ETN-1: A NEW HUMAN ENDOMETRIAL CARCINOMA CELL LINE PRODUCING ASCITES AND DISTANT METASTASES IN NUDE MICE


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A human carcinoma cell line (ETN-1) has been established from a skin metastasis of a moderately differentiated adenocarcinoma of endometrial origin. The cell line has been so far maintained for 27 months through 55 passages, growing as a monolayer as well as in 3-dimensional clusters with a population doubling time of 72 hr. The number of chromosomes per cell varied from 39 to 107 (average number 61.0 ± 19.8), with a modal number of 46–48. Seven clonal marker chromosomes were detected. Flow cytometric analysis revealed a population of pseudo-tetraploid cells (DNA index 2.1) next to a pseudo-diploid population (DNA index 1.1). The epithelial character of the cells was confirmed by a positive immunocytochemical reaction using monoclonal antibodies (MAbs) to different keratins, the epithelial cell markers BW 495/36 and HMFG-2, as well as by the presence of many junctional complexes. The tumour cells retained a positive reaction with the anti-ovarian carcinoma OV-TL 3, OV-TL 10 and OC 125 MAbs, although the reaction was markedly diminished in comparison with the original tumour. Tumour cells inoculated subcutaneously in nude mice produced well differentiated tumour nodules with formation of glandular lumina and basal lamina. Tumour cells injected intraperitoneally produced malignant ascites and regional as well as distant metastases of adenomatous character.

Cell lines derived from human neoplastic tissues are useful for the study of biological characteristics of malignant cells and of their sensitivity to chemotherapeutic and hormonal agents (Wolf et al., 1987; Hill et al., 1987). They also provide a source of tumour-associated antigens against which MAbs can be produced (Poels et al., 1984; Horowitz et al., 1985). A number of well characterized cell lines derived from endometrial cancers have been described (Kuramoto et al., 1972 and 1977; Merenda et al., 1975; Ishiwata et al., 1977 and 1984; Gorodecki et al., 1979; Gal et al., 1982; Way et al., 1983; Richardson et al., 1984; Noumoff et al., 1987). Most of these have been used to study the mechanisms of endocrine regulation of endometrial cancer (Shapiro et al., 1975; Satyaswaroop et al., 1978 and 1980; Suzuki et al., 1980). Little attention has been focused on the malignant potential of these cultures. Most of the cell lines described form local tumours at the site of injection after subcutaneous inoculation in nude mice. Intraperitoneal injection of cultured tumour cells has not been mentioned. We now report the establishment and characterization of a new endometrial carcinoma cell line (ETN-1), which forms local tumours after subcutaneous inoculation, and also gives rise to malignant ascites and widespread metastases after intraperitoneal injection.

MATERIAL AND METHODS

Medical history of donor of ETN-1 cells

A 61-year old female patient presented with carcinomatous lymphangitis of the upper leg and abdominal skin. Histopathological examination of an abdominal skin biopsy indicated localized adenocarcinoma with papillary and tubular structures and psammoma bodies. In her serum CA 125 reached 260 U/ml. Ultrasonographic examination showed an enlarged uterus with normal-sized ovaries and no signs of ascites. Laparoscopy confirmed these findings. Although no intra-abdominal metastases were detected, tumour cells were found in peritoneal washings. Curettage of the uterine body revealed that the primary tumour was a moderately differentiated, partly papillary growing endometrial adenocarcinoma with a minor squamous component. Tumour cells in the skin biopsy had the same morphologic aspect and immunohistochemical reaction pattern as in the curettage material (Table I).

Despite 3 courses of cytotoxic chemotherapy, daily administration of medroxyprogesterone acetate and repeated local irradiations, carcinomatous lymphangitis progressed. The patient's clinical condition deteriorated and she died 14 months after the initial skin biopsy.

Culture methods

Metastatic nodules obtained from skin biopsies (before any treatment was started) were minced into 1–2 mm³ pieces and washed with RPMI-1640 culture medium. The pieces were incubated in the same culture medium supplemented with sodium bicarbonate, HEPES (Dutch Modification—GIBCO, Paisley, Scotland), 15% foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Plastic tissue-culture flasks (Costar, Cambridge, MA) were kept in a humidified atmosphere of 5% CO₂ in air. The culture medium was changed twice a week. Confluent monolayers of tumour cells were dissociated with 0.25% trypsin (Difco, Detroit, MI) and 0.025% EDTA (Merck, Darmstadt, FRG) in Ca²⁺- and Mg²⁺-free Tyrode's solution, re-seeded in new culture flasks at a 1:2 split ratio during the first 10 passages and thereafter at a 1:4 split ratio. The cultures were treated with geneticin (Sigma, St. Louis, MO) to suppress fibroblast growth (Halaban and Alfano, 1984).

Light microscopy

Cells grown on Multispot slides (Flow, Irvine, Scotland) were fixed in methanol and stained with May-Grünwald-Giemsa stain.

Electron microscopy

Monolayers of tumour cells grown in 24-well plates (Flow) were fixed for 10 min in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 420 mOsM) at 4°C, post-fixed in 1% osmic acid in the same buffer and further processed according to standard procedures (Mungyer et al., 1987).

Population doubling time

For cell growth studies, plastic tissue-culture flasks with a growth area of 25 cm² were seeded with 25 × 10⁶ cells. The doubling time was calculated according to the method of van Nierkerk et al. (1988).

Indirect immunofluorescence technique

Cultured cells grown as monolayers on Multispot slides were used.

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(Flow) were fixed in methanol (−20°C) for 1–2 min and processed as described by Ramaekers et al. (1983a). The following mouse MAbS were used: A. RCK 102, RCK 105, RCK 106, RGE 53, OV-TL 12/5 and OV-TL 12/30, specifically directed against different cytokeratins (Ramaekers et al., 1983b and 1987; Broers et al., 1986; van Niekerk et al., 1988); B. vimentin-MAb RV 202 (Quax et al., 1985); C. desmin-MAb RD 301 (Quax et al., 1985); D. MAbs OV-TL 3, OV-TL 10 and OC 125 to ovarian carcino-dermat antigens (Bast et al., 1981; Poels et al., 1986; van Niekerk et al., 1988); E. MAb BW 495/36 to a 200-kDa glycoprotein of epithelial cells (Bosslet et al., 1986); F. epithelial marker HMFG 2 (Taylor-Papadimitriou et al., 1981).

**Cytogetic analysis**

After the tumour cells had been exposed to colchicine (1 μg/ml) for 90 min, they were prepared according to routine cytogenetic procedures (Scheres, 1972). Fifty well-spread metaphases were photographed following conventional Giemsa staining and chromosome numbers were counted from the photographs. Fifteen mitoses were also studied in detail after G-banding of the chromosomes by trypsin-Giemsa. Three of these cells were fully karyotyped.

**Flow cytometric analysis of cellular DNA content**

Suspensions of cultured cells were used for flow cytometric (FCM) analysis according to the method of Feitz et al. (1983). Chicken red blood cells and human lymphocytes were used as internal and external standards.

**Heterotransplantation in nude mice**

From various subcultures 5 × 10⁶ cells were injected either subcutaneously in the flanks of male and female nude BALB/c mice (n = 12) (TNO, Zeist, The Netherlands) or intraperitoneally (n = 12). Animals were observed weekly, and killed either when a subcutaneous tumour with a diameter of 1 cm was observed or when ascites developed. Tumours, ascitic cells and organs were processed for light and electron microscopy and immunohistochemical procedures.

**Receptor analysis**

Estrogen and progesterone receptor levels of cultured ETN-1 cells as well as ascitic tumour cells (3 × 10⁶ cells) were determined by monoclonal enzyme immunoassay (ER-EIA and PR-EIA, Abbott, Chicago, IL), according to manufacturer’s instructions.

**RESULTS**

**Light and electron microscopy of cell cultures**

After the 5th or 6th passage, the cell line exhibited stable morphology. The epithelial growth pattern was characterized by the formation of monolayers, multilayers, and 3-dimensional cell clusters consisting of “tadpole”-like cells as described for normal endometrial cells in culture (Mungyer et al., 1987) (Fig. 1a). Ultrastructurally, cells showed numerous junctional complexes of the fascia adherens type. Intercellular as well as cytoplasmic lumen formations were present.

**Growth characteristics of cell cultures**

The cell line proliferated with a doubling time of 72 hr during logarithmic growth. Cellular growth was not retarded when reaching confluence.

**Immunohistochemical studies of cell cultures (Table I)**

All cells stained homogeneously with the different cytokeratin antibodies and with the epithelial markers BW 495/36 and HMFG 2. Their glandular nature was confirmed by the positive reaction with RGE 53. No reaction was observed with RD 301 (desmin MAb). A positive reaction with the anti-vimentin RV 202 MAb was observed in approximately 30% of the ETN-1 cells. The ovarian carcinoma markers OV-TL 3, OV-TL 10 and OC 125 were expressed heterogeneously, and varied from weak to moderately positive.

**Cytogetic characterization and FCM analysis of cell cultures**

Chromosome counts of 50 mitoses showed a near-diploid chromosome number in more than half of the cells examined, the remaining cells being aneuploid. The chromosome number per cell varied from 39 to 107 (mean = 61.0 ± 19.8 s.d.) with a modal number of 46-48. Structural chromosomal abnormalities were common. Figure 2 shows a G-banded karyotype of a cell with 78 chromosomes. The majority of all chromosome markers studied appeared to occur sporadically, although 7 markers were considered to be clonal and present in each cell analysed. None of the marker chromosomes could be recognized as a HeLa cell marker.

**Flow cytometric analysis of propidium-iodide-stained ETN-1 cells**

Cells showed that the culture consisted of a mixture of 2 populations, corresponding to a pseudo-diploid (DNA index 1.1) and a pseudo-tetraploid (DNA index 2.1) peak, respectively.

**Subcutaneous hetero-transplantation**

A local tumour nodule consistently developed in both male and female mice. About 4 weeks after inoculation the tumour size had reached a diameter of 10 mm or more. Using light microscopy, the tumours localized subcutaneously were encapsulated within fibrous connective tissue (Fig. 1a). The tumour cells grew according to an adenomatous pattern, and abnormal mitoses were frequently observed. Ultrastructurally, the apical cell surface showed long and slender microvilli protruding into the lumina (Fig. 3a). Tumour cells were attached to other
by small desmosomes as well as by junctions of adherent type.
The basal tumour cells were adjacent to an incomplete and often
multiplied basal lamina (Fig. 3b). Occasional tumour cells were also scattered between connective-tissue elements
(Fig. 3c).

Immunohistochemical results were comparable with those
described for cultured cells, although the intensity of the reaction
with OV-TL 10 and OC 125 was diminished (Table I, Fig.
1e–f).

FIGURE 1 – Light microscopy and immunohistochemistry of ETN-1
cells in culture and after heterotransplantation (nude mouse) (scale
bars: 150 μm). (a) Cultured ETN-1 cells in an epithelial growth pat-
tern with elongated "tadpole"-like cells interspersed between larger
polygonal cells. Phase contrast. (b) Part of subcutaneously xe-
ognrafted adenomatous tumour with papillary growing projections.
Asterisks indicate necrotic areas, and arrows indicate the fibrous cap-
sule. Trichrome. (c) Liver metastasis. Trichrome. (d) Lymph node
with remnants of lymphoid tissue and metastasized tumour cells. Tri-
chrome. (e) Distinct immunofluorescence reaction pattern with RCK
106 in xenografted ETN-1 cells. Note the positive reaction in solitary
tumour cells (arrows). (f) A more irregular immunofluorescence stain-
ing reaction with RV 202 in ETN-1 xenograft. Note the negative
reaction of host connective tissue.

FIGURE 2 – G-banded karyotype of an ETN-1 cell with 78 chromo-
somes. M1: der(3)(1;3)(q21;p25). M2: der(1)(1;17)(q11;q21;
p36q21;q21). M3: der(2)(2;3)(q37;p25) M4: der(3)del(3)(q25). M5:

FIGURE 3 – Electron microscopy of xenografts (scale bars: 2.5
μm). (a) Detail of a xenograft in a nude mouse with a glandular lumen
and microvilli of junctioned tumour cells. (b) Basal side of a xe-
ognrafted tumour nodule with irregular duplication of the basal lamina
(arrows), and small protrusions of a tumour cell into the connective
tissue (asterisk). Note local absence of basal lamina (arrowhead). (c)
Solitary malignant cells with glyogen areas (arrows) within the sur-
rrounding fibrous compartment of xenografted tumours. In the neigh-
bourhood, projections of connective-tissue cells (asterisks).
Intraperitoneal hetero-transplantation

Ascites consistently developed about 2 months after intraperitoneal injection in both male and female mice and comprised 15-20 x 10⁶ tumour cells/ml. Light- and electron-microscopy showed essentially the same morphology for ascitic tumour cells and for cultured cells. The immunohistochemical characteristics were comparable with those of the cultured cells. Here again, as for the subcutaneous xenografts, the intensity of the reaction with OV-TL 10 and OC 125 was diminished (Table I).

Carcinomatous peritonitis was consistently observed in these mice with omental and mesenteric tumour localization. Metastases were also found in the liver (Fig. 1c), lymph nodes (Fig. 1d) and lungs. The growth pattern resembled that of the original tumour and of subcutaneous xenografts. Serial intraperitoneal transfer of malignant ascitic cells resulted in the production of ascites and metastases of similar morphological appearance within 2 weeks.

Receptor analysis

The cultured ETN-1 cells had a low oestrogen receptor level of 12 fmol/ml corresponding to 2,118 sites/cell, and a progesterone receptor level of 55 fmol/ml (7.9/99 sites/cell). No oestrogen receptors were detected in ascitic tumour cells, the progesterone receptor level being 62 fmol/ml (13,360 sites/cell).

DISCUSSION

We have described the establishment and characterization of a new endometrial carcinoma cell line ETN-1. Nearly 20 cell lines of endometrial origin have been described and characterized. Like ETN-1, most of these cell lines produced a local tumour nodule after subcutaneous hetero-transplantation into nude mice, their histology usually resembling that of the original tumour. However, intraperitoneal injection of cultured tumour cells has not yet been reported. The originality of our cell line is that it gives rise to malignant ascites with regional and distant metastases after intraperitoneal injection in nude mice.

The epithelial and glandular nature of the newly established cell line has been confirmed by both electron microscopy (junctional complexes, inter- and intracellular lumina) and immunocytochemistry (epithelial markers and anti-cytokeratins). The absence of desmin is in line with previous data, while the presence of vimentin in about 30% of cultured ETN-1 cells can be considered as an adaptation to cell culture conditions, rather than being indicative of the mesenchymal nature of the cells (Franke et al., 1979). With regard to the ovarian-carcinoma-associated antigens, the original tumour reacted positively with OV-TL 3, OV-TL 10 and OC 125. Cross-reactivity with these tumour markers often occurs when frozen sections of endometrial cancer are used (Poels et al., 1986). This may be partly due to the common embryonic origin of ovarian and uterine structures. Upon subculture, the reaction of the tumour cells with OV-TL 3, OV-TL 10 and OC 125 decreased. The variable expression of these tumour markers on cultured cells is well known and may be related to culture conditions rather than being an intrinsic property of the cell line itself (van Niekerk et al., 1988).

Subcutaneously induced ETN-1 tumours in nude mice formed well differentiated adenocarcinomas, based on histological characteristics and keratin filament patterns. Although no distant metastases developed, the presence of an incomplete basal lamina as well as of solitary malignant cells close to the primary nodules in between connective-tissue elements is indicative of the metastatic potential of this cell line. Intraperitoneal injection of cultured cells resulted in malignant ascites, together with regional and distant metastases. Malignant ascitic cells could be serially transferred. Cross-reactivity with MAbs to ovarian-carcinoma-associated antigens decreased further upon hetero-transplantation into nude mice. However, expression of vimentin and cytokeratin remained stable. This has often been noticed in several other gynecological adenocarcinomas (Puts et al., 1987). It may be related to the rapid growth and metastatic properties of this cell line (van Niekerk et al., 1988).

Endometrial carcinoma cells propagated in long-term culture and growing in nude mice are valuable model systems for studying the biology and treatment of endometrial cancer. Subcutaneous and intraperitoneal hetero-transplantation of ETN-1 cells into nude mice obviously results in 2 different human tumour model systems (one with and one without metastatic potential). This provides the opportunity to compare both model systems with respect to biological aspects and responses to chemotherapeutic and hormonal agents.

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