Blood formation in a clonal cell line of mouse teratocarcinoma

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SUMMARY

Pluripotent cells of a teratocarcinoma clonal line differentiate into various tissues when they are cultivated in submerged cultures. We describe here the formation of endodermal vesicles and blood islands in organotypic cultures of such cells. These are the first macroscopically visible tissues to appear in organ culture and they give rise to megalocytes, the ultimate stage of blood cell evolution in the normal mouse embryo yolk-sac. As in the normal embryo yolk-sac, the formation of haemoglobin-containing cells stops after a while, but it is not replaced by adult haemopoiesis in culture.

INTRODUCTION

Teratocarcinomas are transplantable malignant tumours which contain, besides a variety of differentiated tissues, a distinctive cell type known as embryonal carcinoma cell (EC) (Stevens, 1970). The latter is the stem cell of these tumours and shows remarkable similarities to early embryonic cells (Jacob, 1975). Several lines of EC cells have been isolated in vitro (see review of Martin, 1975) and it was shown in vitro as in vivo, that single cells of some clonal lines give rise to differentiated cells of the three primary germ layers (Pierce & Verney, 1961; Lehman, Speers, Swartzendruber & Pierce, 1974; Nicolas et al. 1975).

The present note reports the successive formation in organ culture conditions of endodermal vesicles and blood islands in a EC line isolated from an ascitic embryoid body of a teratocarcinoma-bearing 129/Sv mouse (Jakob et al. 1973). The endodermal structures and the differentiation of blood cells in teratocarcinoma cultures and in mouse embryo yolk-sac are compared.

MATERIAL AND METHODS

Cell cultures

The isolation and properties of the established EC line PCC₃/A/1 have been described previously (Jakob et al. 1973; Nicolas et al. 1975). The culture is

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maintained in an undifferentiated state by serial transplantation every 48 h at
8·10³ cells per cm² in tissue-culture-treated Petri dishes (NUNC). The cells,
dispersed by pipetting, are cultivated in Dulbecco’s modified Eagle’s medium
with 15% foetal calf serum (selected batches). If the cells are kept at confluency,
differentiation occurs and gives rise to a multilayered sheath in which several
tissues appear and very few EC cells persist.

Organotypic cultures

The cells are suspended by gentle pipetting. The cell suspension is centrifuged
at 100g for 2 min and the pellet is separated into small pieces containing about
10⁷ cells which are placed upon the surface of a Millipore filter of 0·22 μm pore
size. The filters, sterilized by immersion in 70% ethanol for 30 min and rinsed
in saline, are placed on the surface of a Trowell-type screen. The medium is the
same as for cell cultures and the explants are kept at gaz-medium interphase.
Both types of culture are incubated at 37°C in 5% CO₂ in air with 100%
humidity. The organotypic cultures are observed daily with a stereomicroscope.

Histochemical characterization of haemoglobin

The zinc-cyanol technique of Dunn (1946) is applied to the explants in toto
after fixation with formalin-ferricyanide (Slonimsky & Lapinsky, 1927); the
fixation converts haemoglobin into methaemoglobin. One percent cyanol solution
is boiled with zinc powder to obtain a cyanol leucodervative. Transfer of
nascent hydrogen from this reagent to methaemoglobin leads to the initial
colour regeneration.

Histological observation

Tissues fixed in Bouin’s or Gendre’s fluid are cut into 5 μm serial sections to
which Masson’s staining procedure modified by Goldner (1938) is usually
applied. The periodic acid Schiff (PAS) technique (Hotchkiss, 1948) is also used
to detect polysaccharides. Presence of glycogen is monitored by the amylase
digestion test.

Electron microscopy

Yolk-sacs of 8½-day mouse embryo and the blood islands which appear in
organ cultures, are fixed: first with 3% glutaraldehyde in phosphate buffer pH
7·2 for 20 min and second with 1% osmium tetroxide in the same buffer at
4°C for 1 h. Tissues embedded in Epon 812 are cut in semi-thin sections and
stained with toluidine blue. Ultrathin sections are double stained with uranyl
acetate and lead citrate (Reynolds, 1963).
RESULTS

Ninety four cultures, divided into two experimental series depending on the stage at which the cells were transferred to organotypic culture, were studied: 33 were made up from undifferentiated cell cultures, 61 from cultures undergoing differentiation obtained after confluence in histiotypic culture after 1, 4, 5, 8 and 30 days. In the latter case, the mechanical dissociation gives rise to a pellet of multilayered fragments. Whatever the duration of the cell culture prior to the transfer to organ culture, the cells grow rapidly and form a thick disc which covers the whole surface of the filter and invades the metallic grid.

The first macroscopic differentiation in the cultures was hollow vesicles, which in most cases (52 out of 94 cultures) rapidly became associated with blood islands (Figs. 1 and 2). In semi-thin sections erythropoietic foci are recognized after toluidine-blue staining. They contained the various stages of erythropoiesis which can be seen in the embryo yolk-sac between the 8th and 11th days of gestation. Primitive erythroblasts, characterized by their cytoplasmic basophilia, can be identified (Fig. 3).

The vesicles associated with the blood islands in culture are ovoid and limited by an epithelium with a PAS-positive basement membrane. They are surrounded by flattened cells which separate the epithelium from tissues either compact or loose in structure. Some necrotic areas are seen in the explants but not in the region of the blood islands. The epithelial cells of the vesicles present features similar to those of the normal yolk-sac endoderm. They are columnar and possess a number of vacuoles containing PAS-positive non-glycogenic material which is released into the vesicle's lumen. They are closely attached, joined by desmosomes and have a microvillus border (Fig. 4).

Various stages in the development of the blood islands can be observed in the cultures. Like the angioblastic cords in the yolk-sac of 8-day mouse embryos, clusters of cells joined by numerous junctional complexes and containing scattered mitochondria and lipid droplets are observed in the vicinity of the endodermal vesicles (Fig. 5). In both primitive erythroblasts are found in the later stages of maturation; cellular and nuclear size as well as the cytoplasmic content of mitochondria, polysomes, Golgi apparatus and RER decreases (Fig. 6). In parallel, the amount of haemoglobin in the cytoplasm increases. The evolution observed in culture (Fig. 7) is similar to the megaloblastic differentiation of the yolk-sac. Nevertheless, in the cultures, the megalocytes are often located at the surface of the explants.

In most cases erythropoiesis becomes apparent within the first week of culture. However, a few cultures show macroscopically visible blood islands, only after the second week.

The blood islands which appear in organ culture are transitory. Despite the healthy state of the tissues, the formation of haemoglobin-containing cells stops
after a while. The mean life span of blood islands is 6 days; on the contrary, endodermal vesicles persist for more than 50 days.

Numerous other tissues also appear in the cultures and the different cell types described after confluency (Nicolas et al. 1975) were observed. The blood seems, however, to be a particularity of organ cultures. No haemoglobin-containing cells can be found by the histochemical technique in confluent tissue culture even after 4, 8, 12 and 50 days.

**DISCUSSION—CONCLUSION**

Transplantation of teratocarcinoma cells from tissue to organ culture results in the formation of blood islands in 55% of the explants. This differentiation, the first to become macroscopically visible when histiotypic cultures are transplanted in mass cultures, appears in close association with hollow vesicles lined by epithelial cells; these vesicles show the characteristic features of yolk-sac endoderm of normal mouse embryo. In addition, the structure of the erythropoietic sites is quite similar to those found in yolk-sac.

It has been previously suggested that erythrocyte differentiation in the chick yolk-sac results from an inductive tissue interaction between the endoderm and the mesoderm (Wilt, 1965). In the same manner, blood islands in teratocarcinoma cultures could proceed from an inductive action of the endoderm. Some other factors seemed to be required, because endoderm-like structures differentiate, in submerged culture (Nicolas et al. 1975: Martin & Evans, 1975) but blood-island formation is not found in these conditions.

The nucleated megalocytes produced in organ culture by blood islands are always located in the upper layers of the culture. This is probably due to the higher oxygen tension in this location than in deeper layers of the culture. This hypothesis can also account for the absence of erythropoiesis in submerged cultures in which the potentialities for blood formation are not lost even if the duration of the culture is 30 days. Probably for the same reason, only some embryoid bodies formed in vivo differentiate blood islands when they are

**FIGURES 1–3**

Fig. 1. Tissue culture harvested 1 day after confluency and cultured for 16 days organotypically at the surface of a Millipore filter. Epithelial vesicle located at the vicinity of a differentiating blood island. The epithelial wall of the vesicle appears regularly thickened at one pole. B, basement membrane. Masson-Goldner trichrome.

Fig. 2. Same experiment as in Fig. 1 (5 days tissue culture transferred to organ culture for 9 days). Picture of the *in toto* culture showing erythropoietic foci. (a) *In vivo.* (b) After formalin-ferricyanide fixation and staining with zinc-leuco reaction to evidence haemoglobin. v, endodermal vesicle; i, blood island.

Fig. 3. Same experiment as in Fig. 1 (30 days tissue culture transferred to organ culture for 6 days). Endodermal vesicle with microvilli on the border facing the lumen. Differentiating red blood cells (arrow) in the blood island associated with the vesicle. Glutaraldehyde-osmium tetroxide fixed, Epon embedded material. 2μm section stained with toluidine blue.
Fig. 4. Epithelial cell of an endodermal vesicle which differentiates in culture. Large vacuoles, a microvilli border and desmosomes. Glutaraldehyde-osmium tetroxide fixation, uranyl acetate and lead citrate staining.

Fig. 5. Angioblastic cord of mesodermal cells, joined by junctional complexes (JC) showing scattered mitochondria and vacuoles. Glutaraldehyde-osmium tetroxide fixation, uranyl acetate and lead citrate staining.
Fig. 6. (a) Primitive erythroblast found at the vicinity of an epithelial vesicle. (b) Ferritin cluster can be seen in the cytoplasm of more mature blood cells. Glutaraldehyde-osmium tetroxide fixation, uranyl acetate and lead citrate staining.

Fig. 7. A mature megalocyte located near the surface of the culture. Note chromatin condensation and electron opacity of the cytoplasm. Glutaraldehyde-osmium tetroxide fixation, uranyl acetate and lead citrate staining.

cultivated in Petri dishes (Hsu & Baskar, 1974). Indeed, availability of adequate oxygen concentration is known to be required for the development of the mammalian embryo in vitro (Hsu, 1971). The organotypic system provides a tridimensional arrangement of cells and an increase of oxygen tension. These two factors may be decisive for blood cell differentiation.

The blood islands which appear in cultures produce the nucleated megalocytes characteristic of vitelline haemopoiesis in mammals. In normal development this is followed by liver erythropoiesis which gives rise to non-nucleated erythrocytes. Neither this second wave of blood cell formation nor liver differentiation occur in culture. As in the normal yolk-sac in vivo, the erythropoiesis which takes place in vitro is transitory: after a while, stem cell activity stops. Whether stem cells capable of further erythroid differentiation are present in the explants cannot be decided from our observations. However, it is of interest to note that teratocarcinoma cells can differentiate into erythrocytes in chimaeric mice resulting from injection of pluripotent cells into normal blastocysts (Papaioannou, McBurney & Gardner, 1975: Illmensee & Mintz, 1976).
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REFERENCES


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