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Differentiation of ICM Cells into Trophectoderm

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It has been established previously that when inserted in the blastocyst E Ca 247 preferentially differentiates into trophectoderm *in vitro*. If the concept that tumors are caricatures of the process of tissue renewal is correct, then some cells from the inner cell mass (ICM), the normal counterpart of embryonal carcinoma, should be able to differentiate into trophectoderm. This has been a controversial issue. Four experiments are now reported that support the idea that ICM can differentiate into trophectoderm: 1) ICM from early blastocysts after classical immunosurgery made blastocysts *in vitro;* 2) ICM obtained from early blastocysts by immunosurgery using antigens other than histocompatibility ones made blastocysts *in vitro;* 3) ICM from early blastocysts, in which the trophectodermal cells had been labeled, contained no labeled cells following immunosurgery; and 4) In reconstruction experiments, polar and mural trophectodermal cells attached to ICM from late blastocysts failed to multiply and make blastocysts when cultured. It is concluded that like the embryonal carcinoma some ICM cells of early blastocysts have the potential to make trophectoderm. This fact is consistent with the concept that tumors are caricatures of the process of tissue renewal; and establishes E Ca 247 as a good model for study of trophectodermal differentiation. (Am J Pathol 1988, 132: 356–364)

CELLS FROM SOME embryonal carcinomas of the mouse, when injected into an early cystic stage of embryonic development (blastocyst), are induced to behave as normal embryonic cells, participate in embryonic development, and, together with the normal embryonic cells, produce chimeric mice.[1-3] Apparently the highly malignant embryonal carcinoma stem cells are converted to normal stem cell lines responsive to homeostatic control. If the mechanism of this regulation of malignant to benign cells could be determined, it might serve as a basis for a noncytotoxic therapy for patients with embryonal carcinoma with metastasis. However, experiments to determine how this regulation of embryonal carcinoma cells by the blastocyst occurs have been frustrated by a paucity of material.[4]

The blastocyst, at 3.5 days after fertilization, is about 80 μ in diameter and contains about 1×10^{-3} lambda of fluid. It has about 52 outer cells (trophectoderm) that will form the placenta and other extra-embryonic tissues, and 12 inner cells (named inner cell mass or ICM) that will form the embryo proper. ICMs from such early blastocysts, when cultured *in vitro,* have been reported to have the potential to differentiate into trophectoderm,[5-11] but ICMs from late blastocysts lack the potential to form trophectoderm and make embryonic structures including endoderm.[3,6-8,12-16,17,18]

The potential for ICM to differentiate into trophectoderm in addition to the 3 germ layers is of great importance to studies of blastocyst regulation of embryonal carcinoma, because it implies that early in blastulation there are 2 types of ICM cells and therefore, because embryonal carcinomas are caricatures of ICM, there should be at least 2 kinds of embryonal carcinoma cells.[19-21] Later in development the potential for making trophectoderm is lost by ICM, which then gains the potential to make endoderm.[7,22,23] Thus, there should also be embryonal carcinomas that are caricatures of ICM cells with the potential to make endoderm. It is conceivable that the regulation of these populations of cells could differ and/or be interrelated.

E Ca 247 is regulated by a soluble factor in blastocele fluid plus contact of the cancer cells with the blastocele surface of trophectoderm,[4] but it does not form

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chimeras when injected into blastocysts (unpublished data) because the cells preferentially differentiate into trophectoderm.[24] In addition, C-145 is an embryonal carcinoma unable to make chimera because it localizes in extra-embryonic endoderm.[25] Could these tumors represent prototypes of ICM suitable for study of their respective differentiations?

Before undertaking a major study of the mechanism of differentiation of ICM into trophectoderm using E Ca 247 as the model, it seemed prudent to reexamine the data supportive of the idea that some early ICM cells could in fact differentiate into trophectoderm. Although there are many reports supportive of the notion,[5-9,11,17] all of these data have been obtained using immunosurgery to destroy trophectoderm.[18] Immunosurgery is a technique that uses antibodies to minor histocompatibility antigens followed by treatment with complement to selectively destroy trophectoderm. The ICM is spared destruction because the immune reactants are unable to gain access to the blastocele because of tight junctions between trophectodermal cells. The ICM can then be separated from the dead trophectoderm by aspiration of the treated blastocysts in small bore pipets.[18] Strong arguments have been presented to the effect that all trophectodermal cells are destroyed by immunosurgery[26] but the most compelling evidence to this effect, generated by Fleming et al,[5] used blastocysts, the trophectoderm of which had been labeled with horseradish peroxidase. After immunosurgery no labeled cells were present on the ICMs. In contrast, Harlow and Quinn[27] isolated ICM by immunosurgery and exposure to a calcium ionophore and concluded that some cells might escape immunosurgery.

While defining optimal concentrations of reagents for immunosurgery, the authors made the observation that the individual trophectodermal cells of a blastocyst acquire their histocompatibility antigens at different times (unpublished). It was thus conceivable that some trophectodermal cells might survive immunosurgery and be the source of the new trophectoderm. In addition, ICMs isolated by microsurgery from early blastocysts, did not form trophectoderm.[10,12-16,28] Those derived by immunosurgery had several more cells than ICM visualized by serial section.[6] Thus the validity of the idea that ICMs of early blastocysts could differentiate trophectoderm was not certain, and 3 experiments were proposed that would test the idea stringently. In the first experiment polar and mural trophectoderm were attached to ICMs from late blastocysts, and cultured to see if they could make trophectoderm. Second, ICMs of early blastocysts, acquired using a technique not dependent upon the presence of minor histocompatibility antigens were cultured to see if they could make blastocysts; and third, ICMs of early blastocysts free of trophectoderm were cultured *in vitro* to determine how they made new blastocysts. The data establish that ICMs from early blastocysts have the potential for differentiating into trophectoderm, and that E Ca 247 can serve as an excellent model of trophectodermal differentiation.

Materials and Methods

Blastocyst

Blastocysts were flushed from the uteri of random bred CD-1 mice (Charles River) on the third day of gestation.[29] They were separated according to age: early blastocysts were defined as those in which the ICM occupied at least one-third of the blastocyst; later ones were defined as those fully expanded with the inner cell mass forming a saucer-shaped aggregate of cells attached to the inner surface of polar trophectoderm. The blastocysts were held in Eagles minimal essential medium plus antibiotics and 10% fetal calf serum (MEM + 10) until used.[29]

Immunosurgery[18] was performed as detailed previously.[4] ICMs were held in MEM + 10. Care was taken in these procedures to have an excess of antibody to blastocysts to ensure maximal reaction with trophectoderm.

Preparation of Labeled Trophectoderm

To prepare labeled polar trophectoderm, 16 cell morulae were incubated overnight in a suspension of fluorescent carboxylated microspheres (0.7 μ CX Green Covospheres, Covalent Technology Corp.) at a concentration of 5.5×10^8 beads per ml of MEM + 10. By morning these morulae had developed into early blastocysts, which were washed 3 times in MEM + 10 to remove unattached spheres. The spheres could not make contact with ICMs, which remained unlabeled, but overlying polar trophectoderm was labeled. The ICMs and labeled polar trophectoderm were amputated as described previously.[4] The ICMs were immediately dissociated in 0.025% trypsin and 0.02% EDTA solutions in calcium- and magnesium-free salt solution, after which the cells were separated by gentle trituration. The cellular suspension was examined under the light microscope and the polar trophectodermal cells were identified by the presence of the microspheres, which appeared as small black dots in their cytoplasm. These labeled polar trophectodermal cells were picked up with orally controlled mi-

cropipets and stored in MEM + 10 until used in experiments.

Trophectoderm was labeled with horseradish peroxidase by placing zona-free blastocysts in a solution of 5 mg/ml horseradish peroxidase type II in Whitten's medium for 3–4 hours at 37 C.[30] They were then washed 3 times in Whitten's medium, the cells were fixed in glutaraldehyde, washed and then placed in a solution of 2 mg diaminobenzidine per ml of Whitten's medium with 2.5 μl of H_2O_2 for 10 minutes at 37 C to develop the reaction product of horseradish peroxidase (Nakane, PK, personal communication). The preparations were then washed 3 times in 0.1 molar Tris HCL buffer.

Wheat Germ Agglutinin Mediated Destruction of Trophectoderm

To confirm the immunosurgical results, trophectoderm was also destroyed by incubation in wheat germ agglutinin (Miles Scientific) followed by treatment with antibodies to wheat germ agglutinin (Dako Corporation) followed by washing and treatment with complement. In this procedure zona-free blastocysts were incubated in a solution of 0.5 mg/ml of wheat germ agglutinin in PBS for 10 minutes on ice. The blastocysts were washed 3 times in PBS, incubated in a 1:10 dilution of antibody to wheat germ agglutinin (Miles Scientific) for 1 hour on ice. They were washed 3 times in PBS and incubated for 30 minutes at 37 C in guinea pig complement diluted 1:8 in MEM + 10. They were then washed 3 times in MEM + 10 and mechanically dissociated to remove dead trophectoderm from the ICMs.

Reconstitution of ICM and Trophectoderm

To attach trophectodermal cells to ICMs, healthy trophectodermal cells and ICMs were placed in a drop of 0.1% phytohemagglutinin in MEM lacking serum. Three to six trophectodermal cells were mechanically placed on the ICMs using orally controlled pipets. After 15 minutes of incubation, the preparations were washed and incubated in MEM + 10 at 37 C in 5% CO_2 and air for 24 and 48 hours, at which time they were examined and scored for formation of blastocysts. Labeled trophectoderm and ICMs were used individually as controls.

Results

In the course of redefining optimal concentrations of reagents for immunosurgery, groups of 5 blastocysts were reacted with serial dilutions of antibody (1:5–1:200). In a group of 5 blastocysts incubated in dilute antiserum a range of reactivity was commonly observed. The trophectoderm of 1 blastocyst might have vesiculated completely (vesiculation is the end point of complement mediated immune cytolysis) whereas in others no vesiculation was apparent. Between these extremes were individual blastocysts in which random trophectodermal cells vesiculated among unaffected ones (Figure 1a–c). It was concluded that there is a variation in the time at which different blastocysts acquire enough histocompatibility antigens to mediate immune cytolysis, and the cells of a blastocyst gain these antigens at different times. However, even under presumed optimal conditions, vesiculation of trophectoderm did not always appear complete (Figure 2).

Consequently, to determine whether or not some trophectodermal cells escaped immunosurgery, blastocysts were embedded in plastic and serially sectioned for electron microscopy using the techniques of adjacent thick and thin sections until the blastocysts in question were examined at all levels. All trophectodermal cells that appeared to have escaped immunosurgery, when observed in this manner with the electron microscope, had indeed undergone immunocytolysis (Figure 2).

This electron microscopic study was prolonged and while it was in process reconstruction experiments were initiated to see if trophectodermal cells, when attached to ICMs could, after appropriate incubation in tissue culture, develop into blastocysts. To this end ICMs from late blastocysts (incapable of forming trophectoderm) had either mural or polar trophectodermal cells attached to them as described in the Methods section. Twenty-eight of these preparations were available for study with mural trophectoderm still tightly attached to the late ICMs after 24 hours of incubation. None of them formed blastocysts. The mural trophectodermal cells formed vacuoles without fail, became distended with fluid, failed to attach to each other, and eventually perished. In these respects they were comparable to those observed in the controls in which single mural trophectodermal cells were cultured in the absence of ICMs. Even ICMs surrounded by as many 6–8 mural trophectodermal cells failed to form blastocysts.

When 6 polar trophectodermal cells labeled with covalent spheres from early blastocysts were attached to each of 45 ICMs from late blastocysts and incubated for 48 hours, none gave rise to new blastocysts. About 30% of the attached cells became distended with fluid and the others could not be identified in the preparations and presumably were lost or became incorporated into the ICM. As in the case of mural

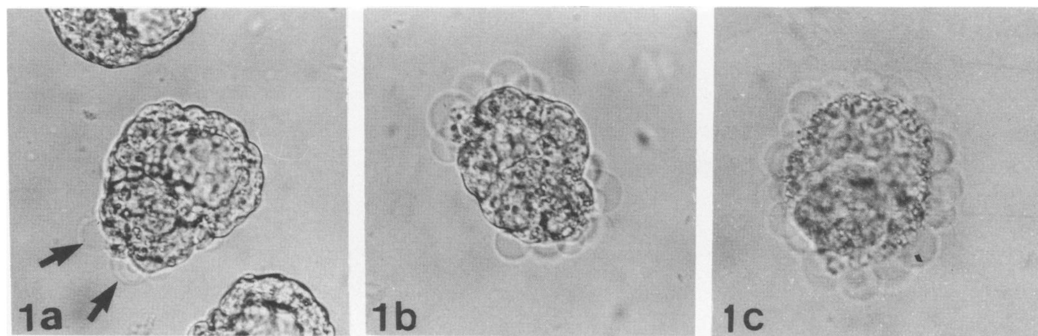

Figure 1—This figure shows blastocysts incubated in dilute rabbit antimouse antibody followed by complement. They illustrate: a—minimal vesiculation of trophectoderm (arrows), b—moderate vesiculation, c—complete vesiculation. This indicates trophectodermal cells acquire transplantation antigens at different times. (×400)

trophectoderm, individual labeled polar trophectodermal cells failed to divide and invariably underwent vacuolization in tissue culture. Thus, under the conditions of the experiment it appeared unlikely that even if a few trophectodermal cells were left on ICMs after immunosurgery they could not regenerate the trophectoderm of a new blastocyst.

In the experiments to test whether or not immunosurgically isolated ICMs from early blastocysts could differentiate trophectodermal cells and form new blastocysts, ICMs were isolated by immunosurgical treatment of early and late blastocysts of CD-1, Balb/C, and C57 black mice using an antibody made previously[4] and another, a gift from Dr. Ivan Damjanov. No significant differences attributable to strain or source of antibody could be detected and the data were pooled. ICM cells from early blastocysts during 48 hours in culture developed into blastocysts in 71 of 128 ICMs cultured (55%) whereas only 8 of 94 ICMs (9%) of late blastocysts formed blastocysts. These data were interpreted as supportive of the idea that early ICMs have the potential to differentiate into trophectoderm, a potential that is lost with time.[5-9,17,18,31]

It was decided to confirm these results using another technique not dependent upon the acquisition of histocompatibility antigens by the blastocyst. Blastocysts were incubated in wheat germ agglutinin, followed by anti-wheat germ agglutinin and complement to kill trophectoderm as detailed in the Methods. Eleven of 50 (22%) ICMs from early blastocysts and 0 of 18 of ICMs of late ones differentiated trophectodermal cells and made blastocysts within 48 hours of culture. Some of these blastocysts were embedded in epon and examined by transmission electron microscopy. They were of small size, and had the typical ultrastructural appearance of normal blastocysts as described by others.[7,22,32-34]

To exclude the possibility that a few trophectodermal cells could survive immunosurgery and form the trophectoderm of new blastocysts in tissue culture, trophectoderm of intact blastocysts was labeled with horseradish peroxidase as described in the Methods section. Although polar and mural trophectoderm were densely labeled in these blastocysts, none of the ICMs derived from them by immunosurgery contained or had labeled cells on their surface. Similarly, when ICMs from blastocysts with horseradish peroxidase-labeled trophectoderm were treated with wheat germ agglutinin and anti-wheat germ agglutinin and complement, no labeled cells were observed in or on the surfaces of the ICMs.

Since these data were overwhelming in support of the observations made by others[5-9,17,18,31] that immunosurgically isolated ICMs of early blastocysts could give rise to trophectoderm, the mechanism by which this differentiation took place was studied. Accordingly, blastocysts undergoing immunosurgery were examined morphologically at 10, 20, and 30 minutes of treatment with complement, and freshly isolated ICMs were similarly examined at 4, 8, 12, 18, and 24 hours of incubation in tissue culture.

The light microscopic appearance of ICMs changed with time during complement-mediated destruction of trophectoderm and the ICMs assumed a lobular appearance not unlike that of the precompacted 8-cell egg (Figure 3a). In exceptional cases, attenuated outer cells that stretched over several inner cells were observed on the surface of the ICMs by the completion of immunosurgery.

The attenuated outer cells of these preparations appeared to be derived directly from a few cells on the surface of the ICM. Stretching of attenuated outer cells over the inner cells continued with time of culture, and most inner cells were completely enclosed by 12 hours of culture. Tight and gap junctions typical of trophectoderm[35] developed between the outer cells, a process that also continued with time. A few small vacuoles of fluid often lay between the outer and inner

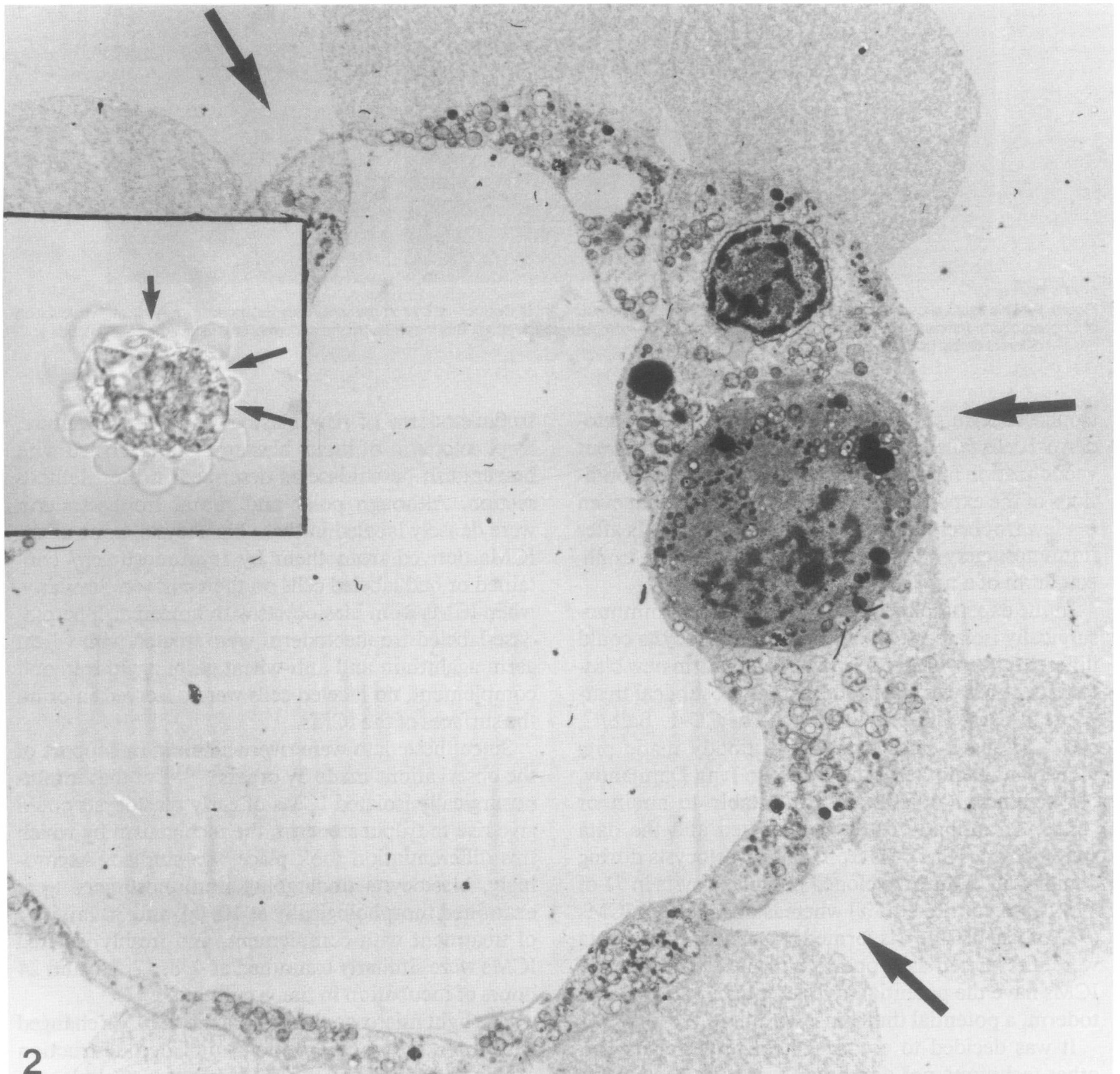

Figure 2—The inset is a light micrograph of a early blastocyst after immunosurgery under ideal conditions (×400). Vesiculation appears incomplete (arrows). When such a blastocyst was examined by electron microscopy, all trophectodermal cells were dead. Vesiculation occurred at focal points, but the spaces apparent in the inset, overlay dead trophectoderm (large arrows). This micrograph was taken through the margins of the inner cell mass. The portions of 2 inner cell mass cells appear embedded in the necrotic trophectoderm. (×3500)

cells by 12 hours, and blastocele formation was often advanced by 18 hours (Figure 3b,c; Figure 4).

In 1 group, 3 of 9 ICMs from early blastocysts differentiated into new blastocysts by 24 hours of incubation in tissue culture. When examined with the electron microscope these were typical small blastocysts. Some of the ICMs that failed to differentiate into a blastocyst matured in tissue culture and eventually formed a flattened layer of outer cells that enclosed the inner cells. These surface cells had abundant rough endoplasmic reticulum containing an amorphous appearing material, and were connected to each other by tight junctions and to inner cells by desmosomes. No basement membrane was present between outer and inner cells. Although these cells closely resembled primitive endoderm, their identity has not been established with certainty. Presumably these ICMs had lost the potential to make trophectoderm and differentiated a layer of endoderm.

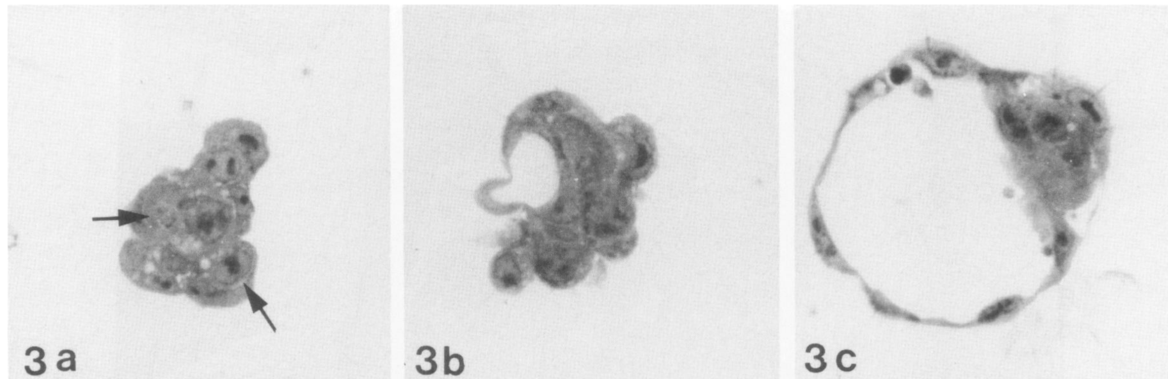

Figure 3a—A lobulated inner cell mass at the completion of immunosurgery. The arrows point to the junction of inner cells and attentuated outer cells. (×600) **b**—An inner cell mass in culture for 18 hours. Note the accumulation of fluid. (×600) **c**—A typical blastocyst formed after 24 hours tissue culture of an inner cell mass from a early blastocyst. (×600)

While these studies were in progress, the experiments of Fleming et al,[5] which demonstrated that labeled trophectodermal cells did not survive immunosurgery, were published. They illustrated the changes in membrane differentiations that developed in the attenuated cells as they formed the trophectoderm of the blastocyst in tissue culture. These observations confirm theirs.

Discussion

E Ca 247 cells preferentially differentiate into trophectoderm when placed in blastocysts.[24] Accordingly, ICMs, which are the normal counterpart of embryonal carcinoma,[36,37] should be able to differentiate into trophectoderm as well as tissues of the embryo, if the concept that carcinomas are caricatures of the process of tissue renewal and specifically, in the case of embryonal carcinoma, a caricature of embryogenesis, is correct.[19,20,24]

On the basis of the data obtained in this study plus those in the literature, the conclusion is now inescapable that ICMs of early blastocysts can differentiate into trophectoderm and thereby form new small blastocysts *in vitro.* This conclusion is based on classical immunosurgery confirmed by complement mediated immune cytolysis of cells using antigen–antibody reactions different from transplantation ones, and by the use of labeled cells to exclude the presence of residual trophectoderm on ICMs after immunosurgery. Finally, reconstruction experiments did not support the idea that a few trophectodermal cells attached to ICMs could regenerate the trophectoderm of a blastocyst.

ICMs in tissue culture develop into blastocysts in a manner somewhat similar to the development of the 8-cell egg into a blastocyst.[22,23,38] In each situation, outer cells surrounded what were to become inner cells. This created different environments for the 2 cell types and the outer ones became trophectoderm and the inner ones became ICM. Then fluid accumulated between trophectoderm and inner cells, and new blastocysts were usually formed by 18–24 hours. Thus, the importance of a positional effect in the differentiation of trophectoderm in the regeneration of a blastocyst from a early ICM might be assumed to be as great as it was in the development of the original blastocyst.[23,38] However, it must be pointed out that in these *in vitro* experiments only occasional surface cells of the ICM attenuated and stretched over the surface of the other cells to form trophectoderm. During this time all of the cells on the surface of the ICM were exposed to the environment. Why only some of these cells should attenuate and form trophectoderm when all were exposed to the environment is not known. The best explanation is that there are a number of surface ICM cells with the potential to form trophectoderm. If these cells adhered to the polar trophectoderm when the ICMs were mechanically removed from the blastocyst, the resultant ICMs would contain fewer cells than those acquired by immunosurgery and they would not be able to differentiate into trophectoderm. This would account for the difference reported in cell number for ICMs from early blastocysts.[6]

The differences in incidence of blastocyst formation by ICMs isolated by classical immunosurgery and by immunosurgery after wheat germ agglutinin, anti-wheat germ aggluinin and complement, and between the data from various published studies is probably the result of variation in the ages of the blastocysts employed. There are strain differences in the length of gestation (unpublished) and probably in time of blastulation. The decision as to what is a late blastocyst and what is an early one is subjective, and it is now

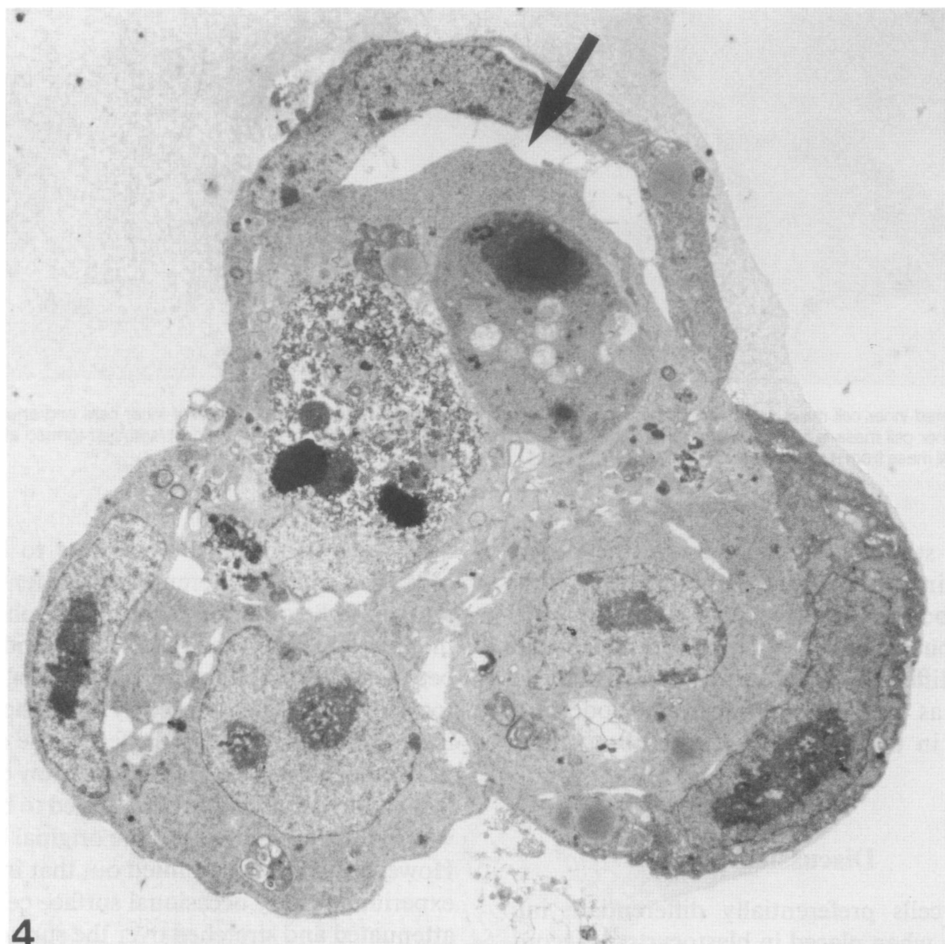

Figure 4—This inner cell mass was cultured for 12 hours. Note the inner cells (one is dead) and the outer ones are flattened and enclose the inner cells. Blastocele fluid is accumulating between the inner and outer cells (arrow). (×3500)

clear that blastocysts amenable to microsurgical removal of the ICM are older than previously anticipated.[31]

It has been shown that maintenance of polar trophectoderm is dependent upon its association with ICM.[12] ICM also stimulates the growth of mural trophectoderm as indicated by the experiments of Gardner in which ICMs were placed in trophectodermal vesicles.[3] In the present experiments, isolated mural and polar trophectodermal cells attached to early ICM failed to proliferate and form new blastocysts. The reason is unknown. They also failed to reattach to each other and form an epithelium, and they failed to proliferate in the presence of the ICM. ICMs injected into trophectodermal vesicles successfully regulate mural trophectoderm and the reconstructed blastocysts successfully implant.[3] This is interpreted to mean that ICMs can reregulate mural trophectoderm to function as polar trophectoderm when the trophectoderm is in a vesicle, but single trophectodermal cells are unable to proliferate and form a vesicle even when in contact with ICM. These single cells become distended with fluid[31,37] and perish.

The concept that embryonal carcinoma is a caricature of the process of preimplantation embryogenesis[19–21] is supported by the observation that like E Ca 247, ICMs of early blastocysts can make trophectoderm. The concept is useful in ordering many facts of neoplasia, and its usefulness is becoming even more obvious in making extrapolations from neoplastic to normal tissues, particularly embryonic ones. For instance, when a previously unrecognized cell was found in an adenocarcinoma of colon, not only was the postulate true that the same cell would be present in the normal tissue, it proved to be the stem cell of a unique and poorly understood cell lineage.[35] In this study the observation that E Ca 247 could make trophectoderm suggested that ICM should have a similar function, which it does. In addition, growth factors can be secreted by tumors as first shown by Braun

in plant gals.[39] In fact, teratomas of plants secrete 2 growth factors which are also synthesized by embryonic plant cells, but not by mature plant cells.[39] This leads to the conclusion that if tumor cells make a growth factor then the normal cell lineage will also make the factor. Confirmation for the idea has been generated by Rizzino, who has shown growth factors produced by embryonal carcinoma are also found in the blastocyst.[40,41] It will be interesting to see if E Ca 247 and ICM cells with the potential to form trophectoderm will react differently to these growth factors than embryonal carcinoma and ICM cells that make embryonic cells.

The concept that tumors caricaturize the process of tissue renewal and development has great predictive value not only for tumor biology but also for developmental biology and therapy.

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