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Amine oxidases, programmed cell death, and tissue renewal

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Embryonal carcinoma cells, with embryonic (ECaE) or trophectodermal (ECaT) potential, have been used in a colony assay to determine regulatory mechanisms in the blastocyst. The mechnism that regulates ECaE and results in chimera formation is dependent upon a soluble factor in blastocoele fluid and contact with trophectoderm. Two mechanisms contribute to the regulation of ECaT: one involves a factor in blastocoele fluid and the other contact with either trophectoderm or inner cell mass which results in differentiation of the cells into trophectoderm, and the other involves the killing of at least 40% of the cells by blastocoele fluid alone. This cytotoxic activity probably causes the programmed cell death that occurs in the inner cell mass during blastulation as it loses the potential to differentiate into trophectoderm. A toxic activity similar to that of normal blastocysts has been obtained from embryoid bodies. This activity is caused by amine oxidase-dependent catabolism of polyamines, and it is postulated that programmed cell death in the embryo and chalone activity in the adult may have similar mechanisms.

Introduction

Thirty years have elapsed since the demonstration of the spontaneous differentiation of embryonal carcinoma into the three primary germ layers (Pierce & Dixon 1959), and subsequently into the benign differentiated tissues of teratocarcinomas (Pierce *et al.* 1960). This unique potential for differentiation suggested that embryonal carcinoma was the neoplastic equivalent of the inner cell mass (ICM) of the blastocyst (figure 1) (Pierce 1967), an idea now confirmed (Evans 1981; Martin 1981). Fifteen years ago, it was shown that the blastocyst could regulate some embryonal carcinoma cells to the extent that they behaved as apparently normal ICM cells (Brinster 1974). The cancer-derived progeny took part in embryonic development with the embryo-derived cells and eventuated chimeric mice (Papaioannou *et al.* 1975; Mintz & Illmensee 1975). There are important unanswered questions concerning the differentiation of neoplastic cells, their regulation by the blastocyst, and the nature of 'cancer-derived normal cells'. For example, these cancer-derived cells are referred to as normal because they respond to developmental and homeostatic signals similarly to embryo-derived cells; but are they the equivalent of initiated cells and only require promotion for malignant transformation? Are they the equivalent of SV40 transgenic cells which when in the host usually behave in a regulated fashion, but quickly transform when placed in tissue culture (Brinster *et al.* 1984). Answers to such questions are urgently needed, as are data concerning the mechanism of blastocyst regulation of embryonal carcinoma cells.

The inherent difficulty of studies on regulation in the blastocyst is the paucity of cells and fluid available for study in the pre-implantation mouse embryo (figure 1). A search was made for other embryonic fields capable of regulating their closely related cancers with the hope that one would be identified that was amenable to biochemical analysis. In this regard, the

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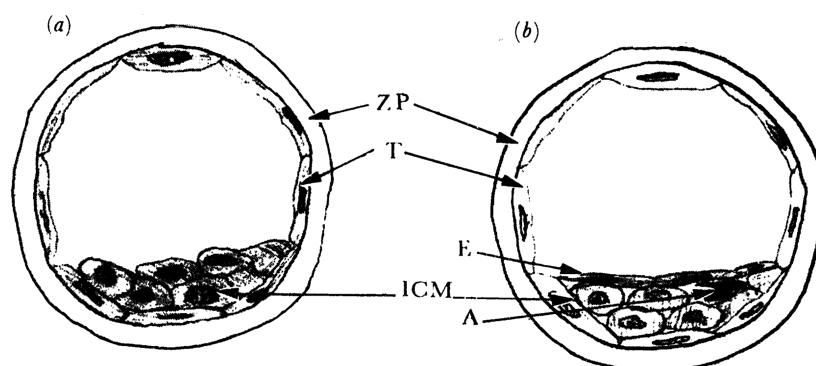

FIGURE 1. (*a*) An early blastocyst of the mouse that illustrates the zona pellucida (ZP) (egg shell), which is lined by trophectoderm (T) that will form the extra-embryonic foetal membranes, including the placenta. The inner cell mass (ICM) will ultimately form the embryo, however, at this stage they also have the potential to form trophectoderm when isolated and cultured *in vitro*. (*b*) A late blastocyst in which endoderm (E) has formed on the surface of the ICM. Programmed cell death, as evidenced by the apoptotic cell (A) has occurred and the ICM no longer has the potential to form trophectoderm when cultured *in vitro*.

malignant phenotype of C1300 neuroblastoma was regulated when the malignant cells were implanted in the neural crest migratory route (Podesta *et al.* 1984) or in the adrenal anlagen (Wells & Miotto 1986), and B16 melanoma cells failed to produce tumours in expected numbers when injected into the embryonic mouse limb on the gestational day when melanoblasts migrating from the neural crest arrived in the skin (Gerschenson *et al.* 1986). Gootwine *et al.* (1982) and Webb *et al.* (1984) injected 10-day-old mouse embryos *in utero* with leukaemia cells; although most of the animals died, two survivors were chimeric in their leukopoietic tissues. Together the data established the concept that appropriate embryonic fields regulate their closely related cancers (Pierce & Speers 1988). Regulation of melanoma by embryonic skin was chosen as the best model for *in vitro* studies on the mechanisms of regulation.

The mechanism of limb bud mediated regulation of melanoma cells in vitro

Because cancer-derived normal cells produce chimeras, media conditioned by or extracts of embryonic limbs were expected to contain inhibitors of proliferation or perhaps agents capable of directing differentiation of the melanoma cells. Surprisingly, the only detectable regulatory activity was serum-dependent cytotoxicity. After demonstrating by ultrafiltration and two-phase organic extractions that a small, strongly basic amine caused the cytotoxicity, the active compound was identified by weak cation exchange HPLC (a biocompatible separation system) as spermidine (figure 2).

Spermidine, like the other polyamines such as putrescine and spermine, is itself non-toxic. However, in the presence of a family of enzymes named amine oxidases, polyamines are oxidized to H_2O_2, aldehydes, and NH_3 (Morgan 1980). Although all of these catabolites are potentially toxic, most of the cytotoxicity is due to H_2O_2 (Henle *et al.* 1986). In our *in vitro* experiments, the amine oxidase was contributed by the serum supplement of the culture medium. When aminoguanidine was employed as an inhibitor of serum amine oxidase (Henle *et al.* 1986), all toxic activity was abolished in limb-bud extracts. From these experiments, it was concluded that the only soluble regulatory molecule detectable in the limb was spermidine and that it killed rather than differentiated melanoma cells, and that an amine oxidase was

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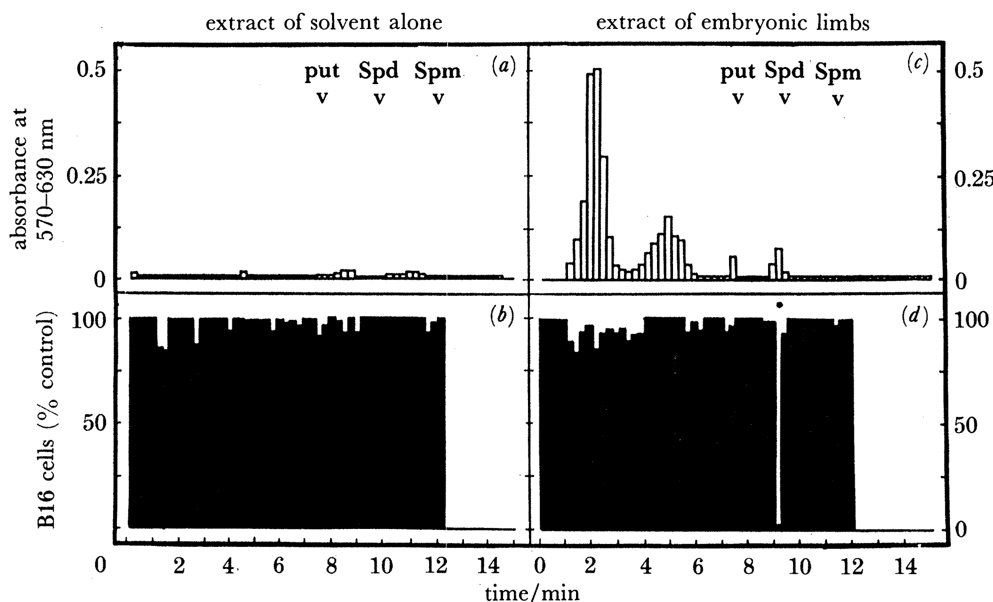

FIGURE 2. Homogenization buffer (left panels) and the soluble fraction of a limb-bud homogenate (right panels) were fractionated by polyaspartate HPLC. Post-column fractions were assayed for amines by ninhydrin (top panels) and for cytotoxicity (bottom panels). The asterisk marks the co-elution of the cytotoxicity with spermidine.

required in the embryonic limb or on the implanted melanoma cells for this mechanism to operate *in vivo*.

The mechanism of blastocyst-mediated regulation of embryonal carcinoma cells

The ICM of the early blastocyst has the potential to make trophectoderm, but during the transition from early to late blastocyst cell death occurs in the ICM and the potential to make trophectoderm is lost. Then the ICM can only differentiate into the three germ layers of the embryo (Fleming *et al.* 1984; Handyside 1978; Hogan & Tilly 1978*a*, *b*; Spindle 1978). Because embryonal carcinoma is the neoplastic equivalent of ICM, and as the regulation of cells with trophectodermal potential might differ from that of cells with embryonic potential, it was decided to employ two lines of embryonal carcinoma in the studies of regulation (Pierce *et al.* 1989). Embryonal carcinoma cells (ECa) 247 were chosen as the neoplastic equivalent of early ICM cells with trophectodermal potential because 90% of labelled ECa 247 cells localized in and differentiated into trophectoderm when injected into the blastocyst (Pierce *et al.* 1987). P19 was chosen as the counterpart of the late ICM cells because 60% of blastocysts injected with these cells were chimeric when analysed at mid-gestation (McBurney & Rogers 1982) (Rossant & McBurney 1983).

Tumour-forming ability and colony-forming ability of ECa 247 and P19 cells were regulated after injection into blastocysts. Thus they could be used to dissect the regulatory mechanisms. Both strains of embryonal carcinoma cells were regulated by trophectodermal vesicles leading to the conclusion that contact with trophectodermal cells, in the presence of blastocoele fluid, was essential for regulation and the development of chimeric mice with ECaE. (Pierce *et al.* 1989).

There is very little blastocoele fluid in a blastocyst to study the regulatory effect of ICM cells

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in the presence of blastocoele fluid. Hence, giant blastocysts were constructed by fusing eight 8-cell eggs (Pedersen & Spindle 1980) and culturing them to the blastocyst stage *in vitro*. These giant blastocysts were large enough to accommodate carriers made from emptied and washed zonae pellucidae. These carriers were injected with either ICM cells and embryonal carcinoma cells, ICM cells alone, or embryonal carcinoma cells alone, and then inserted into giant blastocysts through a triangular hole made in the mural trophectoderm. The preparations were cultured for 24 h in Eagle's minimal essential media with 10% foetal bovine serum (MEM+10), then carriers were rescued, the cells enumerated, examined morphologically, and usually cultured in MEM+10 to test their viability and clonability. The controls for these experiments were carriers containing the appropriate cells that were cultured for the entire time in MEM+10. Regulation of colony formation of ECa 247 cells was achieved by contact of the cells with ICM in the presence of blastocoele fluid but no such regulation of P19 cells occurred (Pierce *et al.* 1989).

However, incubation of ECa 247 cells with ICM cells or trophectoderm in the presence of tissue culture media was not regulatory (Pierce *et al.* 1984). Thus blastocoele fluid was required for regulation. To study the effects of blastocoele fluid, two ECa 247 cells were placed into carriers and incubated in giant blastocysts for 24 h and then the number of cells was counted and compared with the controls that were incubated in MEM+10. On average, two cells were recovered from the giant blastocysts, and four were recovered from the controls, suggesting the presence of an inhibitor of growth for ECa 247 cells. The recovered cells grew *in vitro* as did the controls. P19 cells were unaffected by incubation in giant blastocysts. It was concluded that an inhibitor of cell division might be present in blastocoele fluid for certain populations of embryonal carcinoma, and also for certain populations of ICM cells. This tissue-specific phenomenon resembled chalone-like regulation. However, dead cells were always observed in the carriers incubated in giant blastocysts, suggesting that cell death could be an alternative explanation for the apparent inhibition of proliferation. The experiment was repeated by using single ECa 247 cells to determine whether some of the inhibition of cell proliferation was in fact mediated by cell killing. Surprisingly, 44% of single ECa 247 cells were killed in the presence of blastocoele fluid, compared with 2% that were killed in the control situation (Pierce *et al.* 1989). Single P19 cells were not killed by exposure to blastocoele fluid. Two conclusions were derived from this experiment: there was a toxic factor in blastocoele fluid that selectively killed ECa 247 cells (trophectodermal potential) but not P19 cells (embryonic potential), and that selective killing of part of a population of cells could easily be misinterpreted as inhibition of cell proliferation. This result has implications for regulation of tissue renewal as will be discussed later.

The data also alter the current understanding of programmed cell death. Because tumours are caricatures of the process of tissue renewal and embryonal carcinomas caricature the ICM (Pierce 1967), the selective killing of embryonal carcinoma cells with pretrophectodermal potential would strongly suggest (but not prove) that blastocoele fluid also causes programmed cell death of redundant pretrophectodermal cells in the blastocyst between early and late stages. Such a mechanism would explain why baby mice are born lacking placental tissue in their bodies.

Early ICMs in carriers were exposed to blastocoele fluid in giant blastocysts for 24 h and then removed and cultured in MEM+10. The treated ICMs produced predominantly two-layered embryos whereas the untreated controls regenerated trophectoderm and produced blastocysts.

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The data demonstrated that blastocoele fluid abrogated the ability of ICM cells to form trophectoderm, and dead cells were observed in such ICMs. Although it is difficult to determine what the potential of a dead cell might have been, the data supported the idea that the change in potential of ICMs during the transition from early to late blastocysts was caused by the death of ICM cells with trophectodermal potential (Pierce *et al.* 1989). An alternative explanation, that the sojourn in blastocoele fluid may have changed the developmental potential of the cells from trophectoderm to embryo, cannot be excluded, but because of the direct toxic effect upon embryonal carcinoma cells with trophectodermal potential, we favour the former interpretation.

There was very little normal blastocoele fluid for biochemical determination of the mechanism of cell killing, so as an alternative we analysed the fluid contained in cystic embryoid bodies that at the light microscopic level bore a striking resemblance to blastocysts (Monzo *et al.* 1987). Electron microscopic and immunohistochemical analysis of these embryoid bodies confirmed the presence of an ICM and an outer layer of cells. Unlike the normal blastocyst in which the outer layer of cells is composed of trophectodermal cells only, the outer layer of cells of the neoplastic embryoid bodies is predominantly endoderm and various differentiated derivatives of endoderm. Immunohistochemical studies indicated the presence of embryonal carcinoma and endoderm and cells with features of both endoderm and trophectoderm. Of interest was the presence of dead ICM cells in many of these embryoid bodies, which suggested the presence of a cytotoxin in the cyst fluid. To determine if the putative cytotoxin in cysts might have preferential toxicity for pretrophectodermal cells, single ECa 247 and P19 cells were injected into them and they were cultured in MEM+10. Whereas P19 cells were unaffected by incubation in these vesicles, a significant number of ECa 247 cells were killed (Parchment *et al.* 1990).

However, the fluid, extruded from these embryoid bodies by ultracentrifugation at 450000 *g*, was toxic to both ECa 247 and P19 when cultured in MEM+10. Addition of aminoguanidine to the culture medium abolished the toxic activity, proving its amine oxidase dependency. The identity of the amine oxidase substrate present in the cyst fluid is currently under investigation; preliminary data suggest that it is a polyamine (small and strongly basic). It is believed that the lack of selective killing *in vitro* is because of the presence of high levels of serum amine oxidase in the foetal bovine serum that generates lethal quantities of H_2O_2, killing cells indiscriminately. There is an important warning for tissue culturists in this observation: quite likely the unexplained killing of cells *in vitro*, as in the crisis of spontaneous transformation or in killing attributed to other means, for instance, LAK cell killing, may be the result of generation of toxic levels of H_2O_2 during degradation of polyamines by amine oxidases.

The current hypothesis is that the polyamine in the blastocoele fluid functions as a substrate for an amine oxidase localized to pretrophectodermal ICM and ECa 247 (perhaps on the cell surface via a receptor binding site or internally where it could degrade polyamine taken up by the cells). This amine oxidase may be absent on P19 cells and ICM cells with embryonic potential, or they may lack a cell surface receptor for the enzyme. Alternatively, they may express amine oxidases with different substrate specificities or kinetics. Finally, there may be polyamine conjugates on the cell surface that are substrates for extracellular amine oxidases.

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Programmed cell death and regulation of tissue renewal

Programmed cell death is defined as predictable death of cells during development. The mechanism of programmed cell death is unknown, but the idea is generally accepted that its mechanism is intracellular because no extracellular mediators have been demonstrated. Death of cells in the blastocyst was demonstrated in an elegant ultrastructural study (El-Shershaby & Hinchliffe 1974) and about 10% of the cells die (Handyside & Hunter 1986). The dead cells were recognized as apoptotic (the morphologic appearance of dead cells in programmed cell death) (Kerr *et al.* 1972; Wylie 1981). The decline in numbers of ICM cells has been attributed to programmed cell death (Handyside & Hunter 1986). We believe this programmed cell death destroys redundant pretrophectodermal cells in the ICM and, as a result, ectopic placental tissue is not found within the embryo.

It is postulated that programmed cell death is caused by the catabolism of polyamines and the generation of toxic reaction products by amine oxidases that are regulated in a developmental pattern. A prediction from this hypothesis would be the presence of an amine oxidase in the fluid of the embryoid bodies. Preliminary data from enzymic assays show that the cells and the cyst fluid contain an amine oxidase that catabolizes spermine and, to a lesser extent, spermidine. This type of amine oxidase is quite similar to that isolated from rat liver (Hölttä 1983). We have made antibodies to these tissue-specific amine oxidases and will use them for immunolocalization. If the hypothesis is correct, then the antibody will localize in the dead ICM cells of the cystic embryoid bodies, in about half of ECa 247 cells, and in three quarters of the pretrophectodermal cells of the blastocyst that are expected to undergo apoptosis.

In the *in vitro* system to study melanoma regulation, toxic polyamine catabolites were generated by serum amine oxidase from polyamine either excreted by living cells or released from dying cells into the medium, a situation that could have been dismissed as an artefact of tissue culture. Amine oxidase–polyamine reactions have been labelled as artefacts because of studies on the mechanism of chalone activity (discussed below) (Lenfant *et al.* 1979*a*; Lenfant *et al.* 1979*b*; Rijke & Ballieux 1978). However, the demonstration of selective cell killing in the blastocyst and neoplastic embryoid bodies and the demonstration of polyamines and specific tissue amine oxidases in the cystic embryoid bodies of C44 in the absence of serum amine oxidase lent credence to the idea that polyamine catabolism causes programmed cell death. There is evidence that amine oxidases from bovine serum and porcine kidney cause cell death in about 6 h when microinjected into a target cell (Bachrach *et al.* 1988).

Amine oxidase/polyamine toxicity may also be involved in the regulation of renewing cell populations, that is, chalone activity. Chalones are postulated to be tissue but not species-specific activities synthesized by the differentiated cells of tissues that reversibly inhibit the proliferation of stem cells of that tissue (Bullough 1962). Observations with ECa 241 cells in blastocoele fluid showed that death of a portion of cells in a population produces effects easily misinterpreted as reversible inhibition of proliferation. Thus it is conceivable that the mechanism responsible for programmed cell death in the embryo might be re-regulated to serve as part of the mechanism of tissue-renewal in the adult (Bullough 1962; Langen 1985).

Interestingly, chalone activity co-purifies with polyamines (Barford 1980) and renewing tissues such as epidermis and intestine contain amine oxidases distinct from the serum enzyme

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(Seiler *et al.* 1980). Experiments are currently in progress to examine the role of amine oxidases and their polyamine substrates in regulation of tissue renewal in the epidermis.

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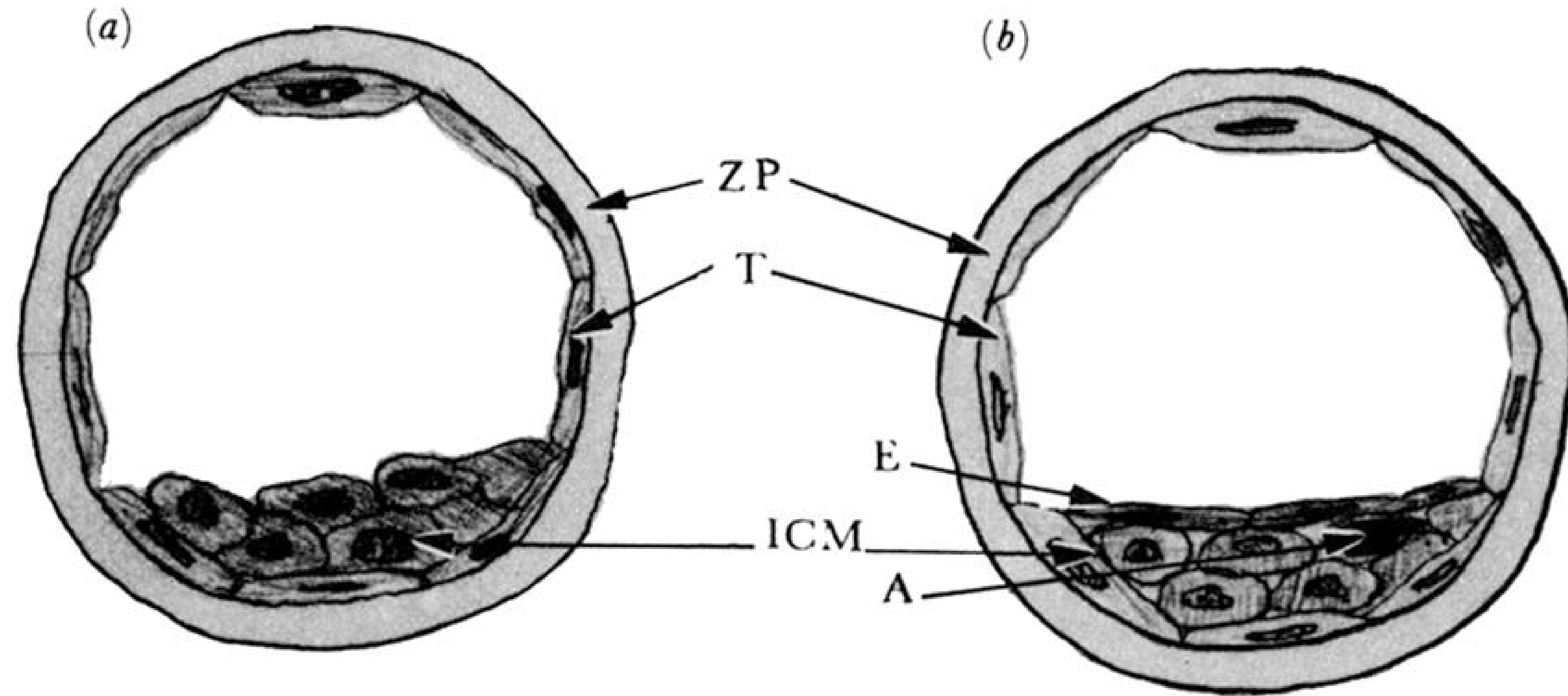

GURE 1. (*a*) An early blastocyst of the mouse that illustrates the zona pellucida (ZP) (egg shell), which is lined by trophectoderm (T) that will form the extra-embryonic foetal membranes, including the placenta. The inner cell mass (ICM) will ultimately form the embryo, however, at this stage they also have the potential to form trophectoderm when isolated and cultured *in vitro*. (*b*) A late blastocyst in which endoderm (E) has formed on the surface of the ICM. Programmed cell death, as evidenced by the apoptotic cell (A) has occurred and the ICM no longer has the potential to form trophectoderm when cultured *in vitro*.