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Cell death in the mammalian blastocyst

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Cell death is a widespread feature in the blastocysts of many mammals. Isolated cells in both the inner cell mass and the trophectoderm undergo cell death. These dying cells appear morphologically to be undergoing apoptosis. In mouse blastocysts, a wave of cell death is seen *in vivo*, suggesting that it plays an important role in normal development. However, cell death is increased under suboptimal culture conditions. There is evidence that levels of cell death are regulated by 'survival' factors produced both by the embryo itself and by the maternal reproductive tract. The role of cell death in development is unknown, but could involve the elimination of abnormal cells, or a sublineage of cells with an inappropriate developmental potential. Work in other systems has demonstrated that cell death is regulated by the activity of apoptosis genes. Whether these genes are implicated in blastocyst cell death, and the reasons for apoptosis in the early embryo, remain to be determined.

Key words: apoptosis/growth factors/inner cell mass/preimplantation embryo/trophectoderm

Introduction

Before implantation, eutherian mammals develop in a similar manner. Preimplantation development spans the period during which the fertilized ovum undergoes a series of cleavage divisions before compacting and cavitating, with the formation of a blastocyst. The blastocyst marks the first differentiative event of development, with the establishment of the trophectoderm (TE), which is specialized for implantation, and the inner cell mass (ICM), from which the fetus is derived.

There has been increasing interest in the importance of programmed cell death, or apoptosis, during development. Cell death is a common feature of development (Glucksmann, 1951) and can serve a variety of purposes, including the removal of unwanted cells. For example, programmed cell death is needed for the removal of the tail of the tadpole during amphibian metamorphosis, and the sculpting of digits in the mammalian limb bud.

Cell death is also observed during very early development, before implantation. The finding of cell death *in vivo* implies that it plays an important, but unknown, role even before gastrulation.

Cell death

There are two forms of cell death, necrosis and apoptosis, which can be distinguished morphologically. Necrosis results from injury and affects large groups of cells, causing cellular swelling and membrane rupture (Wyllie *et al.*, 1980) which generally leads to an inflammatory response in healthy adjacent tissue. Apoptosis, on the other hand, characteristically affects single cells in isolation. Initially, chromatin aggregates into large compact granular masses on the inner nuclear membrane, which then becomes grossly indented. The nucleus fragments, with subsequent degradation of the DNA into oligonucleosome-sized fragments. The cytoplasm condenses, forms blebs and fragments into apoptotic bodies which are either dispersed in the intercellular tissue spaces and extruded from the tissue or phagocytosed by neighbouring tissue cells. This classical sequence of events has been seen in a wide variety of different cell types (Wyllie *et al.*, 1980) and is under physiological control.

Observations of cell death in the blastocyst

Morphology

Light microscope and ultrastructural studies of blastocysts from many species, including mouse, cow, baboon, rhesus monkey and human, describe dead and dying cells within the embryo (El-Shershaby and Hinchliffe, 1974; Copp, 1978; Hurst *et al.*, 1978; Enders and Schlafke, 1981; Enders *et al.*, 1982; Lopata *et al.*, 1982; Mohr and Trounson, 1982; Enders *et al.*, 1990, Hardy *et al.*, 1996). These dying cells are characterized by nuclear and cytoplasmic fragmentation, with condensation of chromatin and loss of the nuclear membrane. Nuclear fragmentation and chromatin condensation have been seen both at the ultrastructural level (El-Shershaby and Hinchliffe, 1974; Hurst *et al.*, 1978; Enders *et al.*, 1982) and with the use of polynucleotide-specific fluorochromes which label chromatin (Handyside and Hunter, 1986; Hardy *et al.*, 1989; Pampfer *et al.*, 1990a,b; Hardy and Handyside, 1996; Lea *et al.*, 1996; Brison and Schultz, 1997).

Further degeneration results in the formation of extracellular debris; the presence of mitochondria and nuclear remnants confirms the cellular origin of this debris (Enders *et al.*, 1982). In healthy cells, the intracellular presence of large phagocytic

vacuoles, often containing entire organelles, suggests that cell remnants are cleared by phagocytosis (El-Shershaby and Hinchliffe, 1974; Enders and Schlafke, 1981; Lopata, 1982; Enders *et al.*, 1982, 1990). Furthermore, confocal microscopy of human blastocysts has confirmed that dying cells can be engulfed by their healthy neighbours (Hardy *et al.*, 1996).

The condensed chromatin, fragmenting nuclei and phagocytosis of cell debris seen in blastocysts are characteristic of apoptosis (Wyllie *et al.*, 1980). Fragmenting DNA in apoptotic nuclei can be demonstrated by the formation of a characteristic ladder following electrophoresis, but this technique is not possible in mammalian blastocysts because there are too few cells. However, the recent development of TdT-mediated dUTP nick-end labelling (TUNEL) allows the assessment of nuclear DNA fragmentation *in situ*. This technique can be used on specimens with only a few cells and is based on the fluorescent labelling of the 3′ end of oligonucleosome fragments. Positive labelling of fragmented nuclei in mouse blastocysts has been demonstrated, providing good evidence that cells in the blastocyst are undergoing apoptosis, and that the DNA is being degraded (Brison and Schultz, 1997).

Many dead cells appear to be cleared by phagocytosis within the blastocyst but some cells which seem to be in arrested development persist. These isolated cells have been observed in human and primate blastocysts in the blastocoel cavity or between the trophectoderm and the zona pellucida (Enders and Schlafke, 1981; Lopata *et al.*, 1982; Hardy, 1993). Their large size, lack of intercellular junctions, poorly differentiated mitochondria and paucity of rough endoplasmic reticulum all suggest that these cells originate during early cleavage (Enders *et al.*, 1982; Lopata *et al.*, 1982). It is interesting that these cells persist in the blastocyst and are not cleared by phagocytosis. Failure to observe phagocytosis in precompaction human embryos, coupled with the intercellular presence of dead cells, led Jurisicova *et al.* (1996) to suggest that phagocytosis does not occur before the blastocyst stage. They believed that this was either because blastomeres have no phagocytic capability, or because dead cells at preimplantation stages do not express the cell surface molecules which promote phagocytosis. The persistence of isolated cells in blastocysts, at a time when phagocytosis is possible, supports the latter hypothesis that these cells arose during early cleavage and thus lack cell surface markers which would promote their ingestion by neighbouring cells.

Localization and quantification of cell death

The histological localization and quantification of dead cells requires the laborious and time-consuming analysis of serial sections. While this has provided valuable information on cell death in mouse blastocysts (El-Shershaby and Hinchliffe, 1974; Copp, 1978), only small numbers can be analysed using this method. An alternative technique is differential labelling of the TE and ICM nuclei of blastocysts with polynucleotide-specific fluorochromes (Handyside and Hunter, 1984). This allows rapid identification, localization and quantification of fragmenting nuclei, indicative of cell death. It involves the antibody-mediated complement lysis of TE cells only, allowing nuclear labelling by propidium iodide, followed by fixation of the whole embryo in the presence of bisbenzimide. Since the emission spectra of propidium iodide and bisbenzimide are different, TE and ICM nuclei can be distinguished by the colour of their fluorescence. Dead cells in either lineage can be identified as discrete clusters of fluorochrome-labelled nuclear fragments (Figure 1). Differential labelling has been successfully used in a variety of species including mouse (Handyside and Hunter, 1986; Hardy and Handyside, 1996; Brison and Schultz, 1997) (Figure 1), human (Hardy *et al.*, 1989), rat (Pampfer *et al.*, 1990a,b; Lea *et al.*, 1996), rabbit (Giles and Foote, 1995) and pig (Papaioannou and Ebert, 1988).

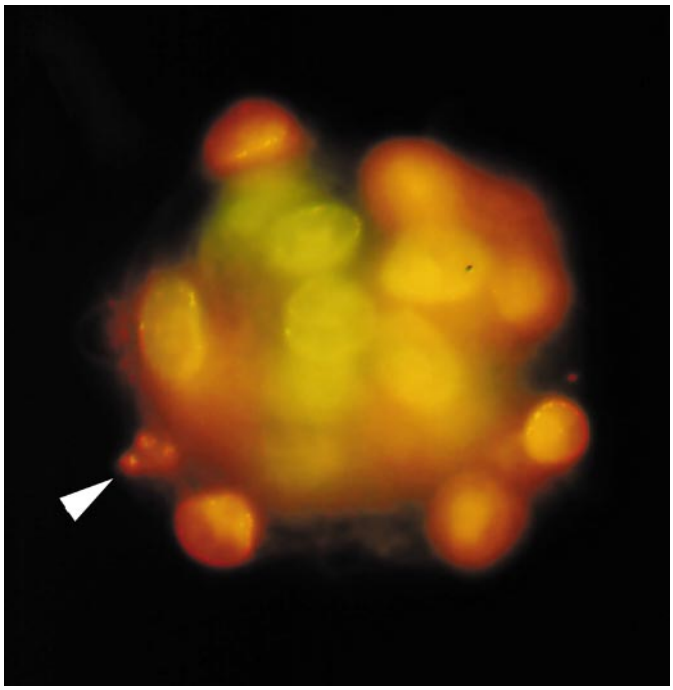

Figure 1. Fluorescence micrograph of a differentially labelled early mouse blastocyst showing a fragmented nucleus in the trophectoderm (arrowed). Trophectoderm nuclei are orange and inner cell mass nuclei are green.

It is difficult to estimate the full extent of cell death in the mammalian blastocyst. It is not known for how long dead cells persist, or how fast dying cells are cleared by phagocytosis. Histological or differential labelling techniques provide only a ‘snapshot’ of the embryo at a specific time. The initial stages of apoptosis appear to be extremely rapid, with cytoplasmic fragmentation often occurring within minutes (Wyllie *et al.*, 1980). It may take 12–18 h for dead cells to be cleared by phagocytosis (Wyllie *et al.*, 1980). Such a rapid disposal of apoptotic cells implies that observation of moderate numbers of dead cells by light microscopy may represent an extensive rate of cell death.

Localization and extent of cell death in mouse blastocysts

Cell death is common in mouse blastocysts. Over 80% of blastocysts freshly flushed from the uterus on day 4 or 5 had one or more dead cells, detected by differential labelling of 139 embryos (F.Devreker and K.Hardy, unpublished observations). Between day 4 and 5, the mean total cell number nearly

doubled from ~50 to over 90. The number of dead cells seen was low, rarely exceeding two in the TE or four in the ICM. However, as <30% of the cells in the blastocyst contribute to the ICM, even a small number of dead cells in this lineage results in the observed dead cell index of 10–20%, compared with a much lower one of <3% in the TE. This confirms other studies where cell death was seen predominantly in the ICM (El-Shershaby and Hinchliffe, 1974; Copp, 1978; Mohr and Trounson, 1982; Handyside and Hunter, 1986; Hardy and Handyside, 1996), with lower levels detected in the TE (Copp, 1978; Hardy and Handyside, 1996; Brison and Schultz, 1997).

Close analysis of blastocyst cell numbers between the stage of cavitation and implantation shows that the increase in the number of TE cells is greater than in the ICM (Handyside and Hunter, 1986). Mitotic figures are common in the ICM and the mitotic index of this lineage is as high, if not higher, than in the corresponding TE. Therefore, the plateau in ICM cell number is not due to cessation of cell division and probably results from the high levels of cell death in this lineage (Handyside and Hunter, 1986). It is of interest that cell death is rarely seen in rabbit blastocysts, and a similar plateau in ICM cell number is not seen (Giles and Foote, 1995).

The degree of cell death varies with the age of the embryo. Analysis of mouse blastocysts *in vivo* flushed at different times following administration of human chorionic gonadotrophin (HCG) showed that a wave of cell death occurred ~97 h post-HCG (Handyside and Hunter, 1986) when they contained 60–110 cells (Copp, 1978). Both these studies also demonstrated minimal levels of cell death at the late blastocyst stage, just before implantation (Copp, 1978; Handyside and Hunter, 1986).

Cell death appears to be correlated with cell number (Figure 2). At any specific time following fertilization there is wide variation in the number of cells in blastocysts, both *in vivo* and *in vitro*. For example, in 155 mouse embryos cultured from the 1-cell stage (F.Devreker and K.Hardy, unpublished data) in KSOM medium (Lawitts and Biggers, 1993), and differentially labelled at 120 h after HCG administration, cell numbers ranged from 28 to 113 (mean 73.1 ± 1.5). In blastocysts with few cells, cell death was variable and ranged from a few to as much as 20% or more. In embryos with a large number of cells, the dead cell index was uniformly low, not exceeding 5% in embryos with over 100 cells (F.Devreker and K.Hardy, unpublished data, Figure 2a). A similar relationship between cell number and cell death is seen in blastocysts freshly flushed from the reproductive tract (Figure 2b).

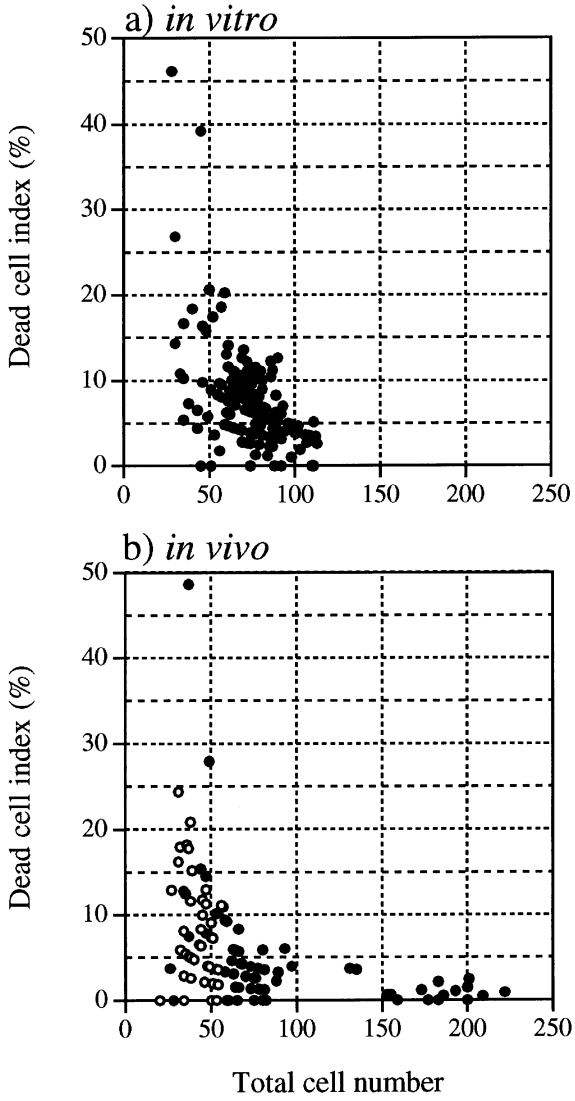

Figure 2. Relationship between dead cell index and total cell number in (**a**) mouse blastocysts at 120 h after administration of human chorionic gonadotrophin (HCG), cultured from the 1-cell stage in KSOM medium and (**b**) mouse blastocysts on day 4 (○) and day 5 (●) *in vivo*.

Localization and extent of cell death in human blastocysts

A large proportion of human blastocysts which have been fertilized and cultured *in vitro* have dead cells. Retrospective analysis of 203 human blastocysts which were differentially labelled during a series of studies over the past eight years shows that ~75% had one or more dead cells on day 6. The mean cell number was 65.6 ± 2.1, with ~40 cells allocated to the TE and 26 to the ICM. Most of these embryos had <10 dead cells, although some embryos had significant levels of cell death of >15%.

Unlike mouse blastocysts, cell death was present equally in the TE and the ICM with a dead cell index of 7–8% in each lineage. We have previously shown that the incidence of cell death appears to be correlated with embryo quality, with the total dead cell index ranging from <10% in day 6 blastocysts of good morphology to 27% in those of poor morphology (Hardy *et al.*, 1989).

As in the mouse, the degree of cell death appears to be correlated with cell number (Figure 3). Blastocysts with low cell numbers had variable rates of cell death, ranging from none to >30%. With greater cell numbers, this variability decreased, and blastocysts with >80 cells rarely had levels of apoptosis of >10% (Figure 3). It is possible that the small population of blastocysts with high levels of cell death and low cell number on day 6 were in the process of arresting,

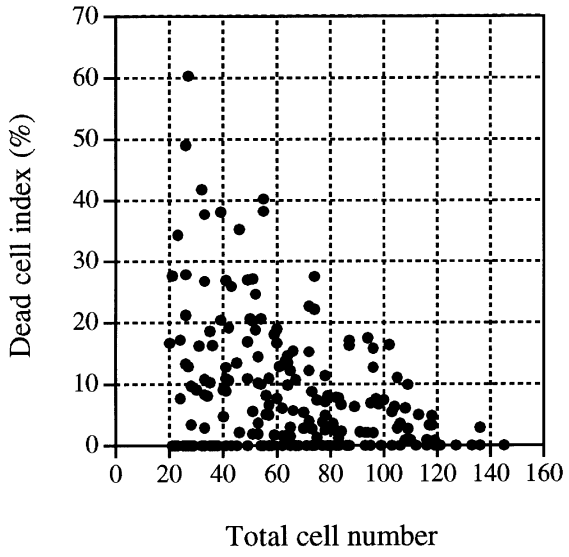

Figure 3. Relationship between dead cell index and total cell number in human blastocysts 6 days after insemination.

and were unlikely to be viable. There may be a critical level of cell death, above which embryo health is compromised.

Cell death in other species

Workers studying the ultrastructure of primate embryos describe cell death as a 'striking feature', present in both the TE and ICM (Enders and Schlafke, 1981; Enders *et al.*, 1990). There appear to be no reports of precise measurements. Rabbit, pig and rat blastocysts have been analysed by differential labelling. Evidence of cell death is rarely observed using this technique in rabbit and pig embryos (Papaioannou and Ebert, 1988; Giles and Foote, 1995). In the rat, as in the mouse, levels of cell death are higher in the ICM (Pampfer *et al.*, 1990a; Lea *et al.*, 1996), although overall levels do not exceed 3% (Pampfer *et al.*, 1990b).

Causes of cell death

Suboptimal culture

Cell death in the mammalian blastocyst is not an in-vitro artefact. It is found in rodent and primate blastocysts which have been freshly removed from the reproductive tract (El-Shershaby and Hinchliffe, 1974; Copp, 1978; Enders and Schlafke, 1981; Handyside and Hunter, 1986; Pampfer *et al.*, 1990a,b; Enders *et al.*, 1990; Brison and Schultz, 1997; Devreker and Hardy, 1997). Furthermore, we have recently observed levels of cell death in mouse blastocysts *in vitro* similar to those seen *in vivo* (Devreker and Hardy, 1997). There have been no studies of human blastocysts derived from embryos fertilized and developed *in vivo*. However, Hertig *et al.* (1954) saw dying cells in several human cleavage stage embryos which were fertilized *in vivo*. These were found fortuitously following hysterectomy, and the presence of dead cells led to their classification as 'abnormal'. The description of the nuclear morphology of these cells, which included multinucleated cells, nuclear fragments, and lobulated nuclei, was indicative of apoptosis. It is possible that, even *in vivo*, cell death is a normal feature of human preimplantation development.

The presence of apoptosis in mammalian blastocysts *in vivo* does not mean that the in-vitro environment has no effect on cell death. Cell death was undetectable in freshly flushed pig blastocysts, but evident in blastocysts which were cultured for a further 24 h (Papaioannou and Ebert, 1986). Brison and Schultz (1997) showed that, in the mouse, levels of cell death *in vitro* were higher than *in vivo*, and that the degree of cell death depended on the density at which embryos were cultured: embryos cultured singly had a higher incidence of cell death than those embryos cultured in groups. The composition of the medium used for culture is also important. Recently we have shown that culturing mouse embryos from the 1-cell stage in KSOM medium lacking glutamine results in dramatically reduced levels of cell death (Devreker and Hardy, 1997) when compared to M16 (Whittingham, 1971), CZB (Chatot *et al.*, 1989) and KSOM with glutamine (Lawitts and Biggers, 1993). Furthermore, culturing rat embryos in increasing concentrations of glucose increases the incidence of cell death dramatically (de Hertogh *et al.*, 1991).

Lack of survival factors

Levels of cell death in embryos cultured *in vitro* can be higher than in embryos *in vivo* (Brison and Schultz, 1997). Moreover, development *in vitro* tends to be slower than *in vivo* (Bowman and McLaren, 1970; Harlow and Quinn, 1984). This suggests that preimplantation development is under regulation by paracrine factors produced by the maternal reproductive tract. There is increasing evidence that embryos themselves also produce factors which act upon development in an autocrine manner. Embryos from several mammalian species have been found to express growth factor receptors; they also secrete a number of growth factor ligands (Watson *et al.*, 1992; Schultz and Heyner, 1993; Zhang *et al.*, 1994; Lighten *et al.*, 1997), many of which have been shown to have beneficial effects on preimplantation development (reviewed by Kaye *et al.*, 1992). In addition, the development of embryos cultured singly is poorer than that of embryos grown in groups (Paria and Dey, 1990; Lane and Gardner, 1992; O'Neill, 1997; Brison and Schultz, 1997). This poor development can be reversed by supplementation of the culture medium with certain growth factors, such as epidermal growth factor (EGF), transforming growth factor (TGF)-α or TGF-β1 (Paria and Dey, 1990; O'Neill, 1997).

These observations clearly demonstrate that preimplantation development is susceptible to the paracrine effects of growth factors, either from the embryo itself, other embryos, or from the cells of the reproductive tract. Recent work strongly suggests that cell death is similarly regulated. Blastocysts from diabetic rats have elevated levels of cell death (Pampfer *et al.*, 1990a), which can be reduced by maternal insulin treatment (de Hertogh *et al.*, 1992). In addition, Brison and Schultz (1997) observed a threefold increase in levels of cell death in mouse blastocysts cultured singly in 25 μl drops of medium. This was reversed by either culturing embryos in groups, or by supplementing the culture medium with TGF-α. These results provide evidence that embryos produce factors which

influence their survival by regulating cell death in a paracrine manner, and that embryos require such factors.

While blastocysts thrive better in the presence of paracrine factors produced by other embryos or by the maternal reproductive tract, they are not essential. In blastocysts cultured singly in the absence of any growth factors, the majority of cells survive (Brison and Schultz, 1997). This suggests that even in the absence of exogenous growth factors, the embryo itself can produce them. This raises the interesting possibility that the decreased levels of cell death seen in mouse blastocysts of higher cell number (Figure 2) result from increased levels of, as yet unidentified, endogenous factors. Attempts to culture individual blastocyst cells in isolation to see if they survive or die could help to clarify the role of growth factors produced by the embryo in the regulation of cell death.

It has recently been proposed that most mammalian cells express the machinery necessary to undergo programmed cell death (PCD), or apoptosis, and that they only survive if they are signalled to do so by other cells (Raff, 1992). Possible signals could take the form of diffusible 'survival factors' produced by other cells. There is evidence supporting this theory, including the observations that several types of cells deprived of serum or extracellular signalling molecules die (Raff, 1992). Furthermore, culture of cells (and blastocysts) in staurospaurine, which inhibits protein kinases and therefore would be expected to inhibit extracellular signalling pathways, induces PCD (Weil *et al.*, 1996). Thus it is possible that the increased levels of cell death seen in mammalian blastocysts *in vitro*, or in blastocysts in diabetic rats, is caused in part by a lack of 'survival' factors. However, this does not explain cell death seen *in vivo*, where it would be supposed that the levels of these factors would be optimal for normal development. This also suggests that cell death may play an active role in the developing blastocyst.

Chromosomal and nuclear anomalies

One possible role for programmed cell death is the elimination of abnormal cells. Embryos from some mammalian species demonstrate a high incidence of morphological and genetic abnormalities. For example, human cleavage stage embryos are characterized by a high incidence of cytoplasmic fragmentation: ~75% show varying degrees of fragmentation (Hardy *et al.*, 1995). Furthermore, labelling of blastomere nuclei with polynucleotide-specific fluorochromes shows that nuclear abnormalities, including binucleate and anucleate cells, and cells with multiple and fragmenting nuclei, are common. Over 60% of preimplantation embryos on day 4 have one or more of these abnormalities (Hardy *et al.*, 1993). It has been proposed that binucleate cells arise during mitosis, resulting from a failure of cytokinesis during telophase, and that these cells subsequently arrest and do not take part in further development. The causes of binucleation are unknown, but a possible fate for the binucleate cells is that they undergo apoptosis (as demonstrated by the presence of fragmenting nuclei). The cell death seen in blastocysts could represent the elimination of these abnormally nucleated cells from the embryo.

Another characteristic feature of human preimplantation embryos is a high incidence (~20%) of gross chromosomal abnormalities (Plachot *et al.*, 1987; Angell *et al.*, 1988; Jamieson *et al.*, 1994). Furthermore, a large proportion (46%) of human embryos are mosaic, with some cells within the same embryo being diploid whilst others are aneuploid, haploid or triploid (Harper *et al.*, 1995). It is possible that these genetically abnormal cells are eliminated by apoptosis.

However, mouse blastocysts also have significant levels of cell death, particularly in the ICM (10–20%), while the incidence of aneuploidy is low and does not exceed 3–4% (Maudlin and Fraser, 1979; Glenister *et al.*, 1987). Both the low levels of aneuploidy and the widespread cell death in one lineage (but not both) suggest that other factors also contribute to apoptosis in the blastocyst.

Elimination of cells with potential to differentiate

One of the functions of apoptosis during development is to eliminate cells that are no longer required. Groups of ICM cells from early blastocysts are able to regenerate trophectoderm cells and form blastocyst like structures, while isolated ICMs from older expanded blastocysts are not able to do so (Handyside, 1978). ICM cells thus lose their potential to form trophectoderm cells during blastocyst expansion. Handyside and Hunter (1986) noted that the wave of cell death seen in mouse blastocysts at 97 h after HCG administration coincided with this period when isolated ICMs lose their ability to regenerate TE cells. They suggested that cell death was a mechanism by which the blastocyst eliminated ICM cells which still have the potential to form trophectoderm. This theory was tested by examining the fate of isolated cells with the potential to form different lineages in blastocoel fluid (Pierce *et al.*, 1989). Individual embryonal carcinoma cells with and without the potential to form TE, were enclosed in an empty zona pellucida (to prevent the cell adhering to host blastocyst cells) and injected into the blastocoel of a giant mouse blastocyst, created by aggregating eight 8-cell embryos. Cells with the potential to form TE frequently died by apoptosis, whereas cells lacking this potential survived (Pierce *et al.*, 1989). This supported the hypothesis that PCD eliminated redundant ICM cells with TE-forming potential and it was proposed that this may reduce the risk of inappropriate ectopic TE expression during germ layer differentiation. Further work suggested that hydrogen peroxide was the active factor in blastocoel fluid which killed the cells (reviewed by Parchment, 1993). The mechanism by which only one lineage is susceptible to hydrogen peroxide damage is not clear. Furthermore, while this hypothesis of cell death eliminating ICM cells with TE-forming potential is attractive, it does not explain the PCD seen in the TE.

Regulation of cell death

In addition to the range of extracellular growth factors which have been shown to modulate cell death (reviewed by Raff, 1992), a number of important genes have been identified which produce intracellular factors involved in its regulation (reviewed by Wyllie, 1995). The genetic analysis of specific cells which undergo apoptosis in the nematode worm *Caenorhabditis elegans* has been instrumental in the identification of

many of these death-regulating genes. The gene *ced-3* is required for PCD in the nematode and encodes a cysteine protease that is homologous to the ICE-family of proteases found in mammals, which are involved in the execution of cell death. The protein encoded by *ced-9*, and its mammalian homologue *bcl-2*, acts as a survival factor and inhibits cell death. Other proteins implicated in the regulation of programmed cell death include the tumour suppressor protein p53, which triggers apoptosis in cells with damaged DNA, and c-Myc, which can either promote or inhibit cell death, depending on the absence or presence respectively of 'survival' factors. In the presence of factors such as insulin-like growth factor (IGF)-1, insulin and EGF, c-Myc promotes cell survival (Evan *et al.*, 1995). This suggests that the increase in cell numbers in mouse blastocysts grown in IGF-1 and insulin *in vitro* (reviewed by Kaye *et al.*, 1992) could be due to these factors reducing levels of cell death rather than by having a mitogenic effect.

The expression of these genes during preimplantation development has not been studied in any mammalian species, and there is clearly a need to examine how apoptosis is regulated in the blastocyst. It is becoming apparent that such regulation is due to a fine balance of endogenous gene products and exogenous signals. However, considerable work still needs to be undertaken to determine the precise mechanisms involved.

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