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Cell Biology

Mechanism of programmed cell death in the blastocyst

(blastocyst regulation of embryonal carcinoma/preimplantation development/apoptosis)

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ABSTRACT **The malignant growth potential of embryonal carcinoma cells may be controlled by environmental factors. For example, embryonal carcinoma cells placed into normal blastocysts may not exhibit the continued growth expected of malignant cells but rather may lose all aspects of the malignant phenotype and become apparently normal embryonic cells. Loss of the malignant phenotype of embryonal carcinoma cells occurs early in these injected blastocysts and has been used as the basis of assays to study the mechanisms of regulation of embryonal carcinoma by the blastocyst. In this regard, P19, an embryonal carcinoma that makes midgestation chimeras, was regulated by blastocele fluid plus contact with trophectoderm but not by blastocele fluid plus contact with inner cell mass (ICM). In contrast, ECa 247, which makes trophectoderm, was regulated by exposure to blastocele fluid plus contact with trophectoderm or ICM. During the course of these experiments, dead embryonal carcinoma and ICM cells were observed, and blastocele fluid was then shown to kill ECa 247 and normal ICM cells of early blastocysts with trophectodermal potential. P19 cells and ICM cells with potential to make the embryo were not killed by blastocele fluid. Programmed cell death occurs in the ICM of the blastocyst during the transition from early (when ICM has the potential to make trophectoderm) to late (when the ICM lacks the potential to make trophectoderm). It is postulated that this programmed cell death is designed to eliminate redundant ICM cells with trophectodermal potential, and its mechanism of action is mediated by epigenetic factors in blastocele fluid.**

Our ultimate goal is to ascertain the mechanism by which the blastocyst regulates embryonal carcinoma cells, which have been injected into the blastocele, to the point that they respond to embryonic signals as if they were normal inner cell mass (ICM) cells as exemplified by the production of chimeric mice (1–7). The blastocyst is a cystic stage of embryonic development occurring in the mouse 3.5 days after fertilization (Fig. 1*a*).

We have previously shown that blastocele fluid plus contact with trophectoderm are required for regulation of embryonal carcinoma (loss of malignant attributes such as the ability to form colonies of malignant cells *in vitro*) (8). However, ECa 247, the embryonal carcinoma cells used in that study, failed to produce chimeras when injected into blastocysts because they preferentially localized in and differentiated into trophectoderm (9). It was decided to compare the mechanism of regulation of an embryonal carcinoma that makes chimeras to that of ECa 247. In contrast to ECa 247, ≈60% of blastocysts injected with P19 cells develop into midgestation chimeras as evidenced by glucose phosphate isomerase assays, but most of them were abnormal and few came to term (10). Nevertheless, the malignant phenotype was at least partially suppressed during the early development of these chimeras.

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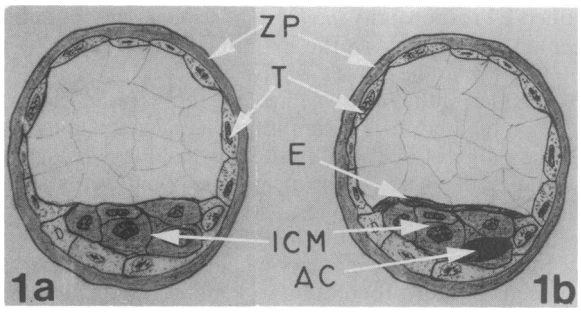

FIG. 1. (*a*) Diagram of a mouse blastocyst 3.5 days after fertilization. The zona pellucida (ZP) is a soft egg shell lined by 52 trophectodermal cells (T), which will form the placenta. The 12 ICM cells will form the embryo, but at this stage they have the potential to form trophectoderm as well. (*b*) Diagram of a late stage blastocyst. Programmed cell death has occurred and one of the ICM cells has become apoptotic (AC). After apoptosis, the ICM no longer has the potential to differentiate trophectoderm; instead it differentiates a layer of endoderm (E). The blastocele cavity contains 1 nl of fluid.

In the course of the current investigation, it was observed that P19 cells, unlike ECa 247 cells, were not regulated by blastocele fluid plus contact with the ICM. Furthermore, some ECa 247 cells, but not P19 cells, were killed during incubation in blastocele fluid. Blastocele fluid also selectively killed some ICM cells, after which the potential of the ICM to make trophectoderm was lost.

MATERIALS AND METHODS

Eight-cell eggs at 2 days of pregnancy and blastocysts at 3.5 days of pregnancy were obtained from 8-week-old CD-1 (Charles River Breeding Laboratories) mice by flushing the oviducts or uteri, respectively, with a few drops of Eagle's minimal essential medium containing penicillin G (100 units/ml), streptomycin (100 μg/ml), 0.1 mM nonessential amino acids, and 1 mM pyruvate (MEM). The eggs and blastocysts were held in MEM, with 10% heat-inactivated fetal bovine serum (MEM + 10) under washed mineral oil.

The methods for injecting blastocysts and producing trophectodermal vesicles have been described (8, 11). Briefly, cancer cells in a drop of MEM + 10 under washed mineral oil were injected into blastocysts by using holding and injecting pipettes attached to micromanipulators. Only blastocysts with a clearly visible cancer cell were used in the assay for cancer cell regulation as measured by abrogation of colony-forming ability (8, 12). As for controls, the cancer cells were cloned in 96-well Linbro plates, where they grew equally well as in the perivitelline space. For production of trophectodermal vesicles containing an embryonal carcinoma cell, separate drops of medium containing either blastocysts or cancer cells were used to obviate transfer of contaminating embryonal carcinoma cells. Blastocysts were injected with a cancer

Abbreviation: ICM, inner cell mass.

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cell, which was placed on the mural trophectoderm opposite the ICM; the ICM was then amputated with a 26-gauge needle. If the tip of the needle was pressed into the bottom of the plastic Petri dish, the edges of the trophectoderm were sealed to form a vesicle. When the vesicles reexpanded, those with a visible cancer cell were assayed for regulation of colony formation (8).

To prepare giant blastocysts (13), the zonae pellucidae of eight-cell eggs were removed by incubation in 0.25% Pronase at 37°C, after which the eggs were washed three times in MEM + 10. Eight eight-cell eggs were fused in a small drop of 0.1% phytohemagglutinin (Sigma) in MEM in 35-mm bacteriologic Petri dishes and incubated for 1–2 min. The dishes were flooded with MEM + 10 to dilute the phytohemagglutin and were incubated at 37°C in 100% humidity with 5% CO_2/95% air on a rocking platform (22 cycles per min). The resultant giant blastocysts were used after either 48 or 72 hr of incubation.

Zonae pellucidae, to be used as carriers of the cellular preparations to be studied in the giant blastocysts, were prepared from blastocysts, morulae, and abnormal eggs unsuitable for other experiments. The blastocysts, attached by gentle suction to holding pipettes, were penetrated by a pipette through which the fluid and cells of the embryo were evacuated (see Fig. 2). The zonae pellucidae were flushed with MEM + 10 by inserting a pipette through the hole used to evacuate their contents and then stored at 4°C in MEM + 10. In contrast to the method of Rossant (14), the method described here has the advantage of usually producing only a single hole in the zona pellucida.

Two lines of embryonal carcinoma were used. ECa 247, which was derived from OTT6050 (15), is aneuploid (a bimodal chromosome number of 57 and 74 chromosomes), grows well *in vitro*, and its neoplastic attributes including tumor and colony formation are regulated by the blastocyst (11, 12). These cells produce typical embryonal carcinomas when injected subcutaneously and fail to make chimeras when injected into blastocysts because they preferentially localize in trophectoderm (9). They can be induced to differentiate *in vitro* and *in vivo* with retinoic acid (16, 17). P19 is an egg-derived embryonal carcinoma of C3H mice (18), which grows as undifferentiated masses of embryonal carcinoma cells. It seldom makes live-born chimeras because most of the chimeric embryos die. However, 60% of 9-day embryos are chimeric based on glucose phosphate isomerase assays (10). The cells were a gift from M. McBurney (18).

ICMs were obtained by immunosurgery of 3.5-day-old blastocysts (19). They were washed three times in MEM + 10, pooled, and single cell suspensions were obtained by gentle trypsin treatment. The ICM cells were then washed three times in MEM + 10 and placed with zonae pellucidae in a drop of MEM + 10 under washed mineral oil in a large Petri dish. The embryonal carcinoma cells to be injected into the zonae pellucidae were placed in a second drop separate from the first one to avoid mixing of the cell types. A zona pellucida was oriented on a holding pipette so that the hole through which the blastocyst had been evacuated faced the injecting pipette. Then, depending on the experiment, embryonal carcinoma cells alone or 2 embryonal carcinoma cells plus 12 ICM cells were injected through the hole into the cavity of the zona pellucida. One-half of the specimens were incubated in MEM + 10 for 7 days in 5% CO_2/95% air with 100% humidity (controls), and half were injected into giant blastocysts. To this end, they were placed in a large drop of MEM + 10 with the giant blastocysts. A giant blastocyst was attached to a holding pipette by gentle suction, and a triangular-shaped hole was made in its wall using three fine glass needles. A zona pellucida carrying the cells to be tested was attached by suction to a pipette and pushed through the triangular hole into the blastocele of the giant blastocyst. The preparations were placed in the incubator until the wound in the giant blastocysts had healed and the blastocele had re-formed. This usually took 1–3 hr. The expanded blastocysts were then placed in 2 ml of MEM + 10 in 35-mm bacteriologic Petri dishes and rocked at 22 cycles per min in the incubator at 37°C and 100% humidity in 5% CO_2/95% air for 24 hr. Rocking prevented attachment of the giant blastocysts to the Petri dish with consequent collapse and loss of blastocele fluid.

After 24 hr of incubation [regulation of ECa 247 occurs within 24 hr (12)], giant blastocysts were returned to a drop of MEM + 10 under washed mineral oil under the micromanipulator assembly. The zonae pellucidae with their contents were removed from the giant blastocysts, and, depending on the experiment, the cells in the zonae pellucidae were either counted, or they were placed in tissue culture (similar to the controls) for 6 days to determine whether they could make colonies of malignant cells.

Table 1. Effect of the blastocyst on colony formation of single P19 cells

Exp.	No. of colonies/no. of attempts: P19 cells in blastocysts	P19 cells cultured alone
1	0/8	14/40
2	5/9	23/40
3	0/8	24/40
4	2/4	13/40
5	0/9	18/40
6	1/13	18/40
Total	8/51 (16%)	110/240 (46%)

$P = 0.001$.

Table 2. Effect of trophectodermal vesicles on colony formation of single P19 cells

Exp.	No. of colonies/no. of attempts: P19 cells in trophectodermal vesicles	P19 cells cultured alone
1	3/12	17/40
2	0/4	20/40
3	4/14	17/40
4	1/6	27/40
5	3/10	25/40
Total	11/46 (24%)	106/200 (53%)

$P = 0.001$.

RESULTS

The results of the first two experiments showed that like ECa 247 (8) P19 was regulated by the blastocyst and by trophectodermal vesicles (Tables 1 and 2). Incorporation into blastocysts reduced the frequency of colony formation of P19 from 46% to 16%; similarly, trophectodermal vesicles, which are devoid of ICM but contain blastocele fluid† surrounded by trophectoderm, reduced it from 53% to 24%. Rossant and Papaioannou (20) did not observe regulation of colony formation of P19 cells by blastocysts or trophectodermal vesicles. Unlike our experiments in which single P19 cells were used, they used clumps of P19 cells, which may have been

†It might be more accurate to refer to the fluid as trophectodermal vesicular fluid, but because many trophectodermal vesicles do not collapse after the amputation of the ICM, the fluid could still have secretions from the ICM; we therefore prefer to designate it as blastocele fluid.

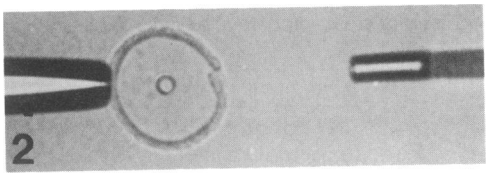

FIG. 2. Cells and fluid have been evacuated from a blastocyst, and a single ECa 247 cell has been injected into the empty zona pellucida through the hole used to evacuate the original contents. (×72.)

large enough to overwhelm the regulatory processes of the blastocysts.

In contrast, blastocele fluid plus ICM could regulate ECa 247 but not P19. Fewer colonies of embryonal carcinoma were formed from two ECa 247 cells and 12 ICM cells in zonae pellucidae (Fig. 2) placed in giant blastocysts for 24 hr (Fig. 3*a*) than were obtained from similar cells in zonae pellucidae (Fig. 3*b*) cultured directly in MEM + 10. Contact with ICM cells plus blastocele fluid decreased colony formation by ECa 247 from 84% to 29% (Table 3), but colony formation by P19 was not affected.

In the course of the experiments demonstrating these differences in regulation of ECa 247 and P19 by ICM and blastocele fluid, changes were observed in the ICM cells with time. Irrespective of experiment or control, the ICM cells first reaggregated into a lobulated ball (Fig. 3*a*), which resembled an ICM acquired by immunosurgery (21, 22). Those cultured in the absence of giant blastocysts usually differentiated a layer of trophectoderm and formed new blastocysts *in vitro* (Fig. 3*b*) as has been reported previously (21–26). Similar ICM cells cultured in blastocele fluid for 24 hr and then cultured in MEM + 10 failed to make new blastocysts; rather, they usually formed two-layered embryonic structures (Fig. 3*c*). The outer layer of cells proved to be endoderm when examined histologically and electron microscopically.

Dead cells were observed in most of these zonae pellucidae, which contained ICM and embryonal carcinoma cells, after incubation in giant blastocysts (Fig. 3*a*); however, dead cells were seldom if ever seen in the controls in which similar cells in zonae pellucidae were cultured in MEM + 10 in the

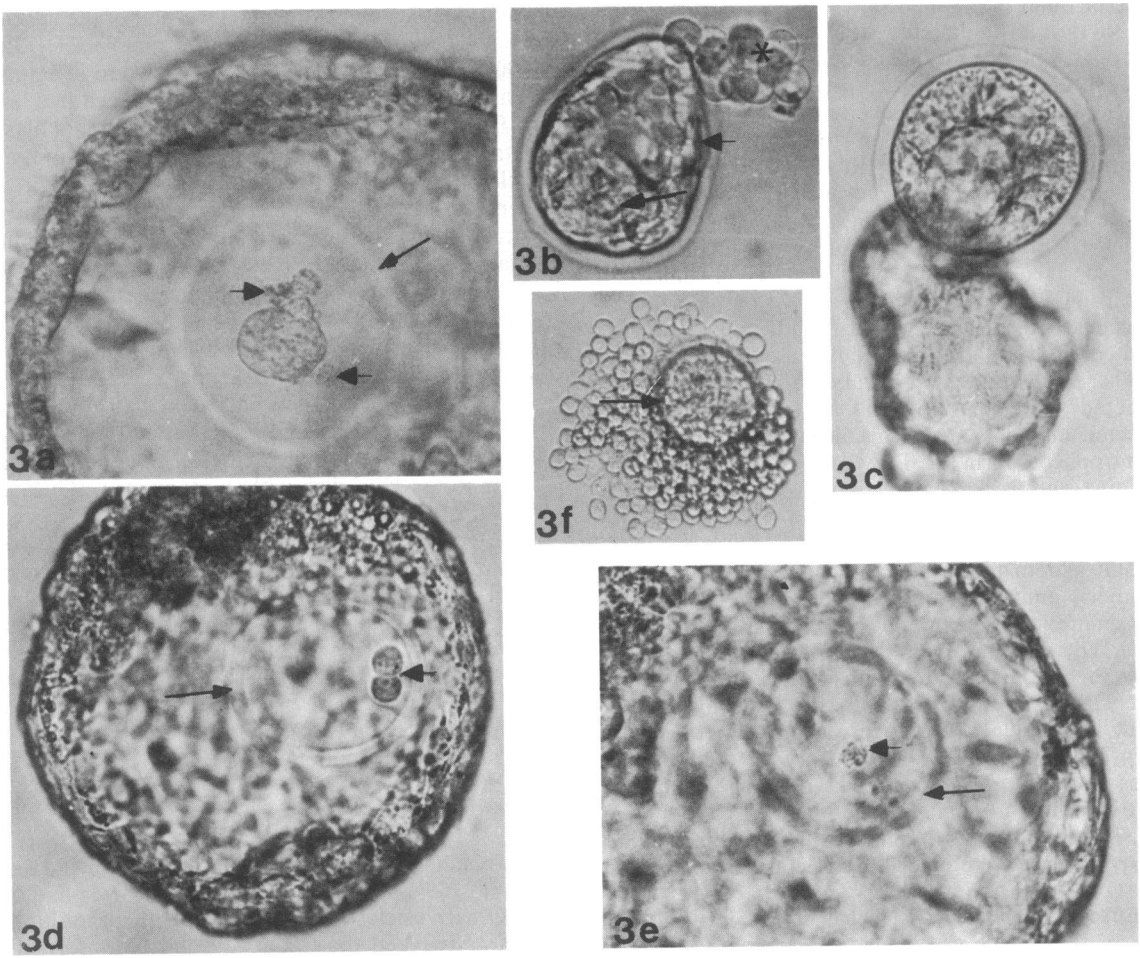

FIG. 3. (*a*) This giant blastocyst was injected with a zona pellucida (long arrow) containing 2 ECa 247 cells and 12 ICM cells and was cultured for 24 hr. The ICM cells have aggregated to form an ICM and several dead cells have appeared (short arrows). The ECa 247 cells in this specimen did not grow out of the zona pellucida to form a colony after rescue. (*b*) This zona pellucida was injected with 12 early ICM cells and 2 ECa 247 cells and was cultured in MEM + 10 for 72 hr. The ICM cells have re-formed a blastocyst, and the cancer cells have proliferated and grown through the hole in the zona pellucida (long arrow, ICM; short arrow, trophectoderm; asterisk, cancer cells). (*c*) This zona pellucida was injected with 12 ICM cells from an early blastocyst and 2 ECa 247 cells. It was incubated in a giant blastocyst for 24 hr and then rescued into MEM + 10 and cultured for 48 hr. Note the thick-walled embryo that not only fills the zona pellucida but has grown out through the hole in the zona pellucida to attach to the substrate (out of the plane of focus of the zona pellucida). (*d*) This giant blastocyst was injected with a zona pellucida (long arrow) containing a single viable P19 cell. After incubation for 24 hr in the giant blastocyst, the P19 cell had divided (short arrow) and no dead cells were evident. (*e*) This giant blastocyst was injected with a zona pellucida (long arrow) containing a single viable ECa 247 cell. After incubation for 24 hr in the giant blastocyst, the cancer cell (short arrow) was dead. (*f*) This zona pellucida (arrow) was injected with a single ECa 247 cell and was cultured in MEM + 10 for 6 days (control). The zona pellucida was packed with cancer cells, many of which have escaped and proliferated on the plastic dish to form a colony. (×120.)

Table 3. Effect of ICM and blastocele fluid on regulation of two embryonal carcinoma cells

Exp.	Tumor	No. of colonies/no. of attempts: Zonae pellucidae incubated in medium and in giant blastocyst		Zonae pellucidae incubated in medium only	
1	ECa 247	0/3		1/1	
2	ECa 247	—		5/5	
3	ECa 247	2/3		5/6	
4	ECa 247	1/1		2/3	
5	ECa 247	0/1		4/5	
6	ECa 247	0/1		1/2	
7	ECa 247	0/2		—	
8	ECa 247	3/5		3/4	
9	ECa 247	—		5/6	
10	ECa 247	0/3		3/3	
11	ECa 247	1/5		7/8	
	Total	7/24	(29%)*	36/43	(84%)*
1	P19	0/3		1/4	
2	P19	2/2		3/3	
3	P19	2/2		3/5	
4	P19	—		3/5	
5	P19	2/3		0/1	
6	P19	2/2		1/2	
7	P19	3/3		2/3	
	Total	11/15	(73%)†	13/22	(59%)†

Two cancer cells and 12 ICM cells injected into zonae pellucidae were incubated in giant blastocysts for 24 hr, rescued, and cultured for 6 days. In the control, similar numbers of cancer and ICM cells were placed in zonae pellucidae and incubated in tissue culture for 7 days.

*$P = 0.001$.

†Differences not statistically significant.

absence of blastocysts. It was postulated that a toxic activity was present in blastocele fluid.

To determine whether a factor(s) toxic for embryonal carcinoma and ICM cells existed in blastocele fluid, a single embryonal carcinoma cell was placed in each empty zona pellucida and incubated in MEM + 10 for 1 hr (Fig. 2). Cells mechanically damaged by the manipulations died during this hour and such preparations were discarded. As a result, only viable cells were present in the zonae pellucidae that were placed in giant blastocysts. After 24 hr of incubation in giant blastocysts, 44% of the ECa 247 cells were dead in comparison to 2% in the controls (Table 4, Fig. 3 *d* and *e*). Viable cells recovered from blastocele fluid grew equally well in MEM + 10 as did control cells (Fig. 3*f*), in confirmation of previous results (Fig. 3*d*) (8).

P19 cells, in contrast to ECa 247 cells, were not killed by blastocele fluid (Table 4, Fig. 3 *d* and *e*). It is concluded that blastocele fluid contains a factor(s) that kills some ECa 247 cells (trophectodermal potential), but does not kill P19 cells (embryonic potential).

The final experiment was to determine whether ICM cells derived from early blastocysts, and therefore having trophectodermal potential, could during or after 24 hr of incubation in giant blastocysts differentiate into trophectoderm of new small blastocysts. Accordingly, 12–15 individual ICM cells from early blastocysts were injected into zonae pellucidae. Half of these preparations were incubated in giant blastocysts for 24 hr and the controls were incubated in MEM + 10 only. Because dissociation of ICMs into single cells might predispose them to the toxic action of blastocele fluid, the experiment was repeated using intact ICMs. The data obtained did not differ and were pooled. As shown in Table 5, in contrast to the controls in which 25/36 specimens differentiated into

Table 4. Killing of single embryonal carcinoma cells in zonae pellucidae carriers by blastocele fluid of giant blastocysts

Exp.	Tumor	No. of zonae pellucidae with dead cells/no. of zonae pellucidae injected: Experimental		Control	
1	ECa 247	2/4		0/5	
2	ECa 247	1/4		1/7	
3	ECa 247	2/5		0/11	
4	ECa 247	2/3		0/3	
5	ECa 247	1/2		0/7	
6	ECa 247	3/5		0/4	
7	ECa 247	0/2		0/6	
	Total	11/25	(44%)	1/43	(2%)
1	P19	0/1		0/5	
2	P19	0/2		0/2	
3	P19	1/2		0/5	
4	P19	0/5		1/7	
5	P19	0/2		0/4	
6	P19	0/1		1/3	
7	P19	0/2		0/5	
8	P19	0/4		0/3	
9	P19	0/2		0/2	
	Total	1/21	(5%)	2/36	(6%)

the trophectoderm of new small blastocysts, only 4/28 specimens in the experimental group cultured in giant blastocysts differentiated into trophectoderm. Those that failed to form blastocysts *in vitro* usually differentiated into two-layered embryonic structures with a thick outer layer of endoderm and an inner layer of embryonic epithelium (sometimes with a central cavity, probably a pro-amniotic cavity). These resembled the embryonic structures illustrated in Fig. 3*c*. Only two of the ICMs in this study died (Table 5). They were small and the cells became vacuolated.

DISCUSSION

The purpose of these experiments was to compare the responses of ECa 247, an embryonal carcinoma that preferentially localizes in and differentiates into trophectoderm and then into extraembryonic tissues when incorporated into blastocysts, with those of P19, an embryonal carcinoma that under similar conditions preferentially differentiates into ICM cells and then into embryonic tissues. Colony-forming ability of P19 was reduced in blastocysts and in trophectodermal vesicles as had been reported for ECa 247 (8, 11). However, colony-forming ability of P19 was not reduced by blastocele fluid alone or by fluid plus contact with ICM in contrast to the results obtained with ECa 247. Similarly, P19 was not susceptible to the toxicity of blastocele fluid, which was lethal for some ECa 247 cells.

The toxic activity of blastocele fluid was also lethal to some ICM cells. When a suspension of 12–15 ICM cells or intact

Table 5. Effect of blastocele fluid on the ability of early ICM to differentiate trophectoderm of blastocysts *in vitro*

Exp.	ICM	Incubated in giant blastocysts	Incubated in MEM + 10 alone
1	12–15 dispersed cells	2/6	4/5
2	12–15 dispersed cells	0/5	3/5
3	12–15 dispersed cells	0/5	4/7
4	Intact	0/1	2/3
5	Intact	0/4	4/6
6	Intact	2/7	8/10
	Total	4/28	25/36

$P = 0.001$.

ICMs from early blastocysts were cultured in zonae pellucidae in giant blastocysts for 24 hr and then in MEM + 10, dead cells were always observed and trophectodermal differentiation rarely occurred in the remaining cells.

These observations are compatible with the conclusion that blastocele fluid contains an activity toxic for cells with trophectodermal potential, whether these cells are embryonal carcinoma cells or ICM cells. Although explanations other than selective killing of ICM cells with trophectodermal potential can be offered, the conclusion that an activity in blastocele fluid, toxic for some ICM and embryonal carcinoma cells with trophectodermal potential, fits the data accurately and also explains why dead cells appear in mid-stage blastocysts. Dead cells have been reported in normal mid- to late stage blastocysts (27, 28) (Fig. 1*b*). In an electron microscopic study, an incidence of about six dead cells per blastocyst was found (29). The dead cells were phagocytosed by adjacent ICM and polar trophectodermal cells and digested in phagocytic vacuoles. The dead cells were believed to be ones that had failed to differentiate normally. Similarly, ≈10% of ICM and polar trophectodermal cells were dead in blastocysts composed of ≈85 cells (30). In a study of cell division and death in the blastocyst, Handyside and Hunter (26) observed a decline in the proportion of ICM cells to trophectoderm, an effect attributed in part to death of ICM cells. Whereas ICMs from early blastocysts have the potential for trophectodermal differentiation, after the death of ICM cells in mid-stage blastocysts, the remaining ICM cells lack the ability to differentiate into trophectoderm and form new blastocysts *in vitro*. However, P19 and ICM cells with embryonic potential remain and differentiate into endoderm, the first of the embryonic structures (1–3).

Toxic activities similar to that of blastocele fluid have been found in embryonic skin at the time premelanocytes arrive in it (unpublished data), and it is postulated that near or at the time of induction of differentiation there is a mechanism to kill cells that do not respond appropriately to developmental signals and are redundant. The advantage to the embryo would be that the phenotype of the developing tissue would be uncontaminated by inappropriate cells. This mechanism could explain the purity of cell type of most tissues and the absence of trophoblast or placental tissues in newborn mice.

This is a clear demonstration of an extracellular mediator of programmed cell death in the preimplantation mouse embryo. Programmed cell death is common in later development—i.e., the death of redundant cells between the digits (31)—but whether or not a toxic factor is responsible is not known. Saunders (31) favors the idea that epigenetic factors are responsible. In the blastocyst, as in most situations with programmed cell death, the cells die by apoptosis (32, 33). Apoptosis is supposedly "death from within," and it describes a mechanism of cell deletion that plays an opposite role to mitosis in regulating cell populations. In the process of apoptosis, cell nuclei and cytoplasm condense with good preservation of ultrastructure. The dead cells are then either phagocytosed by adjacent cells or fragment with phagocytosis of the particles. Review of the micrographs of El-Shershaby and Hinchliffe (29) indicates that the dead ICM cells were apoptotic. Thus, in the blastocyst, programmed cell death is induced by the toxic activity in blastocele fluid. The molecular mediator(s) and the cell(s) of origin of the toxic activity are not known.

Finally, the data obtained in this study are supportive of and extend the concept that carcinoma is a caricature of the process of tissue renewal (34, 35). Embryonal carcinoma is a caricature of subpopulations of ICM cells, and these tumor cells have been used successfully to probe the mechanism of regulation of ICM subpopulations in their embryonic field.

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