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Histochemical and autoradiographic study of the cultured rat visceral yolk sac

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SUMMARY

The proliferation and differentiation potentiality of the rat visceral yolk sac was investigated both in organ culture and after grafting *in vivo*. Using alkaline phosphatase as a marker for germ cells, it was shown that these cells are absent in the 12-day-old visceral yolk sac examined before and after organ culture. Therefore, the only cells that proliferate and differentiate must be of endodermal and/or mesodermal origin. By labelling the cells with [^{3}H]thymidine both the endodermal and mesodermal cells were found to proliferate. After 10 days in organ culture or implantation *in vivo* differentiated tissues (e.g. squamous epidermis, endodermal cysts and giant trophoblast cells) were regularly detected. Several of these differentiated cells contained the radiolabel indicating that they derived from the initial proliferating endodermal and/or mesodermal cells.

INTRODUCTION

The remarkable potentiality of the rat visceral yolk sac to proliferate and to differentiate into adult tissues belonging to derivatives of all three germ layers is well documented. This differentiation potentiality is found both *in vivo* after displacement of this membrane (Sobis & Vandeputte, 1974) and *in vitro* using an appropriate method of organ culture (Lu, Sobis, Van Hove & Vandeputte, 1984). In both cases, proliferation of the endoderm and mesoderm of the visceral yolk sac as well as of poorly differentiated cells is observed (Sobis & Vandeputte, 1975; Sobis, Van Hove & Vandeputte, 1982*a*). The latter display the characteristics of neither endodermal nor mesodermal cells. The nature and origin of these poorly differentiated cells remain unclear however. Only germ cells or early embryonal cells are considered to be able to give rise to derivatives of all three germ layers (Stevens, 1967; Mintz, Cronmiller & Custer, 1978). In previous studies we showed that teratomas develop also after destruction of the primary germ cells during their migration from the visceral yolk sac to the genital ridges (Sobis & Vandeputte, 1976) and from the visceral yolk sac of genetically sterile concepti (Sobis & Vandeputte, 1982). There persists the possibility, however, that some of the primary germ cells do not migrate from the yolk sac but remain sequestrated in it. These sequestrated germ cells could then represent the stem cells that give rise to the different adult tissues. Since germ cells are alkaline phosphatase-positive cells,

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the presence of this enzyme was looked for in the visceral yolk sac *in vivo* and *in vitro* prior to the formation of adult tissues. [³H]thymidine was used to label the proliferating cells in the yolk sac *in vitro* and to verify their differentiation potentiality in organ culture and after *in vivo* implantation.

MATERIALS AND METHODS

Rats of inbred strain R (Wistar albino) were used. 12 days after mating (the day when copulation plug was found was counted as day 0), the uteri with embryos were taken out and put in Petri dishes with PBS. The visceral yolk sacs were dissected free from Reichert membrane, amnion and placenta in a medium composed of PBS supplemented with 5 % heat-inactivated horse serum. Pieces of parietal yolk sac were also used. After washing the yolk sacs in NCTC-135 medium supplemented with 20 % horse serum they were incubated in a roller system for 48 h (Lu *et al.* 1984). Afterwards, part of the membranes was put in organ culture (Lu *et al.* 1984) for 5 days.

To verify the presence of primary germ cells the visceral yolk sacs prior to incubation, after incubation in a roller system and after 5 days of organ culture were fixed in 4 % formaldehyde, dehydrated in acetone and embedded in Technovit (Technovit T7100, Kulzer & Co., GmbH, B.R.D.). Serial sections were stained with Naphtal AS-Mx Phosphate and Fast Blue BB (Pearse, 1968) modified by C. H. Kemper (personal communication). As positive controls the genital ridges fo 15-day-old embryos were used.

Another part of the membranes was transferred to Petri dishes with NCTC-135 medium to which $10 \mu Ci \, ml^{-1}$ [³H]thymidine (Radiochemicals, Amersham) made up to a specific activity of $10.5 \, Ci \, mM^{-1}$ had been added and further cultured at 37°C in an atmosphere of 5 % CO_2 in air.

Preliminary to these experiments we verified that optimal labelling by incubating the yolk sacs for 5, 18 and 24 h with [³H]thymidine and found that a 24 h incubation ensured a sufficient incorporation. Hence, in all experiments described the membranes were incubated for 24 h with the isotope.

After this 24 h labelling the yolk sacs were washed for 15 min in three changes of NCTC-135 medium containing $10 \mu g \, ml^{-1}$ 'cold' thymidine. They were then either fixed immediately (uptake controls), put in organ culture (Lu *et al.* 1984) or implanted in the fat tissue of the supporting ligaments of the uterus of a syngeneic R rat. To verify any possible adverse effect of the incorporation of [³H]thymidine on the viability and/or proliferation and differentiation capacity of the membrane, nonlabelled yolk sacs were cultured or implanted in parallel. After 5 and 10 days in organ culture and 10 days after *in vivo* implantation the pieces of yolk sac were fixed in Carnoy solution. Paraffin blocks were cut serially. The slides were stained either immediately with haematoxylin and erythrosin or after autoradiography with haematoxylin alone.

For autoradiography the sections were hydrated and immediately incubated in a 5 % solution of trichloroacetic acid (BDH Chemicals, Co.) at 4°C for 30 min to remove any unincorporated [³H]thymidine. After washing in running water the slides were placed in distilled water and, in a dark room, covered with AR 10 fine-grain autoradiographic stripping film (Kodak, Co.). The slides were left to expose at 4°C for 4 weeks, developing using D-19 developer (Kodak, Co.) and fixed in a Kodafix solution (Kodak, Co.). When dry, the slides were stained with haemalum and scanned in a light microscope.

Some pieces of the cultured visceral yolk sac were snap frozen and cryostat sections were used to look for Δ^5-3β-hydroxysteroid dehydrogenase using the method described previously (Sobis, Van Hove & Vandeputte, 1982*b*).

RESULTS

Detection of alkaline phosphatase

In serial sections alkaline phosphatase was detected at the brush border of some endodermal cells of the visceral yolk sac. No other cells showed a positive staining

in the visceral yolk sacs examined either prior to incubation, after incubation in a roller system or after 5 days in organ culture. Using the same method, the germ cells in the genital ridges of 13- to 15-day-old embryos could be easily identified (Fig. 1).

Evaluation of cultured yolk sac

Autoradiographs of visceral yolk sacs fixed immediately after incubation with [^{3}H]thymidine (uptake controls) showed that numerous, but not all, nuclei of endoderm and mesoderm were heavily labelled with silver grains (Fig. 2). In the parietal yolk sac some endodermal cells were labelled, while the nuclei of trophoblast giant cells did not show any [^{3}H]thymidine incorporation (Fig. 3).

In the membranes fixed after 5 days of organ culture the grains were present in all proliferating endodermal and mesodermal cells, as well as in cells having neither endodermal nor mesodermal morphological characteristics (Fig. 4). Although the density of labelling was much lower than in uptake controls, all nuclei were covered by a sufficient number of grains to allow the label to be seen easily at a low magnification in the light microscope. In the endoderm of the parietal yolk sac some nuclei were labelled, while all the trophoblastic giant cells were necrotized.

In the visceral yolk sacs fixed after 10 days of organ culture differentiated tissues like epidermal cysts and trophoblast giant cells were observed. The latter cells revealed Δ^5-3β-hydroxysteroid dehydrogenase positivity in frozen sections. Analysis of the cultured membranes by autoradiography showed the presence of silver grains in the basal nuclei of squamous epithelium and of giant trophoblast cells (Fig. 5). Grains were also observed in some of the nuclei of connective tissue, endothelium and mesothelium. In some parts of the visceral yolk sac the nuclei of endodermal cells although heavily labelled showed no signs of proliferation or differentiation (Fig. 6). Histologically, no differences were observed between the yolk sacs cultured in the absence or presence of [^{3}H]thymidine. Their morphology was similar to that described previously (Lu *et al.* 1984) both after 5 and 10 days of organ culture.

Parietal yolk sac was completely necrotized after 10 days of organ culture; only the Reichert membrane persisted.
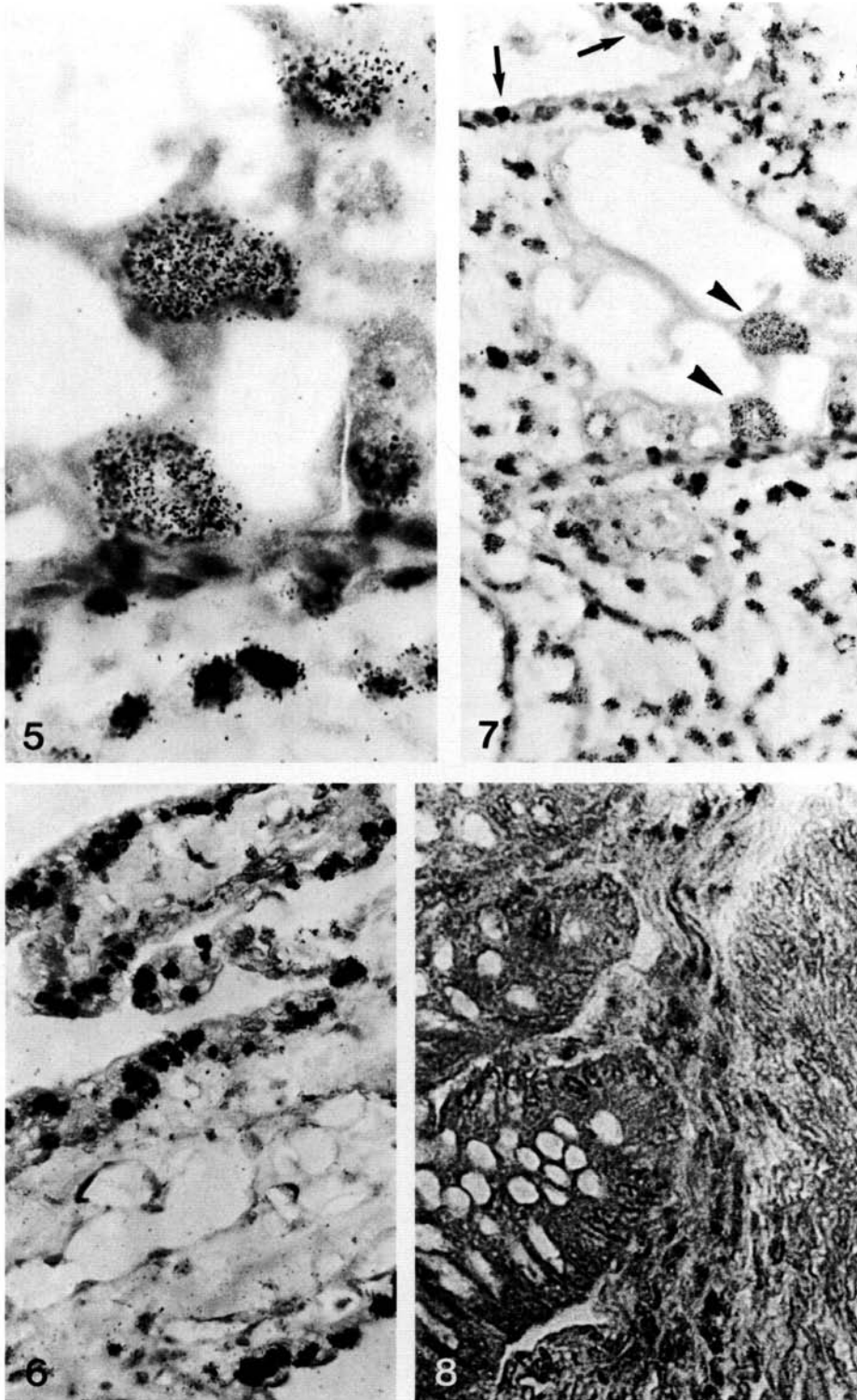
Evaluation of in vivo *transplanted yolk sac*

In the pieces of the visceral yolk sac implanted in the fat tissue of the supporting ligaments of the uterus and examined 10 days later, differentiation into trophoblast giant cells, epidermal and endodermal cysts was observed.

Autoradiographs of these implanted visceral yolk sacs showed a labelling pattern very similar to that observed in the *in vitro* experiments. Silver grains were observed in the nuclei of trophoblast giant cells (Fig. 7), of the basal cells of the epidermis and of the mesenchymal cells in the endodermal cysts (Fig. 8). No grains were found in the nuclei of the surrounding host tissue. There was no difference in proliferation and differentiation capacity between the visceral yolk
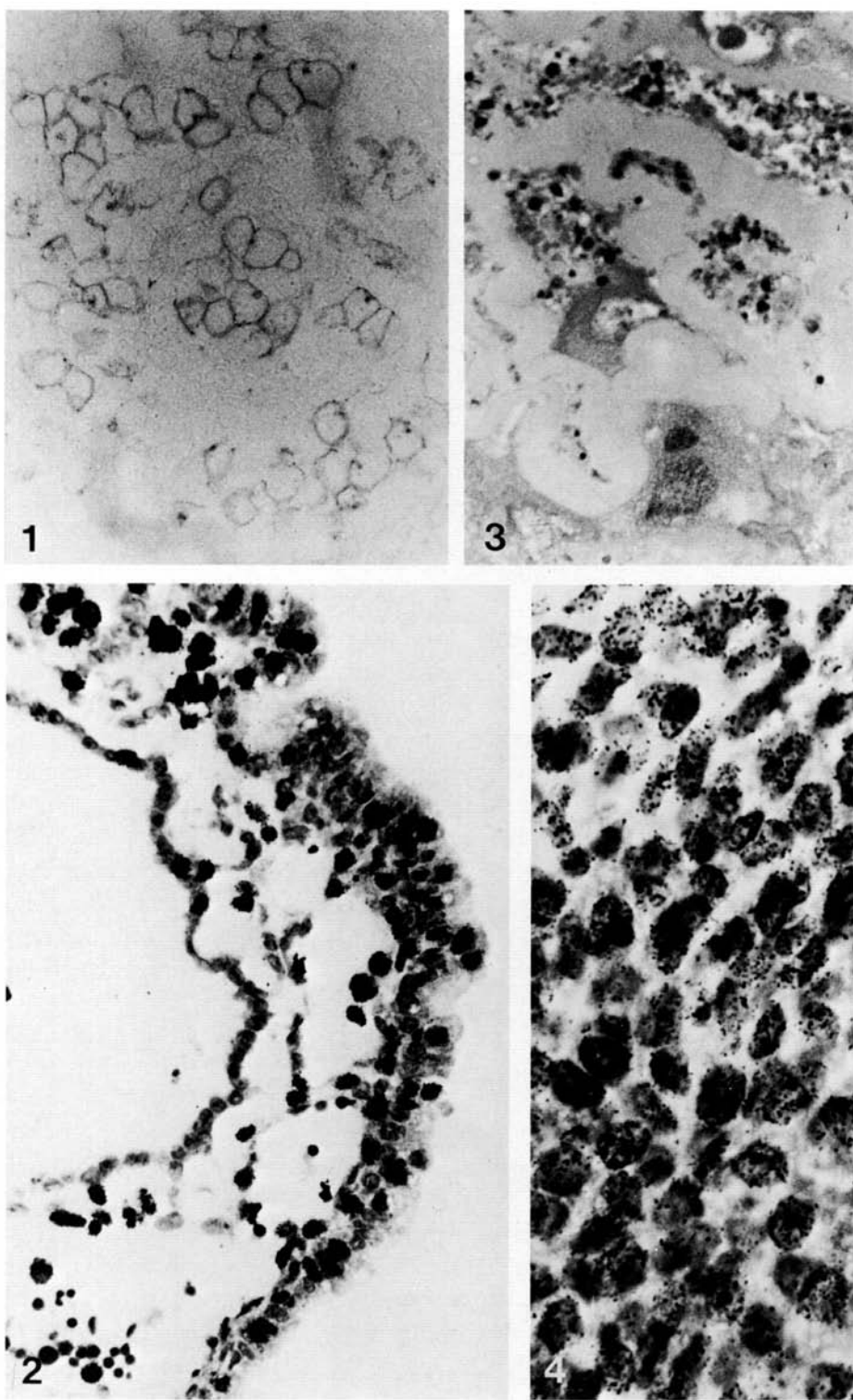
Figs 1–4. For legends see p. 174

Figs 5–8. For legends see p. 174

sacs incubated or not with [³H]thymidine previous to their *in vivo* transplantation. Pieces of implanted parietal yolk sac were no longer detectable 10 days after implantation.

DISCUSSION

In previous studies we described the appearance of proliferating cells in the *in vivo* displaced visceral yolk sac as well as in the yolk sac put in organ culture in the appropriate conditions. We suggested that these proliferating cells derive from endodermal and/or mesodermal cells and not from germ cells and are able to differentiate into various well-differentiated tissues. The present results strongly support this hypothesis. Alkaline phosphatase was only recorded at the apical pole (brush border) of some of the endodermal cells of the visceral yolk sac. No other cells showed a positive reaction either in the freshly dissected yolk sac, after 48 h in the roller system or after 5 days of organ culture. Similar observations were made on the *in vivo* displaced visceral yolk sac (data not shown). These results combined with previous observations (Sobis & Vandeputte, 1976, 1982) make it very unlikely that the proliferating and differentiating cells are of germ cell origin. This is also indicated by the results obtained with [³H]thymidine incorporation. They show that both endodermal and mesodermal cells of the 12-day-old visceral yolk sac incorporate [³H]thymidine in their nuclei. The density of grains found in the nuclei of the cells after 5 days in organ culture was considerably less than seen before the culture (uptake controls), implying that the cells had undergone cell division. After 10 days in organ culture when definite signs of differentiation had appeared, labelled nuclei were observed not only in the endodermal and mesodermal cells but also in trophoblastic giant cells and in the basal cells of the squamous epithelium, tissues that were never seen after 5 days of organ culture. This

Fig. 1. Genital ridge of 14-day-old rat embryo. The cell membranes of the germ cells show a positive reaction for alkaline phosphatase (×1600).

Fig. 2. Visceral yolk sac fixed after incubation with [³H]thymidine-uptake control. Numerous nuclei of endoderm and mesoderm are heavily labelled (×660).

Fig. 3. Parietal yolk sac fixed after incubation with [³H]thymidine-uptake control. Nuclei of some parietal endodermal cells are labelled. No grains are present in trophoblast giant cells (×660).

Fig. 4. Visceral yolk sac fixed after 5 days of organ culture. Numerous silver grains are seen in all proliferating cells (×1600).

Fig. 5. Visceral yolk sac fixed after 10 days of organ culture. Grains are present in the nuclei of trophoblast giant cells and of mesodermal cells (×1600).

Fig. 6. Visceral yolk sac fixed after 10 days of organ culture. Area without signs of differentiation. The nuclei of the endodermal cells show dense labelling as in the uptake control (×660).

Fig. 7. Visceral yolk sac fixed 10 days after *in vivo* implantation. Silver grains are present in mesenchymal, endodermal (arrows) and trophoblast (arrowheads) giant cells (×660).

Fig. 8. Visceral yolk sac fixed 10 days after implantation. Labelled nuclei of mesenchymal cells of the endodermal cyst are seen (×840).

indicates that these tissues derived from the proliferating endodermal and/or mesodermal cells. Indeed, any free [³H]thymidine that might be released from dead cells during organ culture would be considerably diluted by the cold thymidine present in the NCTC-135 medium. The presence of [³H]thymidine in the differentiated tissues in the *in vivo* grafted visceral yolk sacs together with the absence of grains in the nuclei of the surrounding host cells also argues against reutilization of the label. However, these results do not permit any conclusion to be drawn as to the origin of the differentiated tissues. Since both the endodermal and mesenchymal cells incorporated [³H]thymidine we cannot at this stage of the work trace their origin to either the dividing endodermal or mesenchymal cells or to both.

The trophoblastic nature of the giant cells observed in the visceral yolk sac grown *in vitro* or *in vivo* is proven by the presence of Δ^5-3β-hydroxysteroid dehydrogenase. A possible contamination at the time of dissection of the visceral yolk sac by trophoblast from the parietal membrane can be excluded since the giant cells attached to the Reichert membrane did not incorporate [³H]thymidine and completely necrotized during the 5-day organ culture. Moreover, the trophoblastic giant cells were never seen in the visceral yolk sacs examined 48 h after incubation in the roller system or after 5 days in organ culture. Whether the trophoblast originates from the endodermal cells could not be determined. This possibility should, however, be considered as it has been shown to occur in yolk sac carcinoma of rats (Sobis, Van Hove & Vandeputte, 1982*b*; Van Hove, Sobis, Lu & Vandeputte, 1985) and of mice (Damjanov, Damjanov & Andrews, 1985; Gaillard, 1981).

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