 70–90% *of the controls) was observed after continuous exposure to* 1 μmole *tamoxifen. No correlation between the dose response and the content of steroid receptors was found. Even estrogen receptor negative tumor samples showed a maximal antiproliferative effect of tamoxifen.*

INTRODUCTION

THE PREDICTIVE value of the human tumor colony forming assay is limited by the fact that most tumors are composed of different clones with different susceptibility to cytostatic drugs [1–5]. In addition, the same problem might exist for primary tumors and their metastasis. An oncobiogram that is obtained from tumor material from the primary tumor must not necessarily be predictive for the sensitivity of metastatic tumor cells. This discrepancy is demonstrated in several studies with the use of cytostatic drugs [6–10]. In this study we report on our results from experiments with primary ovarian carcinoma cells, abdominal metastases and the malignant cells from ascitic fluids. We have tested the sensitivity of the progestin gestoneron and the antiestrogen tamoxifen as these substances proved to be effective in the *in vitro* and *in vivo* therapy of ovarian carcinomas [6,12–15]. Thirty-five primary ovarian carcinomas were cultured in a soft agar double layer system [13,15–17]. Simultaneous assays for the primary tumors and their abdominal metastases were performed in 12 cases, investigating the growth patterns, the level of steroid receptors and the hormonal sensitivity of these tumor cells.

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‡To whom requests for reprints should be addressed.

MATERIAL AND METHODS

Tumor material

Specimens from untreated human ovarian carcinomas were obtained from patients undergoing gynaecological operations. In 12 out of 35 ovarian carcinomas samples of tumor cells from primary tumor, metastasis and ascitic fluid respectively were cultured simultaneously. 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 [15,16]. The cell suspension was filtered through a nylon gauze sieve (200, 100, 50, 20 μ) to remove cell clumps. After washing the viable cell count was determined by the trypan blue exclusion test. Viability was routinely more than 50–60% if samples were obtained within 1 hr of surgery.

2×10^5 cells/dish were cultured as described by Hamburger and Salmon [16]. Briefly an underlayer was prepared containing 0.5% Bacto-Agar (Gibco) in enriched Mc-Coy'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 medium of enriched CMRL-1066 (Gibco) medium with 15% heat inactivated horse serum containing 0.3% Bacto-Agar (Gibco) and pipetted on the top of the feeder layer. Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. No conditioned medium was used.

Examination of cells and colonies

Freshly plated cultures were examined by an inverted light microscope to ascertain that cell clumps were not present. Colonies were considered as aggregates of more than 30–40 tumor cells [16–18] with a diameter of 80–100 μ or more. Cells from cell suspension prior to plating and cells from colonies plucked by a fine capillary pipette were subjected for further cytological analysis. Cells were stained by the Papanicolaou and haematoxylin/eosin method [19].

Drug testing

Gestoneron (17-α-hydroxy-19-norpregnen-4-en-3,20-dioncaproat, Schering) and tamoxifen (1-p-β-dimethylaminoethoxyphenyl-trans-1,2-diphenylbu-1-ene, Imperial Chemical Industries) were dissolved in absolute ethanol. The medicaments were prepared freshly every 4 weeks and kept in light protected glass vials at −80°C in order not to loose 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 [13].

Cultures were continuously exposed to the hormones at 10^{-8}–10^{-6} moles/l as other authors did not find an inhibition of colony formation with a 1-hr drug exposure [11,13,20].

Steroid receptor assay

The tumor specimens were placed on ice in the operating room as soon as they were excised and within a few minutes frozen at −80°C until the receptor assay.

The samples were analyzed for estrogen and progesterone receptors by the dextran-coated charcoal-method (DCC) as described [21] with or without unlabelled diethylstilbestrol and R5020 respectively. Tissue protein was determined according to the Lowry method [22]. All data were analyzed according to Scatchard analysis [21,23]. A tissue was receptor positive if the receptor concentration exceeded 10 fmol/mg tissue protein [21].

RESULTS

35 samples from patients with metastatic ovarian carcinoma were plated in an agar double layer system. In 12 cases the sufficient colony formation (> 30 colonies/dish) was obtained from primary tumors and their abdominal metastasis in order to perform a simultaneous drug testing. Information about stage, histological diagnosis and the grade of differentiation is listed in Table 1. In addition, the data from simultaneous cultivation and drug testing from another 5 cases of ascitic fluid are provided [Table 2, Figs 1 (c) and 2 (c)]. The plating efficiency of the cells from the primary tumors and their metastases and the ascitic fluid respectively are listed in Table 2. The median plating efficiency of the cells derived from the metastases (0.120%) was higher than that from the primary tumor (0.076%) and that of the cells derived from the ascitic fluid 0.082%. This difference was significant in the paired sample t-test ($P < 0.0532$).

The cytological analysis of cells prior to plating and from cells of the colonies in the agar revealed identic cell morphology. The colonies derived from metastatic lesions could be evaluated 2–4 days

Table 1. Patient information, stage, histological diagnosis and grade of differentiation

Patient Nr.	Age (Year)	Stage [a]	Histology [b]	Grade of differentiation
1	56	III	papillary serous adenocarcinoma	moderate
2	53	IV	adenocarcinoma	poor
3	63	IV	papillary serous adenocarcinoma	moderate
4	70	III	endometrioid adenocarcinoma	poor
5	69	III	adenocarcinoma	poor
6	62	IV	papillary serous adenocarcinoma	mixed poor and moderate
7	68	III	serous and endometrioid adenocarcinoma	moderate
8	49	III	clear cell adenocarcinoma	moderate
9	45	III	papillary serous adenocarcinoma	moderate
10	50	III	endometrioid adenocarcinoma	poor
11	50	III	papillary serous adenocarcinoma	mixed poor and moderate
12	48	III	mucinous adenocarcinoma	poor

a) International Federation of Gynecologists and Obstetricians staging System. The cases include only untreated primary tumors and exclude recurrents.
b) Histology of the abdominal metastasis used to be the same as the histology of primary tumor of the ovary.

Table 2. Plating efficiency (%) and content of estrogen (E) and progesteron (P) receptors (fmol/mg *tissue protein) in human ovarian epithelial carcinomas: (a) primary tumor of the ovary, (b) abdominal site of metastasis mostly from the omentum, (c) ascitic fluid (plating efficiency only)*

	a) Primary tumor			b) Abdominal metastasis			c) Ascitic fluid [2]
Patient Nr.	Plating efficiency %	Steroid receptor [1] E	P	Plating efficiency	Steroid receptor E	P	Plating efficiency
1	0,054	31	55	[redacted]	34	22	
2	0,059	38	neg.	[redacted]	neg.	neg.	0,082
3	0,073	78	112	[redacted]	58	124	0,078
4	0,094	neg.	15	[redacted]	24	37	-
5	0,060	neg.	32	[redacted]	neg.	21	-
6	0,041	82	40	[redacted]	72	64	0,078
7	0,081	44	neg	[redacted]	57	neg.	-
8	0,070	neg.	22	[redacted]	neg.	neg.	0,059
9	0,138	56	109	[redacted]	55	130	0,114
10	0,089	33	139	0,090	48	112	-
11	0,068	49	100	0,085	36	40	-
12	0,086	neg.	neg.	0,142	42	neg.	-

1) Receptor positive ≙ > 10 fmol/mg tissue tissue protein.
2) Content of steroid receptor was not determined.

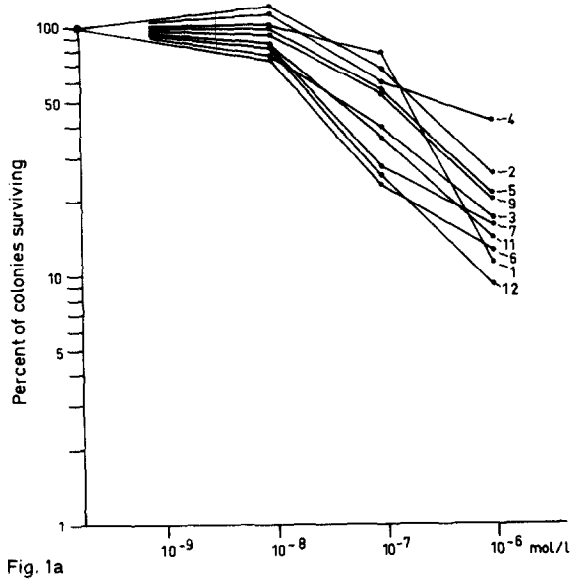

Fig. 1a

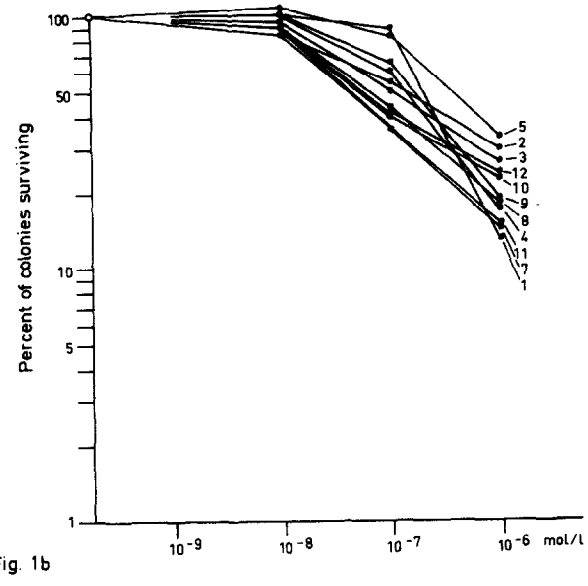

Fig. 1b

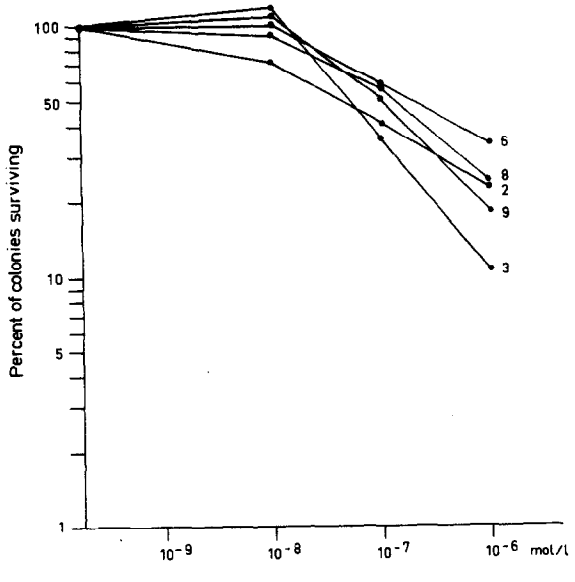

Fig. 1c

Fig. 1. Influence of tamoxifen on the colony growth of human ovarian carcinoma cells from different tumor sites of the same patient (patients numbers 1–12). (a) from the primary tumor of the ovary, (b) from the abdominal metastasis, (c) from the ascitic fluid. Points represent the means of triplicate cultures. The reduction of colony formation at 10^{-6} mol/l *was more than* 50% *in all samples.*

carlier than the colonies from the primary tumor, i.e. when the diameters of the colonies was 80 μ or more [18].

The content of steroid receptors of the tumor samples is listed in Table 2. There was no correlation between primary tumor, metastatic tissue and the content of estrogen or progesterone receptors. There was even no correlation between the plating efficiency and the receptor level.

The dose response curves from tumor colonies are shown in Figs 1 (a)–(c), comparing the effect of tamoxifen on primary tumors, on solid metastases and on ascitic cells. In 10/12 cases the effect of tamoxifen could be evaluated. In 9 cases the tumor colony formation was inhibited to more than 70–90%. The dose response curves for colonies derived from metastases and ascitic cells respectively proved to be similar. With the use of gestoneron 8/10 samples of the primary tumor showed an inhibition of colony formation of 40–60% of control at a concentration of 10^{-6} mol/l [Fig. 2 (a)]. In two samples colony formation was reduced to 20–25% of control. Again, the dose response curves from metastases and ascitic cells proved to show similar response patterns, i.e. at a concentration of 10^{-6} mol/l colony formation was reduced to 40–60% of controls [Figs 2 (b,c)].

No significant correlation between the dose re-

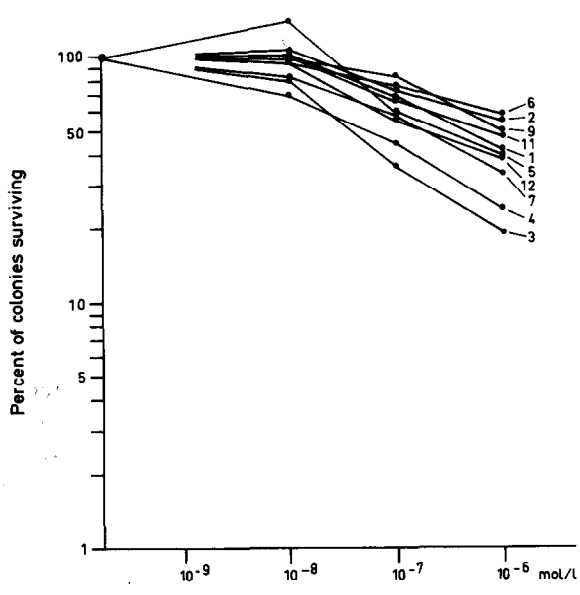

Fig. 2a

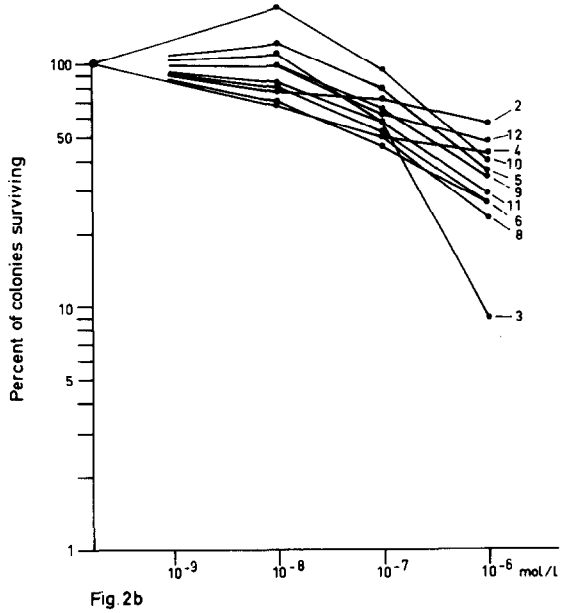

Fig. 2b

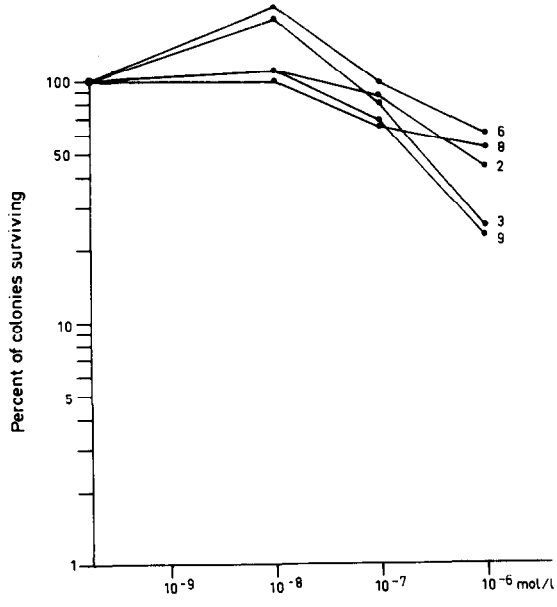

Fig. 2c

Fig. 2. Influence of gestoneron on the colony survival of human ovarian carcinoma cells from different tumor sites of the same patient (patients numbers 1–12). (a) from the primary tumor of the ovary, (b) from the abdominal metastasis, (c) from the ascitic fluid. Points represent the means of triplicate cultures. The reduction of colony formation at 10^{-6} mol/l ranged in all samples between 30% and 80% of the control cultures.

sponse and the receptor level was found. In our experiments even estrogen receptor negative tissues from primary tumor and metastases showed response to tamoxifen and gestoneron, i.e. patient Nr. 4/5/12 in primary tumor and patient Nr. 2/5/8 in metastatic tissues [Figs 1 (a,b) and 2 (a,b)].

DISCUSSION

The clinical application of the human tumor colony forming assay is hampered by the fact that obviously different biological properties of primary tumors and metastases can be tested only in a small number of patients. The results from probes out of the primary tumor must not necessarily predict the drug sensitivity of the metastasis. Schlag *et al.* [6] described that the plating efficiency of the metastatic tumor samples was higher than that from the primary tumor from various tumor types. In contrast, Tanigawa *et al.* [9] could not find significant differences in the plating efficiency when cells from primary tumors and from metastases from different sites were plated. The tumors in those series were breast carcinomas, colon carcinomas and lymphomas.

In the samples from our patients the plating efficiency in simultaneously performed assays was for the primary tumor 0.076%, for the cells from the ascitic fluid 0.082% and 0.12% for the solid metastasis of the abdomen. This significant difference ($P < 0.0532$) is supported by our observation, that the colonies from metastatic tissues could be evaluated earlier than those from the primary tumor. The comparison of the results from Schlag [6], Tanigawa [9] and ours suggests that the growth behaviour is dependent on individual properties of the tumor cells. The morphological differences as reported by other authors [6,24] and the higher cloning efficiency in the metastatic tissue indicates a heterogeneity in the clonable tumor cell compartment of primary tumor and metastasis [24]. The question if this heterogeneity influences the sensitivity for cytostatic drugs or hormonal treatment is still open. Schlag *et al.* [6,10] found that colonies from metastases are probably more sensitive than colonies from primary tumors. The used drugs were adriamycin, nitrosourea (BCNU), 5-fluorouracil and activated cyclophosphamide. Tanigawa *et al.* [9] found no satisfactory correlation of drug sensitivity of primary tumors and metastases using adriamycin, bleomycin, BCNU, 5-fluorouracil, mitomycin, melphalan and *cis*-platinum.

Antiestrogens and progestins are reported to be useful in the treatment of advanced ovarian cancer [13,14,25,26]. It has been shown that the antiestrogen tamoxifen and various progestin are able to inhibit tumor colony formation of ovarian epithel carcinoma *in vitro* [11,13]. Our simultaneously tested tumor samples show similar significant, dose dependent inhibition of colony formation by tamoxifen and gestoneron.

The fact that we did not find a significantly different hormonal sensitivity of primary tumor and metastases may indicate that the patient with advanced ovarian cancer may profit from an endocrine therapy with antiestrogen or progestins.

From the endocrine therapy of breast cancer we know that antiestrogens exert most of the effects through the specific estrogen receptor in steroid receptor positive carcinomas [27–29]. Teufel *et al.* [21] and Schwartz *et al.* [30] report that positive steroid receptors in ovarian cancer have no prognostical meaning or therapeutical value.

Our data suggest, that the likelihood of response to an endocrine therapy with antiestrogens or progestin is independent from the content of steroid receptors [Table 2, Figs 1 (a–c) and 2 (a–c)]. This supports the finding that antiestrogens may act also via an antiestrogenic binding site distinct from the estrogen receptor [31,32].

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