Short Contribution

Differentiation of the mouse embryoid bodies grafted on the chorioallantoic membrane of the chick embryo

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ABSTRACT In order to test the developmental potential of the mouse embryonic-stem-cell-derived embryoid bodies as chorioallantoic grafts, the embryoid bodies were transplanted to the chorioallantoic membrane (CAM) of the chick embryo. The graft implantation was achieved if the embryoid bodies were transferred to the CAM into the blood drop created by gentle laceration of a CAM blood vessel. The resulting tumors were recovered after 10 days, when they were rounded white elevations, up to 1 mm big. Histological analysis showed that they were made of groups of compacted epithelial-like cells and fibroblast-like spindle shaped cells divided by the CAM mesenchyme. The grafting caused angiogenic response of the CAM. Blood vessels converged toward the tumor and spread through the CAM mesenchyme among the groups of condensed cells. Although the embryonic bodies were able to implant to the CAM of the chick, their differentiation did not result in a wide variety of differentiated cell types or in the formation of complex structures resembling morphogenesis. Thus in comparison with *in vitro* differentiation of embryoid bodies on an adhesive substrate, or *in vivo* differentiation under the mouse kidney capsule, the differentiation potential of embryoid bodies as chorioallantoic grafts appeared restricted. However, we suggest that the accessibility of the chorioallantoic graft can be an advantage for future experiments.

KEY WORDS: *ES cells, embryoid body, chorioallantoic membrane, mouse, chick*

The establishment of the culture of embryonic stem (ES) cells from the inner cell mass of the mouse blastocyst enabled the *in vitro* propagation of an undifferentiated cell line at the early stage of embryo development (Evans and Kaufman, 1981). In addition the ES cells were able to resume normal development *in vivo* and generate a mouse completely derived from cultured cells (Nagy *et al.*, 1990). However, ES cells alone can not initiate correct development. They need to be placed within the blastocoel of another embryo by blastocyst injection, or to be aggregated with the host morula (Bradley *et al.*, 1984). Even if the host cells of the morula cannot continue their development, as in case of aggregation with tetraploid embryos, the morula cells are competent enough to instruct ES cells to carry out the further sequence of developmental events (Nagy *et al.*, 1990). It is still unknown what the nature of this induction is, which makes ES cells give rise to the embryo.

The main component that maintains the undifferentiated state of ES cells in a culture is leukemia inhibitory factor (LIF) (Smith *et al.*, 1988; Wiliams *et al.*, 1988). In the absence of LIF, ES cells loose their pluripotency and differentiate into a monolayer of extraembryonic endoderm- and mesoderm-like cells (Heath *et al.*, 1989; Mummery *et al.*, 1990). When cultured in suspension, they form the aggregates that resemble mouse blastocyst, hence they are called embryoid bodies (Martin *et al.*, 1977; Evans and Kaufman, 1981). The attachment of embryoid bodies to the adhesive substrate results in subsequent differentiation of various cell and tissue types such as cardiac and skeletal muscle, neural tissue, visceral yolk sac and hematopoietic cells (Evans and Kaufman, 1981; Martin, 1981; Doetschman *et al.*, 1985; Wiles and Keller, 1991; Wobus *et al.*, 1991; Bain *et al.*, 1995). When transplanted subcutaneously or under the kidney capsule they form teratocarcinomas, which can be composed of highly complicated differentiated tissues corresponding e.g., intestinal epithelium with mucous glands (Chen and Cosco, 1993).

In order to create conditions similar to those of the implantation process of the mouse blastocyst we presumed it would be appropriate to transplant the embryoid bodies to the chorioallantoic membrane (CAM) of the chick egg. The CAM of the chick egg is a thin membrane with a rich vascular network, resembling in this way the endometrium of the uterus. The CAM has been used for a long time for a variety of transplantational approaches where different chick organs, but as well mammalian tissues and tumors, were

Abbreviations used in this paper: CAM, chorioallantoic membrane; ES cells, embryonic stem cells; LIF, leukemia inhibitory factor.

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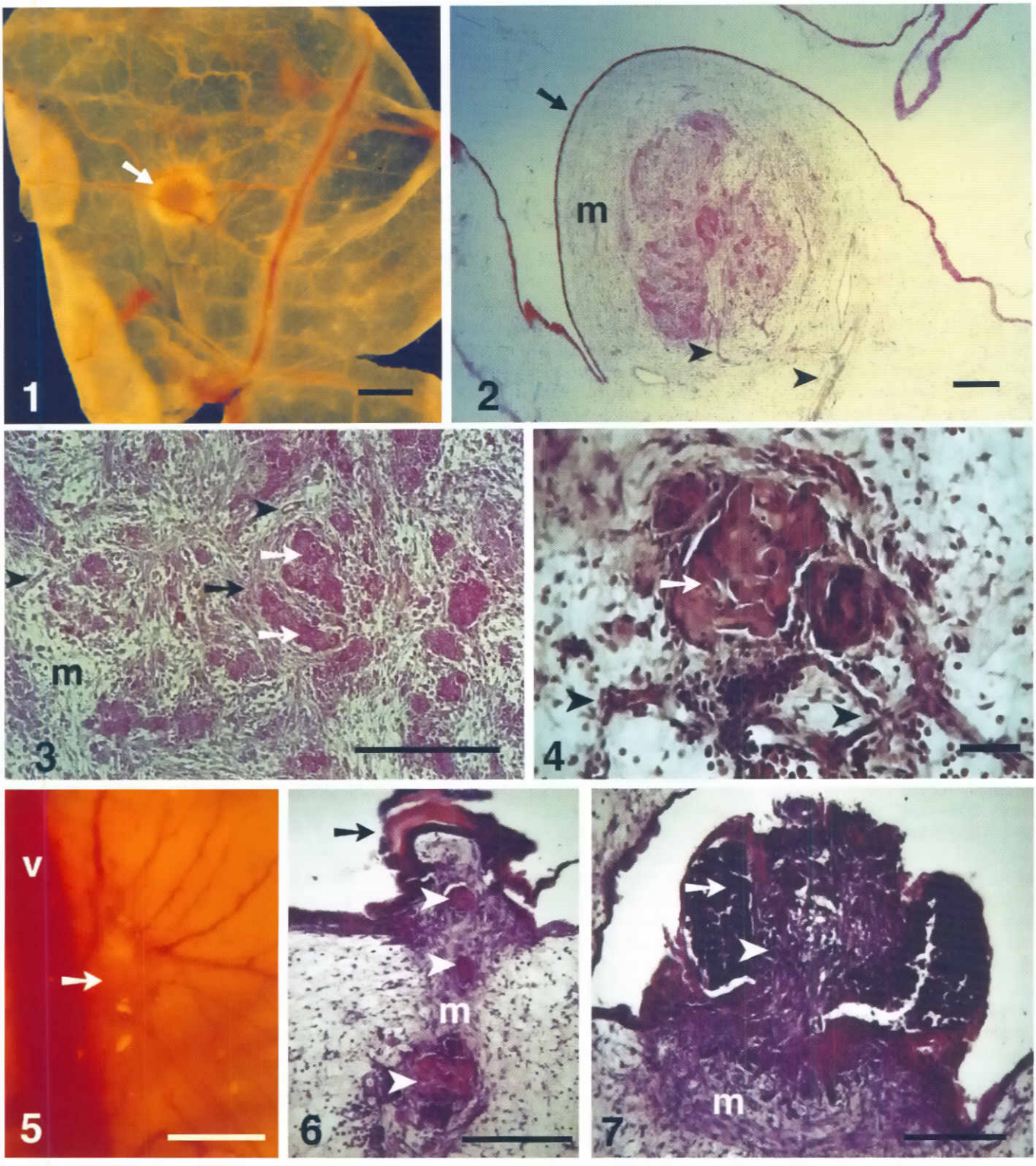

Fig. 1. Isolated CAM of the chick 10 days after grafting containing the embryoid-body-derived tumor (arrow). *Blood vessels can be seen through the CAM as red and white lines. Bar, 1 mm.*

Fig. 2. Histological section stained by neutral red through the CAM and the tumor within. *The CAM elevation caused by tumor is covered by the chorion epithelium (arrow). Tumor is surrounded by a layer of CAM mesenchyme (m). The CAM blood vessels branch and enter the tumor (arrowheads). Bar, 100 μm*

Fig. 3. Histological section stained by neutral red through the center of the tumor. *Condensations of epithelial-like cells (white arrows) are continuous to the densely packed fibroblast-like cells (black arrow). These groups of cells are separated by the loose CAM mesenchyme (m) containing blood vessels (arrowheads). Bar, 100 μm.*

TABLE 1

COMPARISON OF TWO DIFFERENT GRAFTING PROCEDURES OF EMBRYOID BODIES TO THE CAM OF THE CHICK EMBRYO

	Total number of grafted embryoid bodies	Implanted embryoid bodies
Transfer of embryoid bodies to the CAM into a drop of medium	25	1
Transfer of embryoid bodies into a drop of blood caused by gentle laceration of a CAM blood vessel	40	16 (40%)

transplanted (recently reviewed in Ribatti *et al.* 1996). Since it is highly vascularized, the CAM was suitable as well for propagation of viruses and other microorganisms. Although our postulation that such a vascularized membrane will serve as a trigger for simple morphogenesis was not achieved, we showed that mouse embryoid bodies were able to implant and grow on the CAM of the chick.

After 4 days of suspension culture of ES cell aggregates, the resulting embryoid bodies were transferred to the CAM. Two different approaches were applied. In the first one, embryoid bodies were placed to the CAM in a drop of medium, and the egg was sealed. We always intended to place the embryoid bodies as near a large blood vessel as possible. In the second approach, one of large blood vessel of the CAM was gently ruptured by a Wolfram needle in order to cause slight bleeding (Zwilling, 1956). The embryoid body was then placed into the generated blood drop, again as near the ruptured vessel as possible. When the transplantation was done to the bleeding membrane, it was successful in our hands in 40% of the cases (Table 1). The embryoid bodies were implanted into the membrane and produced tumors. When the transplantation was done to the bleeding membrane, embryoid bodies implanted into the membrane and produced tumors. This procedure was successful in our hands in 40% of cases (Table 1).

When eggs were reopened 10 days after grafting, the tumors were present as white elevations on the transparent CAM (Fig. 1). The size of tumors was up to 1 mm, they were rounded, with a regular surface and uniform consistency.

The cells of the tumor were separated in several groups of densely packed cells, surrounded by CAM mesenchyme (Fig. 2). The cells within these groups had epithelial appearance, but surrounded no lumen. They were bigger than neighboring cells, with eosinophylic cytoplasm and centrally positioned nucleus (Figs. 3,4). This epithelial-like condensations were continuous to the densely packed elongated fibroblast-like cells. The groups of densely packed cells were separated by the loose CAM mesenchyme (Fig. 3).

The blood vessels in the surrounding part of the CAM converged toward the tumor, and the vessel density was higher when compared with neighboring areas (Fig. 5). Some of the blood vessels entered into the tumor and spread through the CAM mesenchyme among the epithelial condensations (Figs. 2,3,4). There were no blood vessels within the groups of compacted cells. The elevated surface of the tumor was covered with the chorion epithelium that was in some parts stratified or keratinized (Fig. 6). In some cases the parts of tumor were retained at the outer surface of the epithelium, and one could follow the connection through the epithelium to the implanted parts (Fig. 7). In the vicinity of the tumor the mesenchymal cells were condensed and thickened (Figs. 6,7).

Obviously, the nonspecific reaction of the CAM was important for the graft survival. The successful implantation was achieved by a damage of the CAM, caused by laceration of the CAM blood vessel or, as in one case, by sticking of an egg shell piece to the membrane (Zwilling, 1956). Both, thickening of the chorionic epithelium and condensation of mesenchymal cells, represent a rather common reaction of the CAM to various grafts (Moscona, 1959, Svajger *et al.* 1968). Formation of new blood vessels toward the graft showed that embryoid bodies elicited the angiogenic response of the CAM. The candidates for causing this effect are members of fibroblast growth factor family present during embryoid body differentiation (Rissau *et al.*, 1988). Although Rissau *et al.* (1988) claimed that embryoid bodies themselves undergo vasculogenesis when grafted to the CAM of the quail, we did not achieved it in our experiment.

Apart from epithelial-like aggregates and fibroblast-like spindle shaped cells, we observed no cell types bearing other morphological characteristics. There were no complex differentiated structures. As well, we were not able to see any sign of processes resembling normal embryo development. Compared to the extent and complexity of the embryoid body differentiation *in vitro* when allowed to grow on an adhesive substrate, or *in vivo* when transplanted under the mouse kidney capsule, their developmental potential on the chick CAM appeared rather limited (Martin, 1981; Chen and Cosco, 1993). Although the highly vascularized extraembryonic membrane exposed the transplants to the hormones and other substances carried in the blood stream, our expectations that the CAM would give better positional clues, or trigger some kind of morphogenesis remained unaccomplished.

Although the grafting of embryoid bodies to the CAM did not result in expected differentiation complexity, we suggest that the

Fig. 4. Histological section stained by hematoxylin and eosin through the center of the tumor. *The condensations of epithelial-like cells (arrow) are surrounded by the CAM mesenchyme containing a network of blood vessels (arrowheads). Bar, 10 μm.*

Fig. 5. Photograph through the egg shell window of the CAM containing the embryoid-body-derived tumor (arrow). *The tumor is positioned near the big blood vessel (v) and the network of blood vessels (red lines) converges toward the tumor. Bar, 1 mm.*

Fig. 6. Histological section stained by hematoxylin and eosin. *Thickened and keratinized chorionic epithelium (arrows) at the implantation site of the tumor. Tumor is made of scattered groups of epithelial-like cells (arrowheads). The mesenchymal cells near the tumor are condensed and thickened (m). Bar, 100 μm.*

Fig. 7. Histological section stained by hematoxylin and eosin. *The part of the graft is retained above the chorionic epithelium. It consists of densely packed rounded cells (arrow) and spindle shaped fibroblast-like cells (arrowhead) extending through the thickened chorionic epithelium to the remainder of the tumor not present on this section. The mesenchyme near the tumor contains condensed and thickened cells (m). Bar, 100 μm.*

CAM can be indeed used for this purpose as it enables some advantages not present in other *in vivo* systems. The grafts on the CAM can be constantly monitored through the shell window, and the variety of the chemicals can be applied directly or by impregnated beads during the time course of differentiation (Tickle *et al.*, 1985). This would allow a controllable *in vivo* system where conditions can be modified during differentiation. The scarce differentiation of untreated embryoid bodies would actually turn into advantage, as it would facilitate the interpretation of obtained results.

Experimental Procedures

ES cell culture and embryoid body formation

The R1 mouse ES cell line (kindly provided from A. Nagy) was grown on mitomycin C (100 μg/ml, Sigma) treated primary embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM 4.5 g glucose/l, GIBCO BRL) supplemented with 1 mM sodium pyruvate (GIBCO BRL), 1% of stock solution of non-essential amino acids (GIBCO BRL), 2 mM L-glutamine, 100 μmol β-mercaptoethanol, 500 U/ml leukemia inhibitory factor (LIF, supplied by Amgene) and 20% heat inactivated fetal calf serum (selected batch supplied by GIBCO BRL). Cells were regularly split every 2 days and not kept in culture longer than 10 passages.

Approximately 1.5x106 ES cells (corresponds to quantity of ES cells «ready to split», grown on 3.5-cm-diameter tissue culture dish with feeder layer) were seeded on 10-cm-diameter gelatin (0.1%) coated tissue culture dish. Cells were kept in full ES cell medium as described above. After two days growth on gelatin, embryoid bodies were prepared by careful trypsinization (0.05% trypsin/EDTA solution) preserving the aggregates of ES cells unbroken. The aggregates were resuspended in DMEM medium with 10% serum and transferred into 15-cm-diameter bacteriological dishes. The medium was changed after two days and the resulting simple embryoid bodies were used for transplantation after 4 days of suspension culture.

Eggs treatment and grafting procedure

White Leghorn hens' eggs were incubated at 38°C in a humidified incubator. After 9 days of incubation eggs were candled to mark the place of large blood vessel bifurcation. The air chamber at the blunt end of the egg was punctured with a needle in order to allow the CAM to settle after the rupture of the shell membrane. The marked place was covered with an adhesive tape and a small window sawed with scissors. The piece of egg shell was removed and the shell membrane punctured, what provoked the dropping of the CAM. Single mouse embryoid body was transferred to the CAM of one egg. After enlarging the shell window, the embryoid body was transferred by a glass pipette onto the exposed CAM to a well vascularized spot in an angle between bifurcating blood vessels. One part of grafting was done by gentle laceration of the CAM in order to produce hemorrhage, and the graft was placed over the lacerated area. The shell window was sealed by an adhesive tape and the eggs returned to the incubator.

The graft analysis

The CAM together with the graft was taken out 19th day of incubation (10 days after grafting), fixed in a mixture of 2% formaldehyde and 0.2% glutaraldehyde in phosphate buffered saline for 30 minutes and washed three times in buffer. The specimens were dehydrated in ascending concentrations of ethanol and embedded in Paraplast. Serial histological sections (8-10 μm) were done on the microtome and the sections were stained with neutral red or hematoxylin and eosin.

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