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Establishment of new epithelial carcinoma cell lines by blocking monolayer formation

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Abstract Human endometrial carcinoma cell lines were established by using a new cell culture technique. The malignant endometrial tumors were grossly disaggregated by mechanical means and cultivated in suspension culture. Adhesion to the bottom of the culture flasks was prevented by first coating the flasks with a thin agarose layer. Four cell lines were derived from 17 samples by this new technique. The cell lines obtained in this way were fully characterized, including karyotyping, intermediate filament staining and transplantation to nude mice. This new technique of initial suspension culture may also be applicable to other human tumors that are equally difficult to cultivate in vitro.

Key words Cancer · Cell lines · Endometrial cancer · Establishment

Introduction

The detailed investigation of human cancer requires the establishment of permanent cell lines from clinical tumor biopsies. During recent years, numerous protocols have been developed to define optimal conditions for the establishment of permanent cell lines and for their propagation in vitro. Yet there is no standard cultivation system for meeting the individual growth requirements of all types of tumor cells. Huge efforts have been made to establish breast cancer cell lines suitable for the in vitro investigation of this type of cancer. Yet the success rate has been low in most trials. Vandewalle et al. (1987) only obtained one new cell line from 47 specimens. Approximately 50 mammary cancer lines were described in 1991, of which perhaps 20 are sufficiently well-authenticated to merit continued use (O'Hare 1991). MCF-7, T47D, ZR-75-1 and MDA-MB-453 are the few cell lines that are used in most work dealing with breast cancer. Such a low number of in vitro cell lines does not represent the well-known diversity and heterogeneity of human breast cancer. In other tumor entities the number of cells lines is even smaller and the extensive investigation of endometrial cancer (Gurpide 1991) and some other types of cancer is limited by the lack of suitable cell lines.

It was several years before we were successful in establishing cell lines from endometrial cancer, and numerous modifications of media supplementation did not yield any improvement. Endometrial cancer cells generally adhere to the plastic surface of the culture flasks like many other epithelial cells but do not form continuously proliferating colonies, and we have tried to prevent this adherence. A thin layer of agarose was used to inhibit monolayer formation and spreading of the cells. Single cells and small tumor pieces were kept in suspension culture, and using this new technique of preventing monolayer formation, four new endometrial cancer cells lines were established. The cells were later adopted to standard monolayer cultivation. This technique facilitated the successful establishment of cell lines from endometrial cancer in 24% of all cases and opens new possibilities for the cultivation of epithelial cells from human tumors.

Materials and methods

Characteristics of the patients

All specimens were derived from patients who had been operated on for primary or recurrent endometrial cancer at the Department of Obstetrics and Gynecology of the Philipps University, Marburg. The main characteristics of the patients and tumors are summarized in Table 1.

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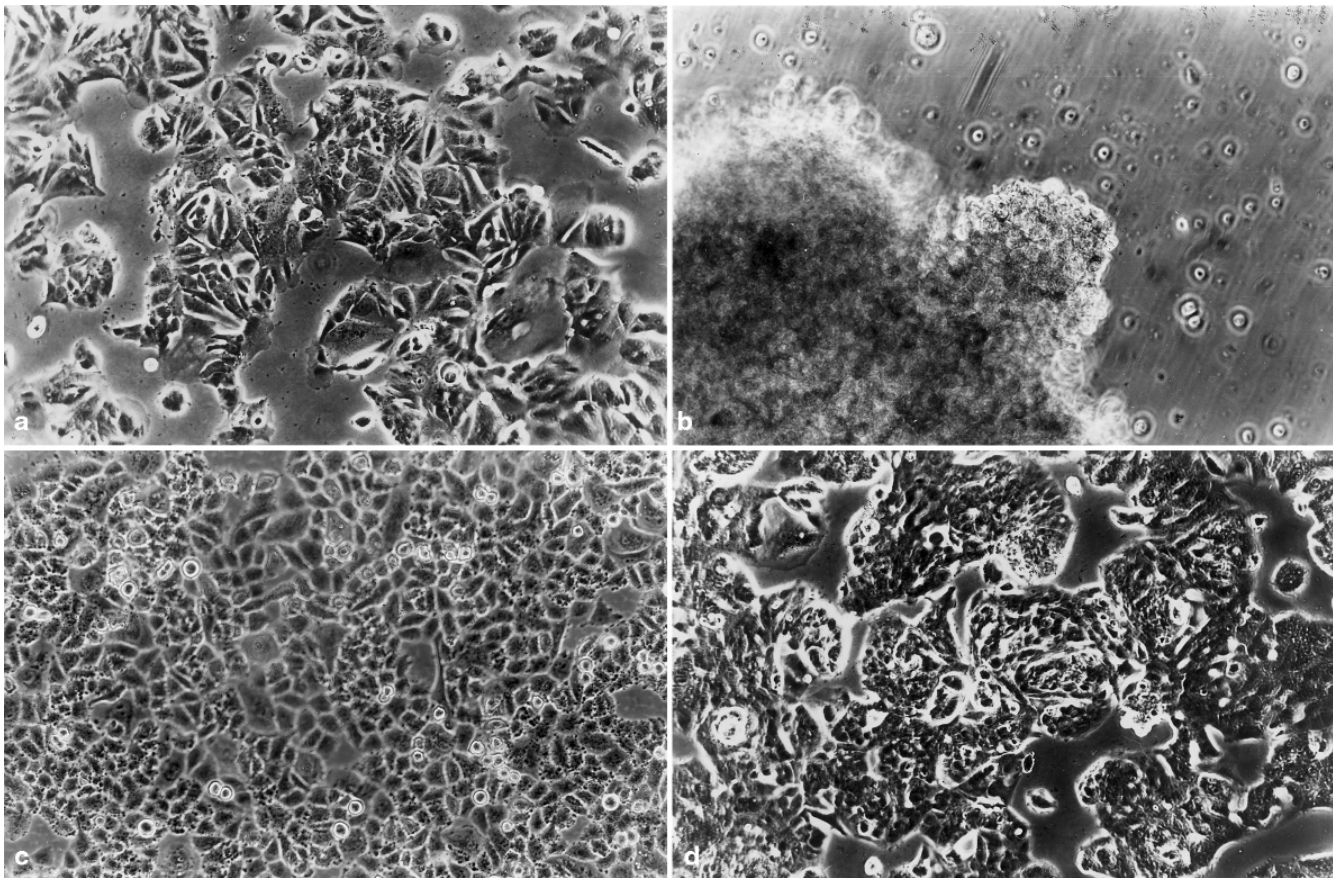

Fig. 1a–d Phase-contrast microscopy of endometrial cancer cells: **a** MFE-280, **b** MFE-294, **c** MFE-296, **d** MFE-319. ×110

Cell culture and media

Standard growth medium was based on Eagle's minimum essential medium and was enriched with 4 mM L-glutamine, 67 mg/l gentamicin sulfate (all from Biochrom, Berlin, Germany), 2.5 mg/l transferrin (Serva, Heidelberg, Germany), 40 IU/l insulin (Hoechst, Frankfurt, Germany), and 10% fetal bovine serum (Boehringer, Mannheim, Germany).

Culture flasks for the cell cultivation under non-adhering conditions by blocking monolayer formation were prepared as follows: 20 ml 0.5% agarose (Seakem, Hameln, Germany) in phosphate-buffered saline was poured into standard cell culture flasks (80 cm^2). After solidifying, the agarose was equilibrated three times with culture medium.

Tumor tissue was transferred to sterile culture medium in the operating theatre. Immediately after arrival in the cell culture unit the tissue was minced with crossed scalpels. The resulting suspension, containing single cells and small pieces of tumor tissue up to 1 mm in diameter, was pipetted into prepared culture flasks and incubated at 37 °C in a humidified incubator. All tumor specimens were also grown as monolayer cultures.

Fresh culture medium (5–10 ml) was added twice a week and cell viability was controlled by phase-contrast microscopy. At regular intervals, the tumor cell suspension was transferred to tubes and centrifuged (5 min at 400 *g*). The supernatant was discarded and the pellet was resuspended and poured back. In most cases single cells regressed within 1 or 2 weeks. Aliqots of proliferating cell cultures were transferred to plastic culture flasks to test their ability to grow under adherent conditions.

Tumorigenicity

Two female athymic nude mice were given s.c. injections of 10^6 cells at early passages. After 6–8 weeks, the tumors growing at the injection sites were removed from the animals and analyzed by standard histological techniques. Animal experiments were performed according to the German Law on the Protection of Animals.

Karyotype analysis

Chromosome preparations were performed as described (Kunzmann and Hölzel 1887; Hild and Fonatsch 1990).

All cell lines were analyzed cytogenetically after 6–12 months of in vitro cultivation.

Table 1 Characteristics of the patients and tumors from which the cell lines were derived. *ER*, estrogen receptor, *PR*, progesterone receptor

Cell line	Primary/recurrent tumor	Histology	Grading	ER (fmol/mg)	PR (fmol/mg)	Age (years)
MFE-280	Recurrent	Adenocarcinoma partly papillary	G3	47	514	77
MFE-294	Recurrent	Adenocarcinoma partly papillary	G2	3	8	69
MFE-296	Primary	Adenocarcinoma moderately diff.	G2	2	4	68
MFE-319	Primary	Adenosquamous carcinoma	G1–G2	171	184	81

Table 2 Patterns of immunocytochemical staining of cytokeratins and vimentin. Semiquantitative score (– up to +++)

Cell line	Cytokeratin				Vimetin
	7	8	18	19	
MFE-280	++	++/+++	+++	++	++
MFE-294	++	–	–/+	++	+++
MFE-296	+/++	++	+	+/++	++
MFE-319	++	+	+++	+	++

Table 3 Steroid hormone receptor content of the new endometrial cancer cell lines

	Receptor level (fmol/mg protein)		
Cell line	Estrogen receptor	Progesterone receptor	Androgen receptor
MFE-280	<5	14	<5
MFE-294	<5	21	<5
MFE-296	<5	7	30
MFE-319	<5	<5	<5

Steroid hormone receptor assays

A standard protocol was employed for the cytosolic receptor analyses of the endometrial cancer specimen and cellular material (EORTC 1980).

Immunocytochemistry of intermediate filaments

Cells were seeded on standard microscope slides in petri dishes and cultivated to subconfluence. The cell layers were fixed with acetone at 4 °C and air-dried. The following antibodies were applied: anti-vimentin Vim-3BH, and anti-cytokeratins 7, 8, 18 and 19 (Boehringer, Mannheim, Germany). For the negative controls the primary antibody was omitted.

Results

Establishment of endometrial cancer cell lines from suspension and monolayer cultures

Twenty samples of endometrial cancer tissue were obtained within 2 years from the operating theatre of our department. All samples were checked for cell viability by the trypan blue exclusion test. Sufficient amounts of vital tissue to start monolayer and suspension cultures in parallel were available in 17 cases. Four permanently growing cells lines (MFE-280, MFE-294, MFE-296, and MFE-319, Fig. 1 a–d) were derived from these 17 malignant endometrial tumors, corresponding to a success rate of 24%. The cell lines MFE-280, MFE-294 and MFE-319 were derived from suspension cultures. The monolayer cultures of these tumors were not successful. The establishment of the cell line MFE-296 was successful in monolayer as well as in suspension culture.

In the case of the cell line MFE-280, tumor cells were transferred from the suspension culture to the monolayer cultivation system every 2 or 3 weeks. But only after 9 months of cultivation in suspension did the MFE-280 cells acquire the ability to grow under adherent culture conditions. Up to this time the cells readily attached to the plastic bottom of the culture flasks but flattened and degenerated within 1–2 weeks. The suspension stock cultures remained in their proliferating state. It was subsequently possible to propagate the cells in monolayer and in suspension culture.

The establishment of the cell line MFE-319 was performed accordingly.

MFE-294 cells did not attach to the bottom of the culture flasks and were permanently grown in suspension culture.

The establishment of the cell line MFE-296 followed the same protocol. This cell line was derived from monolayer cultures using standard techniques. Mesothelial cells were partially removed by fractionated trypsinization. The suspension cultures, which were grown simultaneously, contained viable cells for up to 6 months. After the successful establishment of the cell line from monolayer culture, the suspension cultures were stopped.

Phenotypes of the cell lines

Cells grown on microscope slides were stained with antibodies to intermediate filaments. The stains were evaluated using a semi-quantitative score (– up to +++; Table 2). All cell lines expressed cytokeratins, demonstrating their epithelial character.

Heterotransplantation

All endometrial cancer cell lines formed tumors in female nude mice within 6–8 weeks after inoculation. The animals were not supplemented with hormones. After the tumors had reached 1–2 cm in diameter, they were removed. All the cell lines formed poorly differentiated tumors as shown in Fig. 2 a–d.

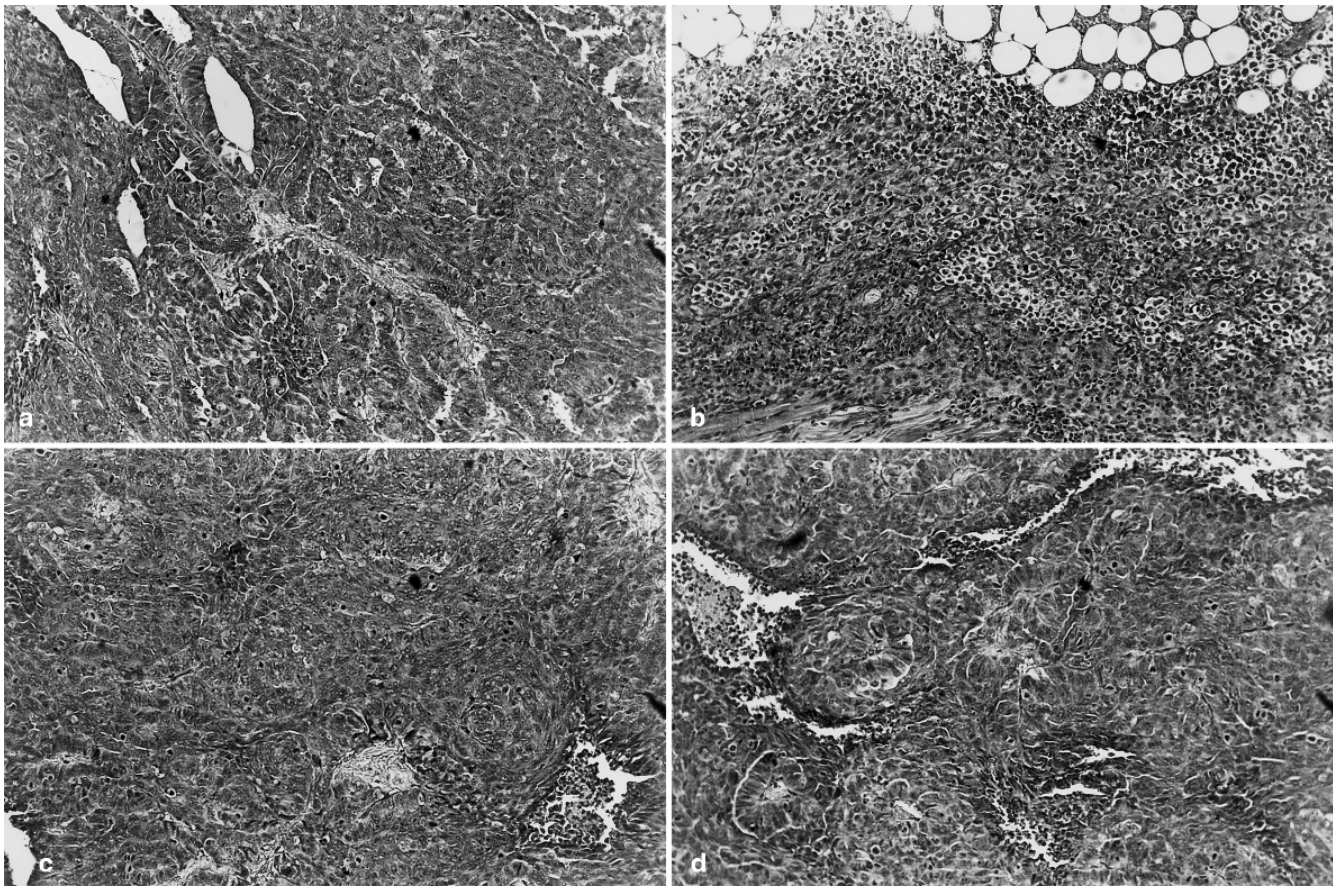

Fig. 2a–d Histological appearance of tumors grown in nude mice: **a** MFE-280, **b** MFE-294, **c** MFE-296, **d** MFE-319. ×110

Karyotype analyses

The chromosomal numbers of MFE-280 cells were widely distributed and showed aneuploidy. The modal chromosome number was in the tetraploid range. Among numerous marker chromosomes and sporadic structural rearrangements it was not possible to identify characteristic cytogenetic aberrations.

All MFE-294 cells showed a normal diploid female karyotype. No rearrangements or marker chromosomes were observed in the 20 metaphases analyzed.

MFE-296 cells were analyzed after 1 year of in vitro cultivation. The chromosome distribution pattern revealed near-tetraploidy for most cells. After trypsin/Giemsa banding, no structural aberrations or marker chromosomes were observed in any of the 10 metaphases analyzed (Hackenberg et al. 1994).

MFE-319 cells showed a karyotypic evolution among diploid and tetraploid cells. All cells were characterized by a reciprocal translocation of the long arms of chromosome 13 and 18. This translocation t(13;18)(q32;q21) represents the primary cytogenetic event in this cell line. Different additional structural rearrangements occurred subsequently as clonal or sporadic secondary chromosome aberrations with a distinct cell-to-cell heterogeneity.

Steroid hormone receptors

The concentrations of estrogen, progesterone and androgen receptors were analyzed using the dextran-coated charcoal method. Progesterone receptors were found in MFE-280, MFE-294, and MFE-296 cells. MFE 296 contained also androgen receptors. Estrogen receptors were found in none of the cell lines investigated (Table 3).

Discussion

The success rates for establishing permanent cell lines from various types of human cancer are between less than 1% in breast cancer (O'Hare 1991) and up to 50% in ovarian (Whelan et al. 1991) and colorectal cancer (Paraskeva and Hague 1991). In comparison with other tumor entities, the number of permanently growing human endometrial cancer cell lines is very small. Several authors have reported the successful establishment of cell lines from human endometrial carcinomas (Gorodecki et al. 1979; Hackenberg et al. 1994; Ishiwata et al. 1984; Kuramoto et al. 1972; Nishida et al. 1985; Richardson et al. 1984). In all of these reports one or two new cell lines were presented, but there are no data

on the number of primary cultures, that were necessary to obtain the new cell lines.

We have been able to establish several cell lines from endometrial, ovarian and mammary malignant tumors (Hackenberg et al. 1988, 1991, 1994; Hofmann et al. 1989) during recent years, but the success rate was very low for endometrial cancer. Numerous modifications of the culture medium and other experimental parameters did not yield a significant improvement. Finally, the combination of monolayer and suspension culture enabled us to establish four cell lines from 17 different tumours (24 %). The three-dimensional growth pattern facilitates direct and close-range cell-to-cell interactions, which may modify cellular metabolism, proliferation and differentiation. The need for cell-to-cell interaction is emphasized by the observation that small tumor pieces kept their viability while single cells degenerated rapidly. The high growth rate of mesothelial cells, which is often a problem in monolayer culture, was not observed in suspension culture. To our knowledge the long-term suspension cultivation of explanted human tumors is new. Short-term suspension culture has been successfully used to establish human sarcoma cell lines; the cells were cultivated in suspension and retransferred to monolayer culture, when the proliferation rate fell (Bruland et al. 1985). In our protocol the primary cells were propagated in suspension culture irrespective of their proliferation rate. Obviously the cells adapt to the in vitro situation within several months and acquire the ability to form monolayer cultures during these initial steps.

Using this new technique we were able to increase to 24% the success rate for establishing permanent cell lines from endometrial cancer.

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