Trophectodermal carcinoma: mouse teratocarcinoma-derived tumour stem cells differentiating into trophoblastic and yolk sac elements

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SUMMARY

The retransplantable tumour line derived from a spontaneous ovarian murine teratocarcinoma (Fekete & Ferigno, 1952) was cloned and characterized using light and electron microscopic and immunohistochemical techniques. Grown in ascites, the tumour consisted predominantly of stem cells and a small number of differentiated derivatives. The stem cells expressed surface reactivity with antibody to SSEA-3 and Forssman antigen, alkaline phosphatase, focal cytoplasmic reactivity with antibody to SSEA-1, and varying amounts of cytoplasmic glycogen and 3 beta-hydroxysteroid dehydrogenase. Their cytoskeleton reacted with antibodies to keratin and vimentin. The differentiated derivatives formed approximately 5–15% of the total cell population in ascites and appeared either as giant cells or were characterized by their reactivity with antibodies to H-2 or a-foetoprotein or intracellular and pericellular laminin or high levels of 3 beta-hydroxysteroid dehydrogenase activity. Solid tumours produced from subcutaneously injected cells had a variegated appearance suggesting, that like the limited differentiation in the ascites, the stem cells can give rise to trophoblastic, as well as parietal and visceral yolk sac elements. On the basis of the presented data the tumour stem cells were considered as representing malignant equivalents of the common precursor of trophoblastic, visceral and parietal yolk sac cells most likely corresponding to trophectoderm. Accordingly, the tumour was designated as trophectodermal carcinoma.

INTRODUCTION

In 1952, Fekete & Ferigno described a spontaneous ovarian teratocarcinoma in a C3H mouse. A retransplantable tumour line was established from the original tumour and has been propagated in ascites form in several laboratories under the name of 'embryonal ovarian carcinoma' (Order, Donahue & Knapp, 1973), or ovarian teratocarcinoma E6496 (Michaelson et al. 1979; Vanhaelen, Fisher, Appella & Ramathan, 1981; Lichtenstein, Kahle, Berek & Zighelboim, 1984). It has been claimed that the stem cells of this tumour cell line have lost their capacity for differentiation (in contrast to the original ovarian tumour which was

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a typical teratocarcinoma composed of different somatic tissues) and that the tumour grows 'as a homogeneous population', either of single cells in the peritoneal cavity or as a solid tumour subcutaneously (Michaelson et al. 1979).

We have recently acquired the tumour E6496 and have started propagating it in vivo. Analysing the tumour morphologically we noticed that the population of cells grown in ascites is not absolutely homogeneous and also that the stem cells apparently can differentiate both in vivo and in vitro. We decided therefore, to clone and characterize this tumour and thus determine the nature of its stem cells. In this report we show that the tumour stem cells have immunohistochemical characteristics of trophectodermal cells from the mouse blastocyst and that the tumour thus represents most likely a malignant equivalent of the trophectoderm, i.e., a trophectodermal carcinoma. As such it provides additional data about the trophectodermal cell lineages in the mouse (Papaioannou, 1982; Gardner, 1982), and could also represent a valuable model for studying trophoblast-specific biological phenomena such as endoreduplication of DNA with giant cell formation (Gardner, 1972; Rossant & Tamura-Lis, 1981), or steroid hormone production (Sheth, Koszalka & Brent, 1978).

MATERIALS AND METHODS

Propagation of tumour

The tumour cell line developed from the spontaneous ovarian teratocarcinoma E6496 of a C3H/Fe mouse (Fekete & Ferigno, 1952) was obtained from Dr. R. F. Ozols, NIH. It has been maintained by serial intraperitoneal (i.p.) transplantations initially in C3H/HeJ mice, and later also in outbred Swiss Webster (SW) mice. In October 1983 the tumour was cloned according to the modified protocol of Kleinsmith & Pierce (1964). To that effect individual small tumour cells were aspirated under a dissecting microscope into a micropipette with an internal diameter of approximately 30 μm, and deposited into the surgically opened abdominal cavity of adult SW mice. Since then all the experiments were performed with a clonal line designated E6496D. Data reported here refer to this clonal tumour line.

Light and electron microscopic and immunohistochemical studies

The cells harvested from ascites fluid were studied either fresh unfixed, washed in phosphate-buffered saline, or fixed in cold absolute acetone (for immunohistochemical studies) or in cold 2.5% glutaraldehyde (for electron microscopy). Prior to fixation for light microscopic studies, the cells were smeared on microscopic slides and air dried for 1 h. Smears were stained with haematoxylin and eosin or PAS with and without diastase pretreatment. Solid tumours grown subcutaneously were also sampled and examined on freshly frozen cryostat sections, paraffin sections or plastic-embedded material prepared for electron microscopy.

Immunohistochemical studies were performed with the previously characterized antibodies recognizing the following antigens: SSEA-1 (Solter & Knowles, 1978), SSEA-3 (Shevinsky, Knowles, Damjanov & Solter, 1982), laminin (Martinez-Hernandez, Miller, Damjanov & Gay, 1982), Forssman antigen (Sterin et al. 1978), keratin (Sun, Shih & Green, 1979), vimentin (Frank & Warren, 1981). Anti-H-2k serum (C57BL/10Sn anti B10.BR) was obtained from Dr. J. Ray, NIH. Polyclonal rabbit anti-mouse alphafoetoprotein (AFP) antiserum was prepared in our laboratory using AFP electrophoretically separated from 16-day pregnant mice amniotic fluid according to the procedure of Dziadek & Adamson (1978). The antiserum was absorbed with adult mouse serum and gave a single precipitation line on Ouchterlony plates when tested against mouse amniotic fluid. A goat anti-mouse amniotic fluid AFP antiserum, provided kindly by
Trophectodermal carcinoma

Dr. E. Ruoslahti was also used. Indirect immunofluorescence and immunohistochemistry on cryostat section of tumours and of cell smears were carried out as previously described (Fox, Damjanov, Knowles & Solter, 1984). Flow cyt fluorimetric analysis of SSEA-1 and SSEA-3 expression on unfixed cells from ascites was performed as described by Andrews et al. (1982), using an Ortho-Instruments cytofluorograph. Fluorescein-isothiocyanate-conjugated second antibodies of appropriate specificities for the assays, were purchased from Cappel Laboratories, Cochranville, PA. Slides were examined with an Olympus photomicroscope equipped for epifluorescence illumination.

**Enzyme histochemistry**

Alkaline phosphatase was demonstrated on formalin-fixed cells according to the technique of Burnstone as modified previously (Damjanov, Solter & Skreb, 1971b). The activity of 3 beta-hydroxysteroid dehydrogenase was demonstrated in unfixed cells according to Botte, Tramontana & Chieffi (1968). Control smears were incubated in media lacking the substrate. Dehydrogenase activity was also inhibited by 20 minute formalin fixation at room temperature.

**Solid tumour formation**

Cells from the ascites were injected into C3H/HeJ or SW mice subcutaneously in a dose of 1–5 (× 10⁶) cells. The tumours were harvested 3–6 weeks thereafter and fixed in glutaraldehyde for electron microscopic studies or freshly frozen for immunohistochemical studies. The light microscopic sections prepared from paraffin-embedded tissues were stained with haematoxylin and eosin.

**Immunodiffusion studies**

Ascites fluid of tumour-bearing mice was harvested, centrifuged at 100 g for ten minutes and the supernatant was assayed by Ouchterlony double diffusion using the rabbit anti-mouse AFP.

**RESULTS**

**Tumour growth**

Tumour line E6496 is easily grown in ascites form. It can be propagated in mice of different strains (Vanhaelen et al. 1981). Initially we have grown the tumour by injecting 10⁶ cells into the abdominal cavity of C3H/HeJ mice; but since the tumour grew equally well in outbred SW mice all the experiments reported here were performed on outbred mice. 10⁶ cells injected into female SW mice will produce up to 30 ml of ascites and kill the host 20–25 days after inoculation. Intravenous injections of 10⁶ cells into 15 mice did not produce any tumours. On the other hand 5 × 10⁶ cells injected subcutaneously invariably produced solid tumours at the site of injection.

The original tumour was cloned by picking up single cells into a mouth pipette and by depositing the cell into the abdominal cavity of adult female SW mice. From 30 mice injected with single cells two formed ascites 35 and 40 days after inoculation respectively. Both of these clonal lines were analysed within the first six weeks after the animals developed ascites, i.e. two to five transplant generations after cloning. The clones were compared with the parent line and since no differences were noticed, one of the lines designated as E6496D was used for all experiments described in this report. The clone designated E6496D reanalysed in March 1984 exhibited the same features as 6 months before.
Cell morphology

In the ascites form, the tumour grew in form of a single-cell suspension or loosely attached aggregates. Most of the tumour cells (85–90%) measured 12–15 μm in diameter (Fig. 1A,B). These cells had large, hyperchromatic, irregularly shaped nuclei surrounded by a small amount of cytoplasm. Cytoplasm of most cells stained with PAS, but the staining could be abolished by diastase pretreatment (Fig. 1A,B). The ascites also contained larger cells many of which were often distinctly vacuolated (Fig. 1B).

Immunocytochemistry

Immunocytochemically, most of the acetone-fixed or unfixed cells reacted in a finely granular cell surface pattern with the antibody to SSEA-3. There were,
Trophectodermal carcinoma

however, also some larger cells that were unreactive (Fig. 2). Approximately 3–5 % cells showed cytoplasmic staining with anti-SSEA-3. Most of the cells exhibited immunoreactivity with the antibody to SSEA-1, usually in the form of a single or several cytoplasmic dots varying in size and shape from one cell to another (Fig. 2B). These cytoplasmic dots were seen only on fixed cells. Except for the occasional reaction of some larger cells, no cell surface immunoreactivity was seen on either acetone-fixed or unfixed cells exposed to the antibody to SSEA-1. These findings were confirmed by flow cytofluorimetry which showed that about 95 % of unfixed cells reacted with antibody to SSEA-3, whereas only a negligible number of cells reacted with the antibody to SSEA-1 (Fig. 3). There was no immunohistochemically detectable H-2k on 85–90 % of tumour cells grown in either C3H/HeJ or SW mice, which obviously correlated with the ability of the tumour to grow in different strains of mice irrespective of their haplotype (Vanhaelen et al. 1981). The remaining 10–15 % cells showed weak reactivity with the antibody to H-2k, which was, however, only slightly stronger than the background reaction obtained in control

Fig. 3 Flow cytofluorimetric separation of cells labelled with antibodies to SSEA-1, SSEA-3 and the immunoglobulin of the PX3 lymphoma as control. Approximately 95 % of cells react with antibody to SSEA-3 whereas a non-significant number reacts with antibody to SSEA-1.
smears incubated with normal mouse serum as the substitute for the primary specific antibody. The immunoreactivity of the serum could be fully absorbed with the spleen cells of C3H/HeJ mice but not the C57BL/10 cells and was thus considered to represent a true weakly positive reaction on some tumour cells.

Most of the small cells (80–85 %) reacted strongly with the antibody to the Forssman antigen (Fig. 4), whereas the larger cells and some smaller cells were unreactive. Antibody to α-fetoprotein reacted with approximately 5 % of smaller tumour cells (Fig. 5). Approximately 3–5 % of cells reacted with the antibody to laminin (Fig. 6). The reaction product was seen either in the form of small cytoplasmic specks or pericellular streaks and small aggregates located between adjacent cells.

Antibodies to vimentin reacted with almost all cells, whereas the antibody to keratin reacted with all smaller cells and most, but not all, larger cells (Fig. 7). The antibodies outlined the cytoskeletal meshwork which varied in appearance from one cell to another, although in general it was coarser in the larger than in smaller cells.

**Electron microscopic findings**

The predominant tumour cells in the ascites had irregularly shaped, deeply indented nuclei and small to moderate amounts of cytoplasm that contained a few mitochondria, a few short profiles of rough endoplasmic reticulum, numerous free ribosomes and quite prominently small aggregates of glycogen (Fig. 8). Glycogen, as it often occurs in glutaraldehyde-fixed cells was frequently washed out leaving small 'empty' spaces in the cytoplasm. The cell surface formed microvilli but no distinct intercellular junctions.

In addition to these stem cells, ascites contained larger cells measuring up to 40–50 μm in diameter. Mostly these cells had abundant cytoplasm and a deeply indented or segmented nucleus (Fig. 9). These giant cells were considered to represent syncytiotrophoblastic cells. Also there were smaller cells containing a large number of clear subplasmalemmal vacuoles, and showing close membrane apposition with adjacent cells of the same type (Fig. 10). These cells were considered to correspond to the AFP-positive, visceral yolk sac carcinoma cells. AFP could be demonstrated by the Ouchterlony technique in the ascites fluid. Also there were somewhat larger cells exhibiting well-developed rough endoplasmic reticulum and containing basement-membrane-like amorphous material in their cytoplasm (Fig. 11) corresponding to parietal yolk sac elements.

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**Fig. 4** Cells reacted with antibody to Forssman antigen. Most of the cells show positive reactivity. ×320.

**Fig. 5** Cells reacted with antibody to alpha-fetoprotein. Approximately 5% react positively. ×320.

**Fig. 6** Cells reacted with antibody to laminin. Note immunoreactive material between some cells in the centre of the photograph. ×420.

**Fig. 7** Cells reacted with antibody to keratin. All cells in this field contain cytoplasmic filaments reacting with the antibody. ×360.
Trophectodermal carcinoma

Figs 4–7
Figs 8–9 for legend see page 134
Trophectodermal carcinoma

Figs 10-11 for legend see page 134
Enzyme cytochemical demonstration of 3 beta-hydroxysteroid dehydrogenase in ascites cells. Some reactivity is seen in the cytoplasm of most cells. Note that the large cells exhibit very high enzyme activity. ×320.

Enzyme cytochemical and supravital staining

Most of the tumour cells exhibited weak cell surface reactivity of the alkaline phosphatase. Some cells displayed strong cell surface as well as cytoplasmic reactivity of alkaline phosphatase. Activity of 3 beta-hydroxysteroid dehydrogenase could be detected in most, but not all, small cells and it was most prominent in the large cells (Fig. 12). Enzyme activity could not be detected in control smears. In accordance with these histochemical findings, all female tumour-bearing animals exhibited signs of increased steroid hormone stimulation evidenced by marked swelling of the uterus and ovaries, which contained numerous corpora lutea.

Morphology of solid tumours

Solid tumours produced from ascites cells injected subcutaneously into mice grew considerably slower than the ascites tumours, and the central portion of each
Trophoblastic carcinoma

Tumour was extensively necrotic. Histologically the tumours had a variegated appearance. In some areas the tumour was apparently composed of cells resembling those in the ascites. These cells were loosely arranged and did not form any distinct pattern (Fig. 13A). Admixed to the small cells there were scattered or grouped giant cells measuring up to 50 μm in diameter. In other parts of the tumour, cells formed a reticulated meshwork of interlacing strands remarkably resembling the so-called endodermal sinuses of the murine placenta and the so-called Schiller-Duval glomeruloid bodies of human endodermal sinus tumours (Fig. 13B). These findings were highly reminiscent of the rat visceral yolk sac carcinoma (Sobis, VanHove & Vandeputte, 1982). Further, some tumours contained broad areas composed of basement-membrane-like hyalin material typical of parietal yolk sac carcinoma of mice and rats (Fig. 13C). This extracellular hyalin reacted strongly with antibodies to laminin (Fig. 14).

Ultrastructurally the cells in the solid tumour resembled those seen in the ascites. Thus, there were glycogen-rich cells corresponding to stem cells; cells rich on rough endoplasmic reticulum surrounded by basement membrane material corresponding to parietal yolk sac cells; cells with abundant lysosomes, lipid droplets and cytoplasmic vacuoles corresponding to visceral yolk sac cells; giant cells corresponding to syncytiotrophoblastic cells; and also cells of intermediate phenotype that could not be properly classified.

DISCUSSION

The tumours derived from mouse embryos, such as embryonal carcinoma or parietal yolk sac carcinoma have been compared with normal ectodermal cells in the peri-implantational mouse embryo or the parietal yolk sac cells respectively (Solter & Damjanov, 1979). In the present paper we describe the phenotype of yet another malignant cell type which, we believe, could be used for the study of differentiation along the pathway leading to the formation of placental and yolk sac structures in the mouse. Evidence presented in this paper indicates that: 1) the tumour stem cells are distinct from embryonal carcinoma cells; 2) morphologically and immunochemically they correspond to trophoblastic cells in the early blastocyst; 3) developmentally they may represent the malignant equivalent of a putative common precursor of parietal and visceral yolk sac and trophoblastic cells.

E6496 stem cells are not embryonal carcinoma or endodermal (yolk sac) carcinoma cells

Tumour line E6496 originated from serial retransplantations of a pluripotent teratocarcinoma described by Fekete & Ferigno (1952). It would thus be quite logical to assume that the stem cells of this tumour line correspond either to typical embryonal carcinoma cells (Solter & Damjanov, 1979) or developmentally restricted embryonal carcinoma cells, which like the cell line F9 have lost their
Fig. 13 Histologic appearance of solid tumour produced from ascites tumour cells injected subcutaneously. (A) Part of the tumour is composed of small dissociated cells and giant cells. (B) Reticulated meshwork of tumour cells with formation of glomeruloid bodies. (C) Broad zones of basement-membrane-like extracellular hyalin. ×240.
pluripotency and have retained only a limited capacity of differentiation into pari-
etal and visceral endoderm (Hogan, Barlow & Tilly, 1983). However, E6496 cells
differ significantly from either pluripotent or developmentally restricted embryonal
carcinoma cells, and also from closely related primitive endodermal cell lines
(Searls & Edidin, 1982; Kahan & Adamson, 1983). In contrast to typical murine
embryonal carcinoma cells which express cell surface SSEA-1 (Solter & Knowles,
1978) and do not express cell surface SSEA-3 (Shevinsky et al. 1982), E6496 cells
express prominently surface SSEA-3 and lack surface SSEA-1. In contrast to the
cytoskeleton of typical murine embryonal carcinoma cells which is composed of
vimentin only (Paulin, 1981), these stem cells contain filamentous keratin, as well
as vimentin. Stem cells of E6496 tumour differ from parietal yolk sac carcinoma in
that they do not produce extracellular laminin (Howe & Solter, 1980). Unlike
visceral yolk sac carcinoma the stem cells of the present tumour do not synthesize
α-foetoprotein (Adamson, Evans & Magrane, 1977). In contrast to primitive en-
dodermal carcinoma TerC (Searls & Edidin, 1982) stem cells of E6496 do not
express H-2.

Ultrastructurally, E6496 differed from various endoderm-like cell lines or typical
embryonal carcinoma cells (Damjanov, Solter, Belicza & Skreb, 1971a). The fairly
conspicuous aggregates of glycogen seen in most E6496 tumour stem cells are not
a feature of either embryonal carcinoma cells or other related mouse cell lines.
Activity of 3 beta-hydroxysteroid dehydrogenase was not recorded in any of these
cell lines either. Steroid-hormone synthesis may occur in rat yolk sac carcinoma
(Sobis et al. 1982), but apparently only in large terminally differentiated syncytio-
trophoblast-like giant cells.
**E6496 stem cells represent malignant equivalents of trophodermal cells from early blastocyst**

Tumour cells forming the bulk of the cell population in ascites were considered to represent the stem cells of E6496 tumour. Ultrastructurally, these cells appeared 'primitive' and resembled stem cells from other tumours. Clonal lines derived from individually selected cells had the same characteristics as the non-cloned tumours indicating that single tumour cells have indeed the features of stem cells, i.e. can give rise to other more undifferentiated cells and also replicate in an undifferentiated form.

The following evidence suggests that the stem cells of E6496 correspond to trophodermal cells from early blastocysts: 1) like the trophodermal cells of early blastocyst E6496 stem cells express surface SSEA-3 (Shevinsky et al. 1982), alkaline phosphatase (Vorbrodt, Konwinski, Solter & Koprowski, 1977), Forssman antigen (Willison & Stern, 1978); 2) in the cytoplasm of these cells, as in the trophoderm, there are visible cytokeratin filaments (Brulet, Babinet, Kemler & Jacob, 1980) and a few endocytotic vacuoles (lysosomes) (Solter, Damjanov & Skreb, 1973); 3) SSEA-1, an antigen present on the surface of inner cell mass cells (Solter & Knowles, 1978) and in the cytoplasm of visceral endodermal and trophodermal cells (Fox, et al. 1981) as well as in the cytoplasm of acetone-fixed trophodermal cells in the blastocyst (Damjanov, unpublished), is present in the cytoplasm of most tumour cells of E6496; 4) like the trophoderm of mouse blastocyst, especially under influence of oestrogen (Naeslund, Lundkvist & Nilsson, 1980) these cells contain glycogen granules and even aggregates of glycogen-like trophoblast-like cells derived in vitro from the inner cell mass (Hogan & Tilly, 1978 cf. Fig. 5); 5) finally stem cells of E6496 show small but definitive potential for steroid synthesis which suggests most strongly that these cells are indeed trophodermal in origin since this enzyme is found only in trophodermal cells lineages (Chew & Sherman, 1975).

**E6496 stem cells differentiate into trophoblastic, visceral and parietal yolk sac cells.**

Stem cells of E6496 undergo limited spontaneous differentiation in the ascites. Giant cells were interpreted as representing trophoblastic giant cells (Rossant & Tamura-Lis, 1981). High activity of 3 beta-hydroxysteroid dehydrogenase (Botte et al. 1968; Sobis et al. 1982) confirmed their trophoblastic nature. The cells associated with extracellular laminin were interpreted as parietal endodermal cells, whereas alpha-foetoprotein was taken as an indicator of visceral yolk sac differentiation (Hogan et al. 1983). Cells showing similar signs of terminal differentiation were seen in solid tumours produced from stem cells injected subcutaneously into mice as well as in vitro.

Since the tumour line E6496D was derived clonally from a single cell and since this line showed the same tridirectional differentiation one can confidently conclude that the stem cells of this tumour represent a common precursor of visceral
Trophectodermal carcinoma

and parietal yolk sac cells on one side and trophoblastic cells on the other. If one accepts the previously presented evidence that the stem cells of E6496 represent trophectodermal cells, it would then follow that trophectoderm in the mouse represents a common precursor of both trophoblast and yolk sac cells. However, since the cell lineage analysis does not provide support for the existence of a common precursor for trophoblast and yolk sac cells in the normal mouse embryo (Papaioannou, 1982; Gardner, 1982), it remains to be determined how relevant the present data are for the understanding of normal embryogenesis.

In view of the possibility that the tridirectional differentiation of E6496 stem cells represents a unique feature of some malignant teratocarcinoma or embryo-derived cells, it should be noted that the present tumour is not unique and that some of the findings pertaining to this tumour have been described in other tumour models as well. Thus, Nicolas et al. (1976) developed a cell line which they called trophoblastoma. Photograph of a solid tumour produced from this trophoblastoma cell line published by Nicolas et al. (1976) resembles, to an extent, E6496. The peculiar endodermal sinus pattern seen in our tumour was not illustrated, and it was not stated whether yolk sac elements were also present. The trophoblastoma cell line, like E6496, contains keratin (Brulet & Jacob, 1982). Sobis et al. (1982b) reported placenta-derived rat yolk sac tumours with histologic features identical to those seen in solid tumours produced from E6496 cells in the present study. The present report shows, however, that mouse tumours could also show the morphologic features typical of rat tumours and that extraembryonic (yolk sac-trophoblastic) elements could indeed all originate from a common precursor as postulated by Gaillard (1973, 1981).

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REFERENCES

I. DAMJANOV, A. DAMJANOV AND P. W. ANDREWS


Trophectodermal carcinoma


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