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The mode of cell death associated with cavitation in teratocarcinoma-derived embryoid bodies

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SUMMARY

Cell death occurring in embryoid bodies derived from the embryonal carcinoma cell line, PSA4, which undergo cavitation, and in those from the related cell line S2, which do not undergo cavitation, was classified as apoptosis or necrosis by ultrastructural criteria. Both modes of cell death were seen in PSA4 embryoid bodies while apoptosis alone was seen in S2 embryoid bodies. No significant difference was found between PSA4 and S2 embryoid bodies either in apoptotic incidence score or in the spatial distribution of apoptotic events. We therefore conclude that although apoptosis and tissue modelling coexist in PSA4 embryoid bodies, necrosis rather than apoptosis is causally related to formation of the cavity.

INTRODUCTION

The significance of cell death in early development has long been recognized (Glücksmann, 1951; Bellairs, 1961; Saunders, 1966). Study of its mechanism is hindered however by the small size of the early embryo, the admixture of the dying cells with their proliferating and differentiating neighbours, and the limited range of experimental manipulation which can be applied to early embryonic tissues. Cell lines derived from the pluripotent embryonal carcinoma (EC) cells of teratocarcinomas afford more plentiful and accessible material than embryonic tissues, and have permitted elucidation of several basic questions in developmental biology (Martin, 1980). When cells of certain embryonal carcinoma lines are grown in suspension culture, two-layered cell aggregates are formed in which a core of EC cells is surrounded by an outer layer of endoderm. In these 'embryoid bodies' reproducible and focal cell death associated with cavity formation has been described (Martin, Wiley & Damjanov, 1977). As EC cells are thought to be developmentally analogous to epiblast cells of the early post-implantation embryo, it has been suggested that the cavity formation in the embryoid bodies may parallel the formation of the pro-amniotic cavity. This suggestion is supported by the observation that further differentiated cell types appear in the embryoid body concurrently with, or subsequent to, cavity formation. EC cell lines exist whose embryoid bodies neither develop cavities nor differentiate beyond the two-layered stage in suspension. Comparison of related

cavitating and non-cavitating cell lines should therefore allow the degree of association between cell death and cavity formation to be precisely assessed.

Although there have been many observations on cell death in developing systems, only recently has the classification of death been formalized to emphasize the existence of two, apparently discrete, morphologically defined pathways – *necrosis* and *apoptosis* (Kerr, Wyllie & Currie, 1972; Wyllie, 1981). Whereas necrosis is characterized by cellular swelling and rupture of the plasma and internal membranes, apoptosis is characterized by cell shrinkage, with conservation of the integrity of cytoplasmic organelles, but widespread condensation of nuclear chromatin, and cleavage of chromatin into oligonucleosome chains (Wyllie, 1980). Extensive review of the literature suggests that apoptosis rather than necrosis is associated with developmentally regulated death (Wyllie, Kerr & Currie, 1980). Only in phylogenetically primitive organisms has necrosis alone been described unequivocally as a homeostatically regulated mode of cell death (Bowen & Ryder, 1974). The cell death associated with cavity formation in embryoid bodies has been interpreted as apoptosis (Solter & Damjanov, 1979). In this paper we present data which do not support this contention. We describe and quantify cell death in embryoid bodies of the EC cell line PSA4, which form cavities, and in those of the related EC line S2, which do not. We show that whilst apoptosis occurs in both cases, necrosis appears exclusively in the PSA4 embryoid bodies, in association with cavity formation.

MATERIALS AND METHODS

Cell culture

PSA4 and S2 EC cell lines are derived respectively from primary and secondary cultures of the transplantable tumour line OTT5568 (Martin & Evans, 1975*a*). PSA4TG12 is a thioguanine-resistant variant of PSA4 which behaves identically to its parent with regard to cavitation and differentiation (Slack, Morgan & Hooper, 1977). Stock cultures of these pluripotent EC cell lines were maintained in their undifferentiated state by frequent subculture on a feeder layer of mitomycin C-treated mouse STO fibroblasts (Martin & Evans, 1975*b*), in Glasgow-modified Eagle's medium supplemented by non-essential amino acids (each at 0·1 mM), 1 mM-sodium pyruvate, 0·1 mM-β-mercapto-ethanol, and 10 % newborn calf serum (selected batches). Cultures were maintained at 37 °C in a humidified atmosphere of 95 % air, 5 % CO_2. Embryoid body formation was induced as described by Martin & Evans (1975*b*). Cells were seeded at $2{\cdot}5\times10^6$ per 5 cm diameter tissue culture dish in medium as above but lacking non-essential amino acids, pyruvate and β-mercapto-ethanol. On the fifth day after seeding, nests of cells formed and were gently detached by blowing fresh medium across the surface. These aggregates were subsequently grown in suspension in bacteriological dishes.

Morphological studies

Embryoid bodies on the day of detachment and 1, 3, 5, 7 and 9 days subsequently were transferred to universal containers and allowed to sediment under gravity. They were then washed twice in Dulbecco's phosphate-buffered saline solution A (PBS A) and fixed in Bouin's fluid. 5 μm paraffin sections were stained with Mayer's haematoxylin and eosin or by the Feulgen reaction. Feulgen-stained preparations were scanned at 250× magnification using a Tektronic (4050 series) Graphic Information System to determine the following parameters in each embryoid body section: (1) area; (2) area of cavity; (3) number of apoptotic cells. From these data a score proportional to the incidence of apoptosis could be derived. This 'apoptotic incidence score' was defined as the number of apoptotic cells per unit area of embryoid body section. It is recognized that the absolute incidence of apoptosis in embryoid bodies is a complicated function of this simple score, requiring knowledge of the duration for which an apoptotic body, once formed, remains identifiable by light microscopy. Further, an absolute score of incidence requires information about the effective diameter of apoptotic cells relative to the section thickness, as this affects the probability with which they are recognized (Aherne, 1967). However, since these additional factors are likely to be constant in embryoid bodies, the simple score defined above permits analysis of changes in incidence of apoptosis.

For electron microscopy, embryoid bodies were fixed in 3.1 % glutaraldehyde and 0·1 M-osmium tetroxide, embedded in Araldite and stained with uranyl acetate and lead citrate. Thin sections were viewed in a Jeol 100/S electron microscope.

RESULTS

As described by Martin *et al.* (1977) PSA4 EC cells formed embryoid bodies with eccentric cavities. The cavities were first observed on days 4–5 after detachment, as areas of cellular debris. They were lined by cells which had a radial polarity, contrasting with the apparently random orientation of the EC cells in the rest of the embryoid body core. Over the next 4–5 days the cavities enlarged (Figs 1, 2) and differentiated cells appeared elsewhere in the embryoid bodies. S2 cells formed simple embryoid bodies in which neither cavity formation nor morphological evidence of differentiation beyond the two-layered stage was observed.

Two types of cell death were identifiable in embryoid bodies. The first was observed in both PSA4 (cavitating) and S2 (non-cavitating) embryoid bodies and bore the characteristics of *apoptosis*. Light microscopy showed that the affected cells were scattered amongst apparently viable neighbours (Fig. 3). Their nuclei were rounded, sometimes fragmented, and stained homogeneously in the Feulgen reaction. Ultrastructurally some of these cells appeared as membrane-

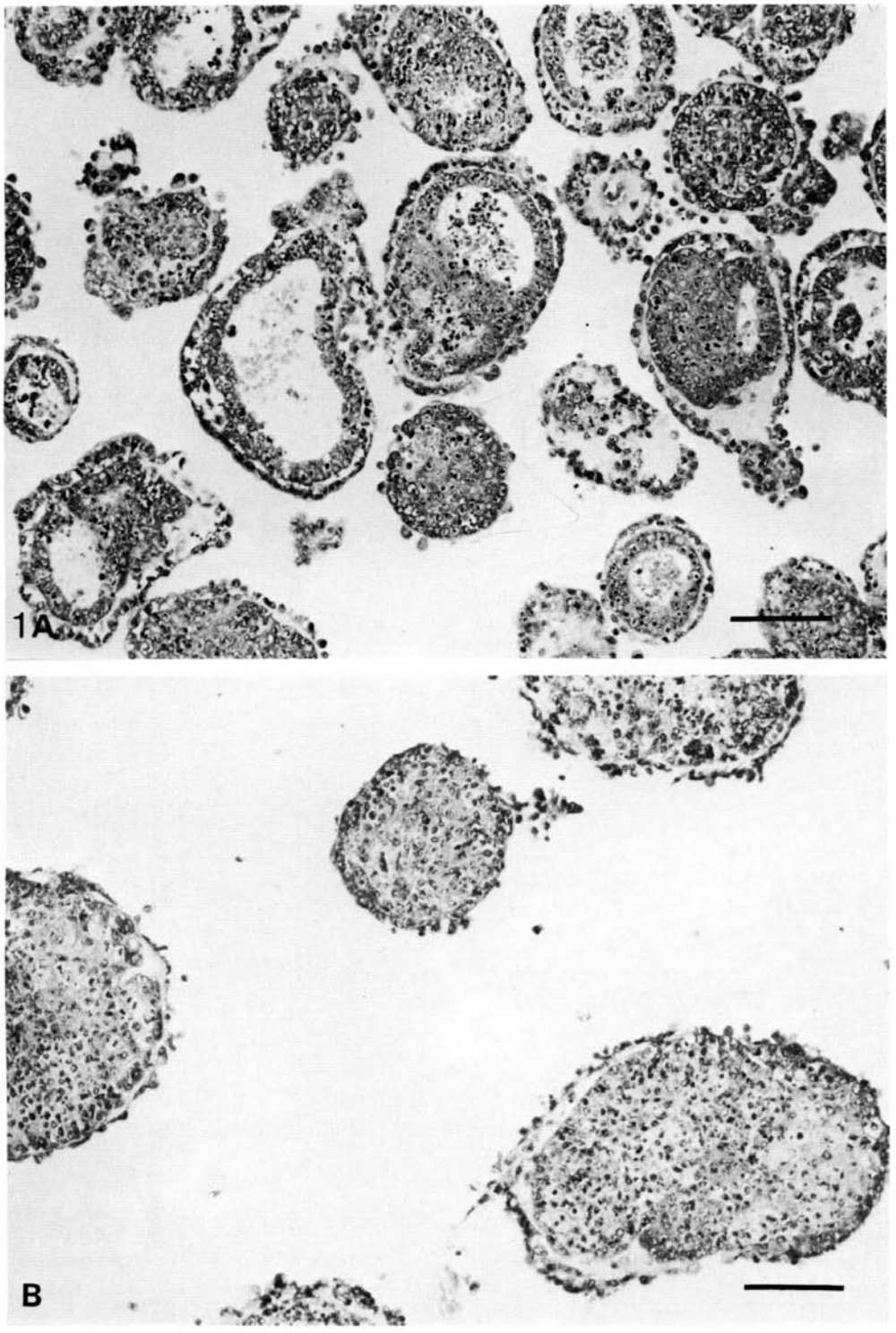

Fig 1

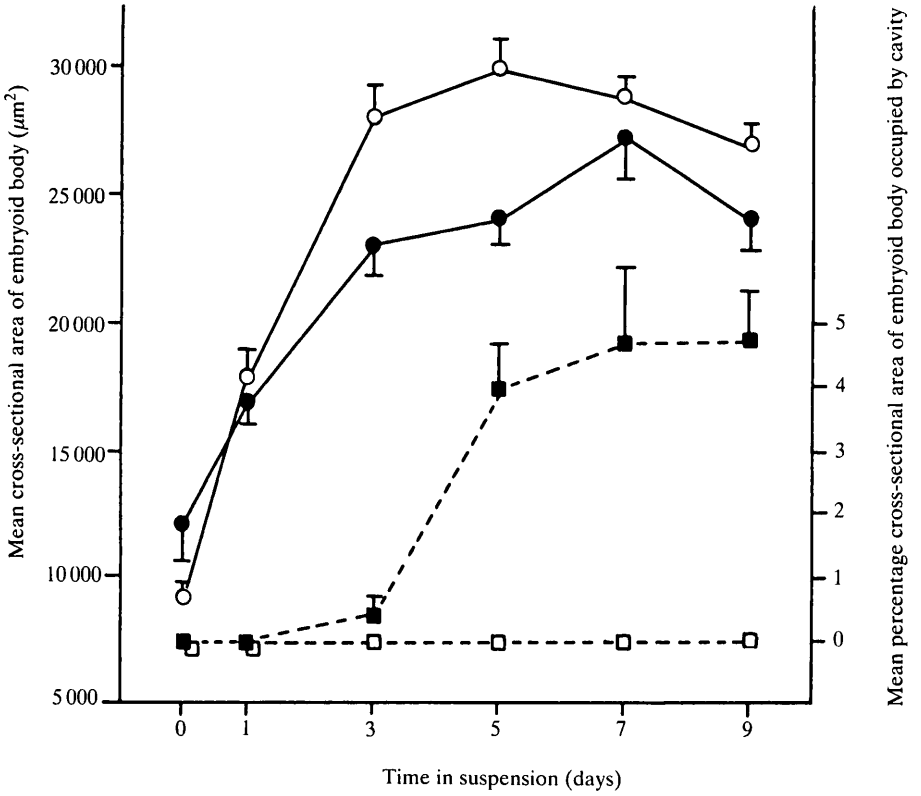

Fig. 2. Time course of embryoid body growth and cavitation in PSA4 and S2 embryoid bodies, showing cross-sectional area (left ordinate) of PSA4 (●) and S2 (○) embryoid bodies, and percentage of embryoid body cross-sectional area occupied by cavity (right ordinate) for PSA4 (■) and S2 (□). Data are presented as the mean determined from five separate experiments, with the error bar indicating one standard error of the mean.

bounded fragments including compacted cytoplasmic organelles and nuclei with condensed chromatin (Fig. 4B). In other cells, presumably at an earlier stage, the condensation of nuclear chromatin was localized to a hemilunar cap (Fig. 4C). Although occasionally observed free in the intercellular space (Fig. 4B), apoptotic bodies were more often found undergoing degradation within large heterophagosomes in viable EC cells. Apoptotic incidence scores were closely similar in PSA4 and S2 embryoid bodies (Fig. 5). Around the onset of cavity formation in PSA4 embryoid bodies there was no increase in apoptotic incidence. In both types of embryoid body the apoptotic cells showed similar spatial distributions (Fig. 3) with no focal grouping in the vicinity of developing cavities.

Fig. 1. Embryoid bodies from (A) PSA4TG12, at day 5 after detachment; (B) S2, at 7 days after detachment (both panels ×144; scale bar, 100 μm).

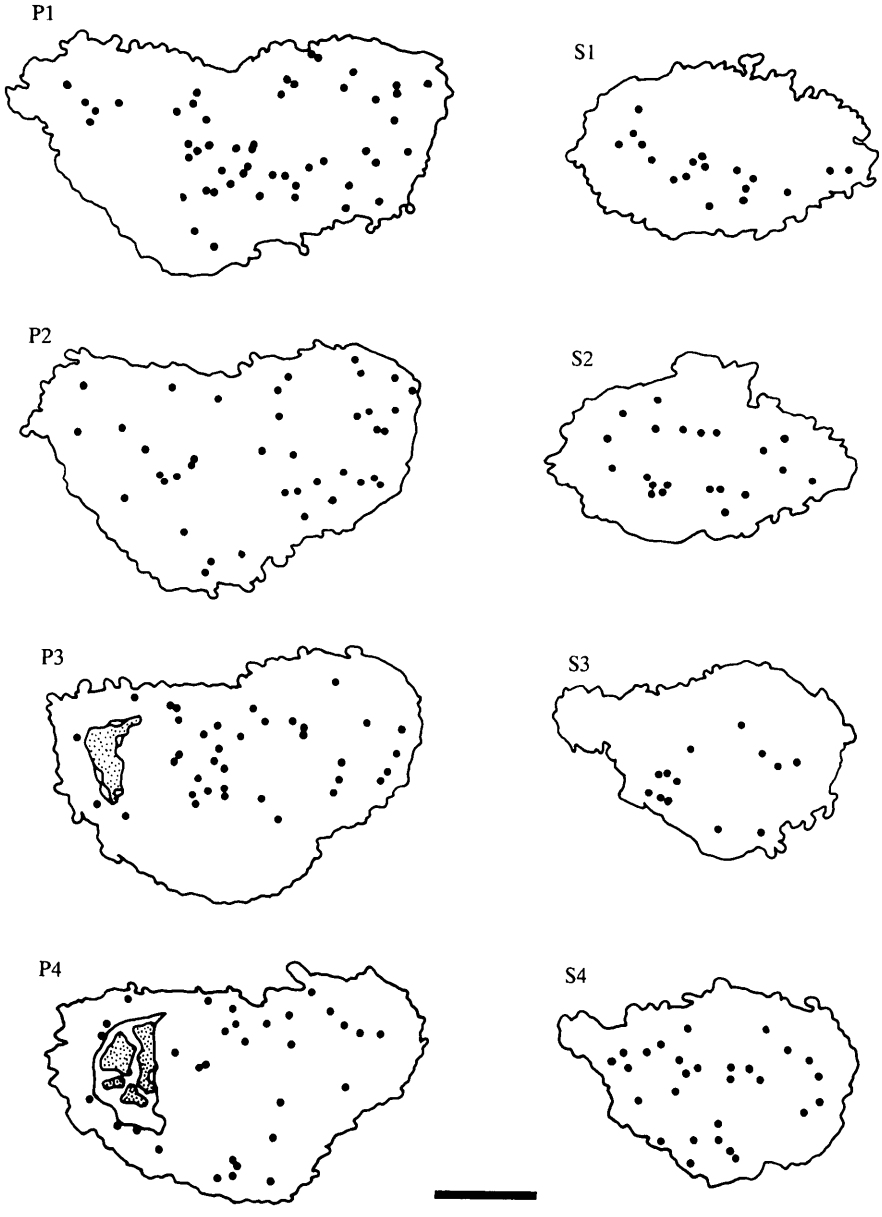

Fig. 3. Spatial distribution of apoptotic cells within embryoid bodies of PSA4 and S2. Outlines of sections taken at approx. 20 μm intervals from serial sections of a PSA4 embryoid body at day 3 after detachment (P1–P4), an S2 embryoid body at day 3 (S1, S2) and an S2 embryoid body at day 4 (S3, S4). Apoptotic cells are shown by dark circles (●). In sections P3 and P4 a developing cavity is present, within which are necrotic cells (stippled areas). ×135; scale bar, 100 μm.

The second type of cell death was observed only in PSA4 embryoid bodies, and showed the features of *necrosis*. At light microscope resolution the dead cells were represented by debris in the developing cavities. The earliest recognizable cavities were often completely filled with these necrotic cells. Ultrastructurally, nuclei were swollen and included heterochromatin aggregates in approximately the same distribution as in normal nuclei (Fig. 4D). Cytoplasmic swelling was gross and frequently associated with ruptured plasma membranes, 'high amplitude' swelling of mitochondria, or extensive disruption of ER membranes (Figs 4D, E).

DISCUSSION

The results show conclusively that apoptosis alone is not responsible for cavity formation in cystic embryoid bodies. Although all the characteristic features of apoptosis were identified in embryoid bodies during culture, the spatial and temporal distribution of apoptotic cells within the embryoid bodies was unrelated to the appearance of cavities. Moreover apoptosis was as frequent in non-cavitating S2 as in cavitating PSA4 embryoid bodies. Cell death, however, did occur during the development of the cavities but with the morphology of necrosis. In contrast to apoptotic cells, the necrotic cells showed focal grouping and at the onset of cavity formation the entire region of the developing cavity was frequently packed with necrotic cells. The earliest morphological stigmata we could identify – nuclear and cytoplasmic oedema – excluded the possibility that these dying cells were undergoing degeneration secondary to an initial phase of apoptosis as has been described elsewhere (Wyllie *et al.* 1980). This result was unexpected as, unlike apoptosis, necrosis is in general associated with pathological cell states, often involving grossly unphysiological environmental conditions (Kerr *et al.* 1972; Wyllie, 1981).

Cell death has been described in the early mouse embryo at the blastocyst stage (El-Shershaby & Hinchliffe, 1974; Copp, 1978), in the egg cylinder (Bonnevie, 1950; Poelmann & Vermeij-Keers, 1976), and in association with neural tube formation (Schlüter, 1973; Poelmann, 1980). Bonnevie (1950) stated that cell death occurred at the inner surface of extraembryonic and embryonic ectoderm, beginning after a proamniotic cavity was already evident. In contrast Poelmann & Vermeij-Keers (1976) observed cell death (with the morphology of apoptosis) at about the time of proamniotic cavity formation but with no obvious clustering at the prospective site of cavity formation. Despite these differences in detail, in neither case was cell death associated spatially and temporally with proamniotic cavity formation, nor is there evidence of this in other published studies of the egg cylinder at the light-microscope level (Reinius, 1965; Theiler, 1972; Poelmann, 1975). In contrast, in embryoid bodies the prospective cavity is first recognizable as an area of dying cells (Martin *et al.* 1977; Fig. 3). While this does not prove that the necrosis is the primary event in cavitation (we cannot

exclude the possibility that necrosis and cavitation coexist as parallel results of a common stimulus) it does suggest that embryoid body cavitation may not provide a model which can be applied in detail to the specific case of proamniotic cavity formation in the embryo. Although Martin *et al.* (1977) found that the development of embryoid bodies *in vitro* paralleled normal embryonic development in a general way, they, too, pointed out discrepancies in detail, notably in endoderm and mesoderm formation, and noted that embryoid body development

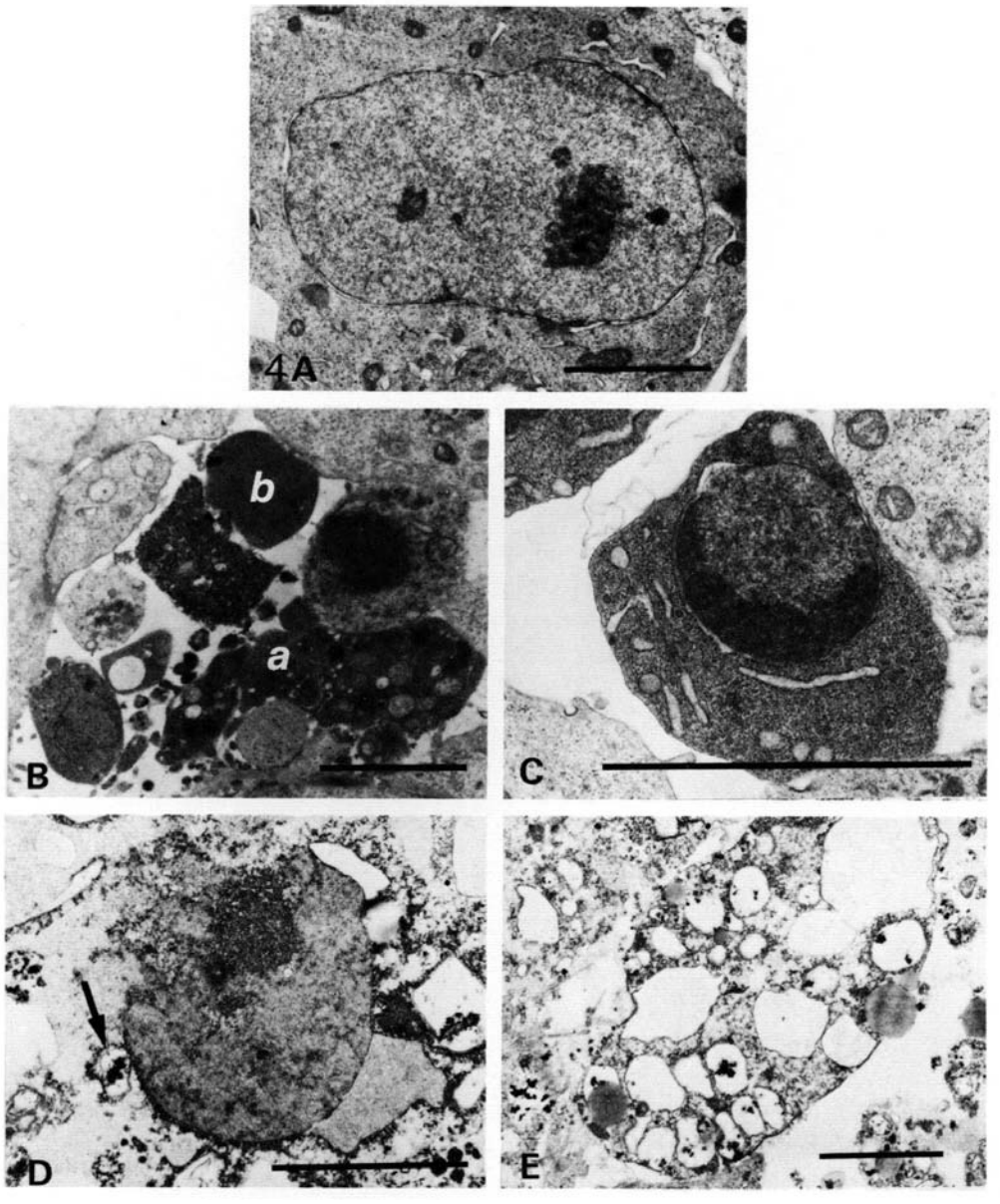

Fig. 4

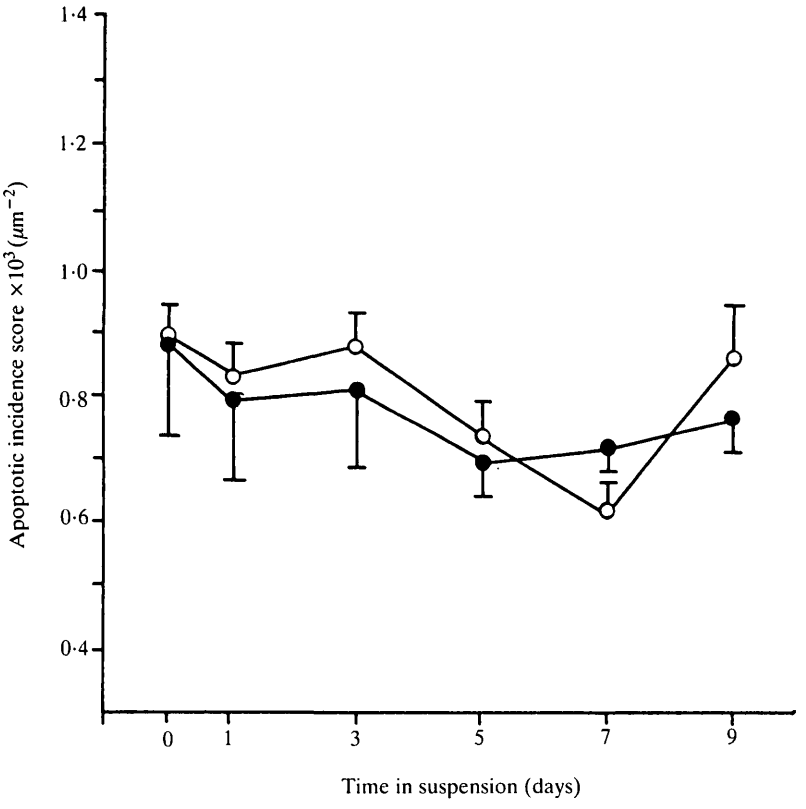

Fig. 5. Time course of apoptotic incidence in PSA4 (●) and S2 (○) embryoid bodies. Data are presented as the mean determined from five separate experiments, with the error bar indicating one standard error of the mean.

Fig. 4. Ultrastructure of normal, apoptotic and necrotic cells in embryoid bodies.

(A) Normal EC cell from an S2 embryoid body. Note the dispersed nuclear chromatin, prominent nucleoli and cytoplasm rich in polysomes (×3800).

(B) Fragments of an apoptotic EC cell lie in the intercellular space in a PSA4 embryoid body. One fragment shows the characteristic compaction of cytoplasmic organelles (a) whilst another consists almost entirely of condensed chromatin (b) (×3800).

(C) Higher magnification of an apoptotic EC cell from a PSA4 embryoid body. The smooth plasma membrane, cytoplasmic electron density, vesicular ER, and nucleus with prominent cap of condensed chromatin are characteristic features. In contrast to the surface of the apoptotic cell, that of adjacent normal cells shows microvilli and active endocytosis (×9600).

(D) A necrotic EC cell from a cavitating PSA4 embryoid body. Mitochondria, in contrast to those in Fig. 4B, show disrupted cristae and prominent matrix densities (arrow). The nuclear chromatin remains dispersed, and a residual nucleolar structure is present despite the advanced stage of degradation of this cell (×5100).

(E) Cellular debris, some without bounding membranes, from the necrotic centre of a PSA4 embryoid body cavity (×3200).

In all panels, scale bar = 5 μm.

more closely paralleled that of normal inner cell masses explanted in culture (Wiley, Spindle & Pedersen, 1978). Embryoid body cavitation is nevertheless a developmentally significant tissue-modelling process, since further differentiation of the core EC cells is restricted to those embryoid bodies which show cavitation, and the first cells to differentiate are aligned along the edge of the cavity.

Two facts argue against the hypothesis that necrosis in PSA4 embryoid bodies is merely a trivial culture artefact, due, for example, to inadequate diffusion of oxygen or substrate to the central cells in the embryoid body. Firstly, necrosis is always absent in S2 embryoid bodies, even at diameters twice as great as those found in PSA4 embryoid bodies with internal necrosis, and despite the similar overall growth rates of cavitating and non-cavitating embryoid bodies. Secondly, cavitation in PSA4 embryoid bodies is always eccentric, and frequently very markedly so, whereas hypoxic or nutritional necrosis is centrally disposed in 'spheroids' of other cell types growing in suspension culture (Franko & Sutherland, 1979) or occurs at a remarkably constant, predictable distance from blood vessels in tissues (Thomlinson & Gray, 1955; Tannock, 1970).

Two classes of hypothesis could account for the differing incidence of necrosis in PSA4 and S2 embryoid bodies. (1) S2 EC cells may be incapable, perhaps by virtue of intrinsic metabolic differences, of responding to lethal stimuli equally present in PSA4 and S2 embryoid bodies. (2) Conversely, lethal stimuli may be engendered only in PSA4, and not in S2 embryoid bodies. The latter situation could arise, for example, because of differences in the nature and extent of differentiation prior to cavitation. At least one such difference in differentiation is recognized prior to cavitation: PSA4 embryoid bodies form both visceral and parietal endoderm on their surfaces, whereas S2 embryoid bodies form only parietal endoderm (Martin *et al.* 1977). The question remains open whether the wider spectrum of differentiation which develops subsequently in PSA4 embryoid bodies is a consequence of cavitation, or whether the primary events in differentiation precede cavitation. It may be possible to distinguish between hypotheses (1) and (2) by study of mosaic embryoid bodies formed from mixtures of cells from cavitating and non-cavitating lines; in our laboratory we are currently developing the appropriate lineage markers to permit analysis of such mosaic embryoid bodies.

In conclusion, the data presented here emphasize the need for caution in interpreting the role of cell death in morphogenesis. Apoptosis and cavity formation are not causally related in embryoid bodies, although they coexist in the PSA4 line. Necrosis, however, is closely associated with cavity formation, but we have not established which is the primary event.

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REFERENCES

AHERNE, W. (1967). Methods of counting discrete tissue components in microscopical sections. *J. Roy. microsc. Soc.* **87**, 493–508.

BELLAIRS, R. (1961). Cell death in chick embryos as studied by electron microscopy. *J. Anat.* **95**, 54–60.

BONNEVIE, K. (1950). New facts on mesoderm formation and proamnion derivatives in the normal mouse embryo. *J. Morph.* **86**, 495–505.

BOWEN, I. D. & RYDER, T. A. (1974). Cell autolysis and deletion in the planarian *Polycelis tenuis* Iijima. *Cell Tissue Res.* **169**, 193–209.

COPP, A. (1978). Interaction between inner cell mass and trophectoderm of the mouse blastocyst: I. A study of cellular proliferation. *J. Embryol. exp. Morph.* **48**, 109–125.

EL-SHERSHABY, A. M. & HINCHLIFFE, J. R. (1974). Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscope study of dead cells and their fate. *J. Embryol. exp. Morph.* **31**, 643–654.

FRANKO, A. J. & SUTHERLAND, R. M. (1979). Oxygen diffusion distance and development of necrosis in multicell spheroids. *Radiat. Res.* **79**, 439–453.

GLÜCKSMANN, A. (1951). Cell death in normal vertebrate ontogeny. *Biol. Rev.* **26**, 59–86.

KERR, J. F. R., WYLLIE, A. H. & CURRIE, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239–257.

MARTIN, G. R. (1980). Teratocarcinomas and mammalian embryogenesis. *Science* **209**, 768–775.

MARTIN, G. R. & EVANS, M. J. (1975*a*). The formation of embryoid bodies "in vitro" by homogeneous embryonal carcinoma cell cultures derived from single cells. In *Roche Symposium on Teratomas and Differentiation* (eds M. I. Sherman & D. Solter), pp. 169–187. New York: Academic Press.

MARTIN, G. R. & EVANS, M. J. (1975*b*). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. *Proc. natn. Acad. Sci., U.S.A.* **72**, 1441–1445.

MARTIN, G. R., WILEY, L. M. & DAMJANOV, I. (1977). The development of cystic embryoid bodies *in vitro* from clonal teratocarcinoma stem cells. *Devl Biol.* **61**, 230–244.

POELMANN, R. E. (1975). An ultrastructural study of implanting mouse blastocysts: coated vesicles and epithelium formation. *J. Anat.* **119**, 421–434.

POELMANN, R. E. (1980). Differential mitosis and degeneration patterns in relation to the alterations in the shape of the embryonic ectoderm of early post-implantation mouse embryos. *J. Embryol. exp. Morph.* **55**, 33–51.

POELMANN, R. E. & VERMEIJ-KEERS, C. (1976). Cell degeneration in the mouse embryo: a prerequisite for normal development. In *Progress in Differentiation Research* (ed. N. Müller-Bérat *et al.*), pp. 93–102. Amsterdam: N. Holland Publishing Company.

REINIUS, S. (1965). Morphology of the mouse embryo from the time of its implantation to mesoderm formation. *Z. Zellforsch. mikrosk. Anat.* **68**, 711–723.

SAUNDERS, J. W. (1966). Death in embryonic systems. *Science* **154**, 604–612.

SCHLÜTER, G. (1973). Ultrastructural observations on cell necrosis during formation of the neural tube in mouse embryos. *Z. Anat. EntwGesch.* **141**, 251–264.

SLACK, J. R., MORGAN, R. H. M. & HOOPER, M. L. (1977). Action potentials recorded intracellularly from differentiated mouse teratocarcinoma cells in culture. *Expl Cell Res.* **107**, 457–459.

SOLTER, D. & DAMJANOV, I. (1979). Teratocarcinoma and the expression of oncodevelopmental genes. In *Methods in Cancer Research*. Vol. 18 (eds W. H. Fishman & H. Busch), pp. 277–332. New York: Academic Press.

TANNOCK, I. F. (1968). The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Brit. J. Cancer* **22**, 258–273.

THEILER, K. (1972). The house mouse. Springer-Verlag, Berlin.

THOMLINSON, R. H. & GRAY, L. H. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. *Brit. J. Cancer* **9**, 539–549.

WILEY, L. M., SPINDLE, A. & PEDERSEN, R. (1978). Morphology of isolated mouse inner cell masses developing *in vitro*. *Devl Biol.* **63**, 1–10.

WYLLIE, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature (Lond.)* **284**, 555–556.

WYLLIE, A. H. (1981). Cell death: a new classification separating apoptosis from necrosis. In *Cell Death in Biology and Pathology* (eds I. D. Bowen & R. A. Lockshin), pp. 9–34. London and New York: Chapman and Hall.

WYLLIE, A. H., KERR, J. F. R. & CURRIE, A. R. (1980). Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**, 251–306.

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