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Apoptosis in the human embryo

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Preimplantation human embryos are characterized by various degrees of cytoplasmic fragmentation, and a high incidence of developmental arrest before the blastocyst stage. This review examines the current morphological and biochemical evidence that apoptosis plays a role in early human development and embryonic loss. Embryos examined 24 h or more after arrest often show characteristic features of apoptosis including cytoplasmic, nuclear and DNA fragmentation. In contrast, embryos of good morphology that appear to be developing normally show no evidence of apoptosis before compaction. However, at the morula and blastocyst stages, scattered cells with fragmented nuclei and DNA characteristic of cells undergoing apoptosis are common features. Apoptosis may result from suboptimal culture conditions, or may be involved in the elimination of abnormal cells. However, the causes, role and regulation of apoptosis in the human preimplantation embryo remain to be determined.

Human preimplantation embryos are characterized by highly variable morphology and developmental potential that are reflected in low implantation (25%; Dawson *et al.*, 1995) and pregnancy rates (15%; Human Fertilisation and Embryology Authority, 1997) after *in vitro* fertilization (IVF), and significant numbers of arrested embryos (approximately 50%) during the first week *in vitro* (Hardy, 1993). The reasons for this high attrition rate during early development are unclear. Both arrested and developing embryos contain different proportions of cells with the classic features of apoptosis, including cytoplasmic, nuclear and DNA fragmentation (Hardy, 1997). Although the presence of similar cells *in vivo* (Hertig *et al.*, 1954) indicates a role for apoptosis in normal development, it may also play a role in embryonic arrest. However, the causes and roles of apoptosis before implantation remain unknown.

Apoptosis

There are two forms of cell death, necrosis and apoptosis, which are morphologically distinct. Necrosis results from injury and affects large groups of cells, causing cellular swelling and membrane rupture (Wyllie *et al.*, 1980) that invoke an inflammatory response in adjacent healthy tissue. In contrast, apoptosis characteristically affects single cells in isolation, and there is no associated inflammation. Apoptosis involves a series of consecutive morphologically distinct phases. Initially, chromatin aggregates into large compact granular masses on the inner nuclear membrane, the cytoplasm condenses and the nuclear and cytoplasmic membranes become grossly indented. The nucleus undergoes fragmentation, and the whole cell blebs and fragments into membrane bound apoptotic bodies which may contain nuclear fragments. These bodies are either dispersed in the intercellular tissue spaces and extruded from the tissue or are phagocytosed by neighbouring tissue cells. Apoptotic cells that are not phagocytosed, for example those shed from an epithelium into a duct lumen, undergo secondary necrosis, which is characterized by an increase in membrane permeability and distension of cytosolic membrane structures. This classical sequence of events (summarized in Fig. 1) has been observed at the ultrastructural level in a wide variety of different types of cell (Wyllie *et al.*, 1980; Wyllie, 1997; Harmon *et al.*, 1998) and is under physiological control.

In addition to the classic morphological features described here, which distinguish apoptotic cells from those undergoing necrosis, biochemical changes in cell surface morphology and DNA structure have been associated with apoptosis (Fig. 1). Apoptosis is heralded by cell rounding, indicating that changes in cell adhesion occur (Wyllie *et al.*, 1980; Collins *et al.*, 1997). Other cell surface changes that occur early in apoptosis include redistribution of membrane phospholipids within the lipid bilayer. In healthy cells, phosphatidylserine is normally confined to the inner cytoplasmic leaflet. An early feature of apoptosis is the loss of this asymmetry and exposure of phosphatidylserine on the outer leaflet (Martin *et al.*, 1995). Exposed phosphatidylserine may act as the signal on apoptotic cells, promoting their phagocytosis by neighbouring healthy cells. In the nucleus, DNA degradation is frequently observed in which the DNA between nucleosomes is cleaved by endonucleases into oligonucleosomal fragments (Wyllie *et al.*, 1980; Arends and Wyllie, 1991). Agarose gel electrophoresis of such DNA shows a characteristic 'ladder' pattern with bands at 180–200 bp intervals. In contrast, DNA from necrotic cells is cleaved randomly and forms a 'smear' on agarose gels. Structural changes in the nucleus are caused by the activation of caspases (proteases) that cleave a range of proteins including those involved in the cytoskeleton, DNA repair, nuclear envelope integrity and cell cycle control (Wyllie, 1997). It is thought that caspase activation is regulated by proteins of the BCL-2 family.

Apoptosis is an essential feature of many normal processes and pathological conditions, and serves a variety of purposes, including tissue homeostasis and remodelling, and the removal of unwanted cells (Wyllie *et al.*, 1980). It may result from

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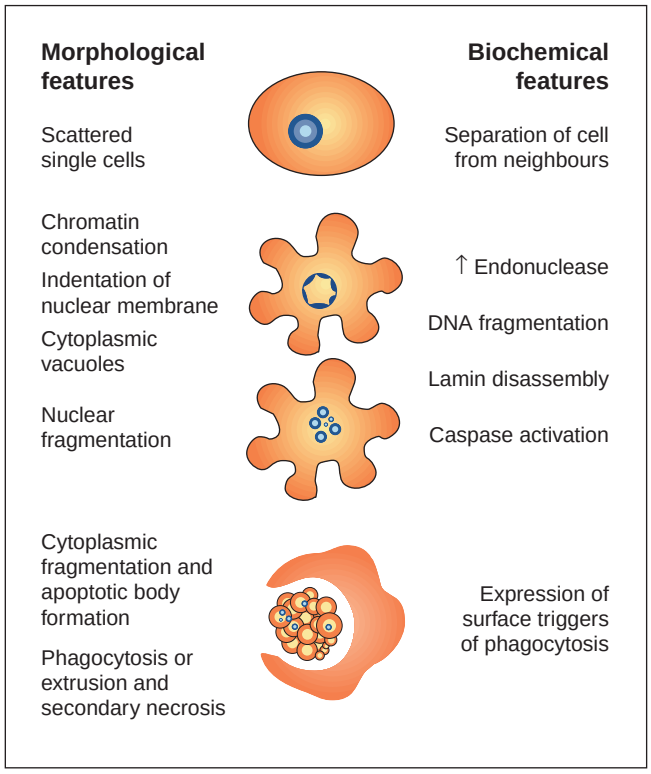


Fig. 1. Diagram outlining the main morphological and biochemical features of apoptosis.

activation of an endogenous programme or be induced by stimuli. Over recent years there has been increasing interest in the importance of apoptosis during development. Examples of developmental events that require extensive apoptosis include the removal of the tail of the tadpole during amphibian metamorphosis, and the sculpting of digits in the developing limb bud. During early rodent development, apoptosis plays a crucial role in amniotic cavity formation soon after implantation (Coucouvanis and Martin, 1995). It appears that cell death may also play a role before implantation, as soon as the totipotent cells start to differentiate (for review, see Hardy, 1997).

Evidence for apoptosis in mammalian blastocysts

Since the mid-1970s, there have been ultrastructural descriptions of dead and dying cells in preimplantation embryos in a number of species, including mouse (El-Shershaby and Hinchliffe, 1974; Copp, 1978), cow (Mohr and Trounson, 1982), baboon (Enders *et al.*, 1990), rhesus monkey (Enders and Schlafke, 1981) and human (Lopata *et al.*, 1982; Mohr and Trounson, 1982; Sathananthan *et al.*, 1982). These dying cells are characterized by blebbing of the nuclear membrane, chromatin condensation, cytoplasmic vacuoles and nuclear and cytoplasmic fragmentation. Nuclear fragmentation has also been seen in approximately 75% of mouse (Handyside and Hunter, 1986; Brison and Schultz, 1997; Hardy, 1997) and human (Hardy *et al.*, 1989; Hardy, 1997) blastocysts using polynucleotide-specific fluorochromes to label chromatin.

These observations, and the increasing interest in the importance of apoptosis in early development, have prompted investigation of cell death in the preimplantation embryo, particularly in humans (Hardy, 1997).

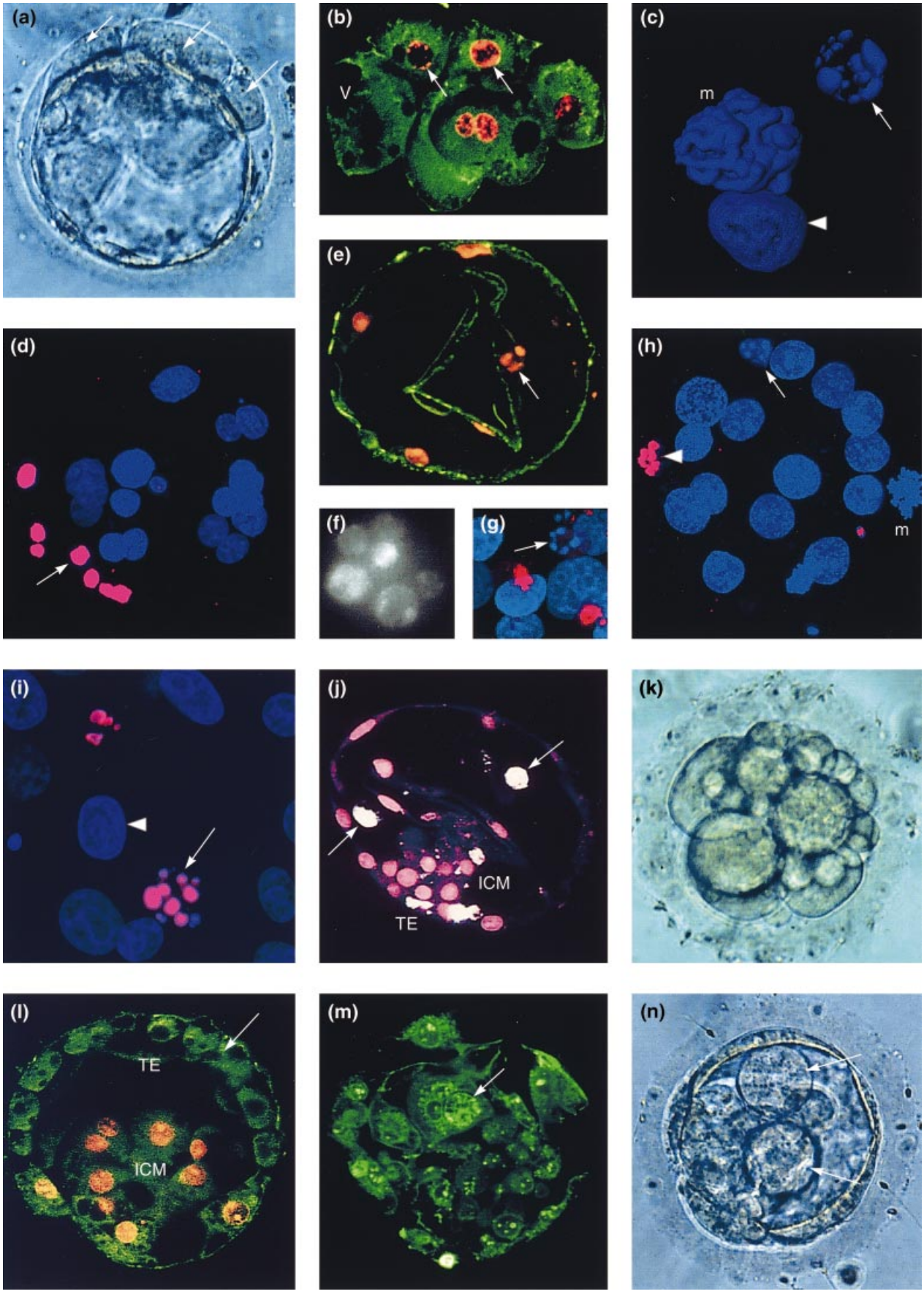
Evidence for apoptosis in the human preimplantation embryo

Membrane changes

The earliest events in apoptosis involve the cell membrane; the apoptotic cell rounds up and separates from neighbouring cells (Wyllie, 1997). At compaction and blastocyst formation, it is not uncommon to see excluded cells between the developing embryo and the zona pellucida (Fig. 2a). Immunocytochemical examination of these embryos shows that excluded cells have poor gap junction communication with the embryo (Hardy *et al.*, 1996).

Another early event of apoptosis, which has also been described in other types of cell, is a redistribution of the phospholipid phosphatidylserine in the cell membrane. In a healthy cell, phosphatidylserine is distributed asymmetrically and is confined to the inner plasma membrane leaflet. The anticoagulant protein annexin V has a specific and high affinity for phosphatidylserine. Healthy cells incubated in fluorescein isothiocyanate (FITC)-conjugated annexin V remain unlabelled, as annexin V cannot access the phosphatidylserine in the inner leaflet of the plasma membrane. However, cells induced to

Fig. 2. Light, fluorescence and confocal micrographs of the morphological and biochemical features of apoptosis in human preimplantation embryos. Nuclei are labelled with propidium iodide (b,e,j,l) or 4′,6-diamidino-2-phenylindole (DAPI) (c,d,g,h,i). (a) Day 6 blastocyst with excluded cells (arrows). (b) Day 4 ten-cell embryo with cytoplasmic vacuoles (v) and condensed chromatin (arrows). (c) Three-dimensional confocal reconstruction of nuclei from a day 6 blastocyst showing nucleus with condensed chromatin (arrow), healthy interphase nucleus (arrowhead) and a nucleus in mitosis (m). (d) Day 4 morula containing 23 nuclei labelled with DAPI (blue), including seven TUNEL-labelled condensed nuclei (pink, arrow) that were excluded from development. (e) Single confocal section of an expanded blastocyst labelled for IGF-I receptor (green) showing a fragmented nucleus (arrow). (f) Fragmented nucleus from a non-fixed day 3 seven-cell embryo labelled with Hoechst 33342. (g) Partial image of a day 6 hatching blastocyst with a fragmented nucleus and no TUNEL labelling (arrow). (h) Day 4 morula with 14 cells showing TUNEL-labelled fragmented nucleus (arrowhead), fragmented nucleus with no TUNEL labelling (arrow) and mitosis (m). (i) Day 6 blastocyst with fragmented TUNEL-labelled nucleus (arrow) and healthy nucleus (arrowhead). (j) Single confocal section through day 6 blastocyst showing TUNEL-labelled nuclei in the inner cell mass (ICM) and polar trophectoderm (TE), and two isolated cells in the blastocoel (arrows). (k) Day 3 embryo showing cytoplasmic fragmentation. (l) Single confocal section of blastocyst showing rim of anucleate sequestered fragments (arrow) lying between the zona pellucida and the TE (nuclei lying out of plane of focus). The ICM is also shown in part. (m) Day 5 blastocyst showing engulfed cell (arrow). (n) Blastocyst showing two large cleavage stage blastomeres in the blastocoel cavity (arrows) that were excluded from development.



undergo apoptosis show a marked increase in annexin V binding, indicating that phosphatidylserine is translocated to the outer leaflet (Martin *et al.*, 1995). This change is an early event in apoptosis and precedes other morphological changes such as nuclear condensation and cell shrinkage. Therefore, annexin V can act as a marker for the early stages of apoptosis before these characteristic morphological changes occur.

Membrane integrity is maintained until the late stages of apoptosis (Wyllie *et al.*, 1980; Martin *et al.*, 1995; Collins *et al.*, 1997). It is important to use a marker of membrane permeability in addition to annexin V labelling to differentiate between cells undergoing apoptosis and those undergoing necrosis. One such marker is the polynucleotide-specific fluorochrome propidium iodide, which can only enter cells with 'leaky' membranes and labels the DNA in the nucleus. Although cells in the early stages of apoptosis label with annexin V but not with propidium iodide (confirming that these cells still have intact membranes), necrotic cells with permeable membranes allow both propidium iodide and annexin V to enter (Martin *et al.*, 1995) and label the nucleus and the phosphatidylserine in the inner leaflet, respectively.

A few studies that have examined annexin V labelling in early human embryos and oocytes (Levy *et al.*, 1998; Van Blerkom and Davis, 1998; Antczak and Van Blerkom, 1999) produced conflicting results. Levy *et al.* (1998) reported extensive annexin V labelling in arrested and fragmented embryos. However, as the nuclei also labelled with propidium iodide, indicating that the plasma membrane was permeable, it cannot be concluded unequivocally that these embryos were undergoing apoptosis. In contrast, Antczak and Van Blerkom (1999) examined 63 embryos with various degrees of fragmentation at the two- to eight-cell stages and found no annexin V or propidium iodide labelling, indicating that fragmentation was not correlated with apoptosis. This result confirmed earlier studies with aged and fragmented human oocytes (Van Blerkom and Davis, 1998).

Cytoplasmic vacuoles

Translucent vacuoles are frequently associated with the cytoplasmic condensation typical of cells undergoing apoptosis (Wyllie *et al.*, 1980). Vacuoles are a common feature of human preimplantation embryos and have been observed by light (Hardy *et al.*, 1996), electron (Sathananthan *et al.*, 1982) and confocal (Fig. 2b) microscopy.

Chromatin condensation

The earliest morphological change indicative of apoptosis is the condensation of chromatin on the inner nuclear membrane. This process has been detected in human embryos by labelling DNA with polynucleotide specific fluorochromes such as propidium iodide, Hoechst or 4',6-diamidino-2-phenylindole (DAPI). Nuclei with clumped chromatin have been observed in blastocysts using standard fluorescence microscopy (Hardy *et al.*, 1989) and in cleavage stage embryos and blastocysts using confocal microscopy (Hardy *et al.*, 1996; Levy *et al.*, 1998) (Fig. 2b,c).

Nuclear fragmentation

The same approach of labelling DNA enabled observation of condensed and misshapen nuclei in fragmented arrested human embryos (Jurisicova *et al.*, 1996; Levy *et al.*, 1998) and in developing embryos (S. Spanos and K. Hardy, unpublished; Fig. 2d). These may be precursors of the fragmented nuclei that are observed in cleavage stage embryos (Hardy *et al.*, 1993; Levy *et al.*, 1998) (Fig. 2f), morulae (Fig. 2h) and blastocysts (Hardy *et al.*, 1989, 1996) (Fig. 2e,g,i). These nuclei appear as discrete clusters of labelled fragments that are smaller than intact healthy nuclei (Fig. 2i). Importantly, pyknotic and fragmented nuclei have been described in human embryos *in vivo* (Hertig *et al.*, 1954).

A large proportion of human blastocysts fertilized and cultured *in vitro* have fragmented nuclei. Retrospective analysis of 203 human blastocysts labelled with DNA-specific fluorochromes during a series of studies (for review, see Hardy, 1997) showed that approximately 75% of blastocysts had one or more dead cells on day 6. Most of these embryos had fewer than ten dead cells, although some embryos had a significant incidence of cell death of > 15%.

Cell death was present equally in the trophectoderm (TE) and the inner cell mass (ICM), and the dead cell index was between 7 and 8% in each lineage. The incidence of cell death appeared to be correlated with embryo quality; the total dead cell index ranged from < 10% in day 6 blastocysts of good morphology to 27% in those of poor morphology (Hardy *et al.*, 1989).

DNA fragmentation

The condensed chromatin and fragmenting nuclei seen in human preimplantation embryos are characteristic of apoptosis. Another feature is the degradation of DNA into oligonucleosomal fragments. Owing to the small number of cells in preimplantation embryos, it is impossible to use electrophoretic techniques to look for DNA laddering that is typical of apoptotic nuclei. However, the development of TdT-mediated dUTP nick-end labelling (TUNEL) (Gavrieli, *et al.*, 1992) enables 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. In addition, TUNEL has the advantages of enabling both localization and quantification of the percentage of nuclei with DNA fragmentation (Gavrieli *et al.*, 1992). Positive labelling of fragmented nuclei in mouse blastocysts has been demonstrated, providing evidence that DNA is being degraded (Brison and Schultz, 1997; Moley *et al.*, 1998).

Arrested embryos. Jurisicova *et al.* (1996) examined a large series of over 200 arrested cleavage stage human embryos using the TUNEL technique. A small proportion (11%) of these embryos showed no morphological evidence of apoptosis, that is, no cytoplasmic, nuclear or DNA fragmentation. The remaining embryos showed various degrees of cytoplasmic fragmentation, and 75% of these also showed evidence of nuclear changes: either condensed nuclei, TUNEL-labelled nuclei (indicating DNA fragmentation), or both (Jurisicova *et al.*, 1996). In a separate study, 30% of fragmented arrested embryos showed TUNEL labelling, while unfragmented arrested embryos did not (Levy *et al.*, 1998). Prolonged culture of arrested embryos for up to 72 h caused an increase in the proportion of TUNEL-labelled and fragmented nuclei (S. Spanos and K. Hardy, unpublished).

Fragmented developing embryos. Normally developing human embryos have also been examined with TUNEL labelling. Antczak and Van Blerkom (1999) found negligible TUNEL labelling in fragmented non-arrested embryos between the two- and eight-cell stages. In contrast, Yang *et al.* (1998) found TUNEL-labelled nuclei in 16/21 fragmented embryos before the eight-cell stage.

Unfragmented embryos. Pre-compaction embryos with intact blastomeres and minor or no fragmentation show no TUNEL labelling (Yang *et al.*, 1998; S. Spanos and K. Hardy, unpublished). In a recent series of 66 normally developing embryos between the four-cell and blastocyst stages, TUNEL labelling was observed only after compaction (Fig 2d,h) and increased after the blastocyst stage (S. Spanos and K. Hardy, unpublished) (Fig. 2j). At the morula stage, 7/12 embryos had one or more TUNEL-labelled nuclei and the overall percentage of labelled nuclei was 7%. Eleven of 17 blastocysts had one or more TUNEL-labelled nuclei and overall 11% of nuclei were labelled.

Approximately 40% of embryos of good morphology (with intact blastomeres and minor fragmentation) arrest development before the blastocyst stage (Fig. 3), mostly at the eight-cell and morula stages (Hardy 1993). Thus, a proportion of embryos fixed for TUNEL labelling would be expected to arrest if allowed to develop further. However, there was no evidence of DNA fragmentation in any embryos before compaction, indicating that apoptosis is a consequence, rather than a cause, of embryonic arrest during early preimplantation development.

A number of workers advocate caution in the interpretation of TUNEL results. It has been proposed that DNA strand breaks may occasionally occur as artefacts of fixation and specimen preparation (Collins *et al.*, 1997). In addition to labelling apoptotic nuclei, positive TUNEL labelling has also been observed in cells undergoing necrotic cell death (Bicknell and Cohen, 1995; Charriaut-Marlangue and Ben Ari, 1995; Grasl-Kraupp *et al.*, 1995), so it is important to confirm apoptosis using other morphological markers such as nuclear morphology. In necrotic cells, the nature of the TUNEL labelling is different: the signal is not confined to the nuclei but is seen as diffuse staining in the cytoplasm as well (Charriaut-Marlangue and Ben Ari, 1995; Jurisicova *et al.*, 1996). Furthermore, morphological features of apoptosis are not always associated with DNA fragmentation (Cohen *et al.*, 1992). Fragmented nuclei without a positive TUNEL signal occur in human blastocysts (S. Spanos and K. Hardy, unpublished; Fig. 2g,h). Conversely, TUNEL-positive nuclei are sometimes observed in the absence of morphological features of apoptosis (Sanders and Wride, 1996), indicating that DNA fragmentation is an early event in apoptosis. When TUNEL labelling is observed in arrested embryos, it is possible that DNA degradation is occurring in cells undergoing the early stages of secondary necrosis, rather than apoptosis.

Cytoplasmic fragmentation

The observation that over 75% of embryos generated during IVF have various degrees of cytoplasmic fragmentation provides the most compelling visual evidence that apoptosis occurs in human preimplantation embryos (Fig. 2k,l). These fragments resemble apoptotic bodies seen in other types of cell. Ultrastructural examination of fragmented embryos showed that these membrane-bound fragments contain organelles (Jurisicova *et al.*, 1996; Yang *et al.*, 1998). Fragmentation has also been observed in human embryos *in vivo* (Hertig *et al.*, 1954; Pereda and Croxatto, 1978; Buster *et al.*, 1985), indicating that fragmentation is not an *in vitro* artefact.

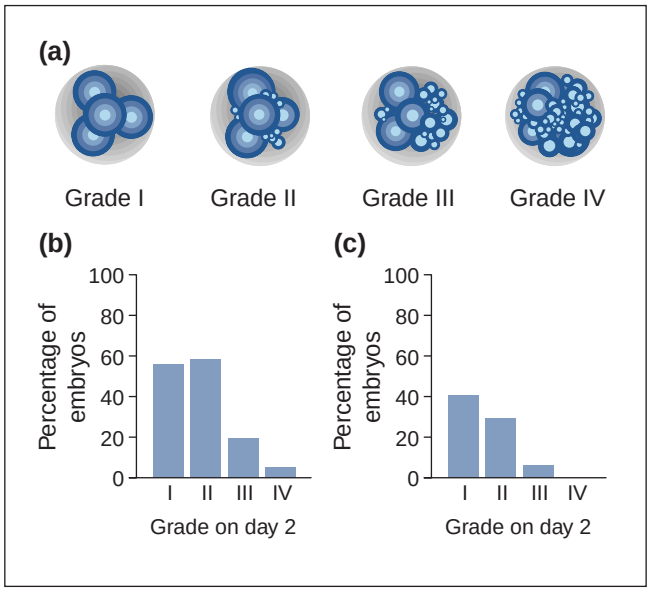


Fig. 3. (a) Diagram outlining morphological grading of human embryos. Grade I: even and symmetrical blastomeres and no fragments; Grade II: all blastomeres intact, but some cytoplasmic fragmentation with or without unevenly sized cells; Grade III: asymmetry and one blastomere completely fragmented; Grade IV: asymmetry and more than one blastomere completely fragmented (Dawson *et al.*, 1995). (b) Percentage of embryos with and without extensive fragmentation reaching the blastocyst stage by day 6 (n = 980). (c) Implantation rate (as defined by raised serum βhCG at 14 days after oocyte retrieval) after transfer of a single embryo (n = 273; data courtesy of K. Dawson, S. Duffy and B. Lavender).

Only a small proportion of human embryos have even and symmetrical blastomeres and no fragments (Grade I). Most embryos have various degrees of asymmetry and fragmentation, ranging from embryos with all the blastomeres intact, but some cytoplasmic fragmentation with or without unevenly sized cells (Grade II), to embryos with one (Grade III) or more (Grade IV) completely fragmented blastomeres (Fig. 3) (Dawson *et al.*, 1995).

The poor potential of embryos with extensive fragmentation to form blastocysts and undergo implantation (Fig. 3) (Giorgetti *et al.*, 1995) indicates that most embryos in which one or more blastomeres are fragmented have an intrinsic defect. Certainly, extensive fragmentation has been associated with a higher incidence of chromosomal abnormalities (Pellestor *et al.*, 1994). Jurisicova *et al.* (1996) proposed that these fragments represented apoptosis. However, failure of the majority of fragmented embryos to show either TUNEL labelling (indicating DNA fragmentation) or annexin V labelling (indicating phosphatidylserine redistribution in the plasma membrane) led

Antczak and Van Blerkom (1999) to postulate that fragmentation was not part of, or a consequence of, apoptosis. The failure to observe complete cell loss by fragmentation in Grade II embryos with minor fragmentation, coupled with the lack of DNA or nuclear fragmentation, indicates that, in these embryos, cytoplasmic fragments are not apoptotic bodies. Indeed, minor fragmentation is not associated with reduced potential to develop to the blastocyst stage (Fig. 3). Furthermore, even the presence of more extensive fragmentation is not always associated with death of the whole embryo or embryonic arrest. Blastomeres in extensively fragmented embryos can continue to divide and, although the viability of these embryos is reduced, even quite fragmented embryos can form blastocysts, implant (Fig. 3) and give rise to babies.

Video time-lapse microscopy is a useful tool for observing the kinetics of apoptosis. This technique revealed violent membrane blebbing in TE and ICM cells over a period of up to 1 h, followed by rupture of the cell and its contents into the blastocoel cavity (S. Spanos and K. Hardy, unpublished). Similar cytoplasmic blebbing has been observed in other types of cell undergoing apoptosis using time-lapse cinematography (Collins *et al.*, 1997; Wyllie, 1997).

Phagocytosis

Further degeneration results in the formation of extracellular debris (Wyllie *et al.*, 1980) and the presence of mitochondria and nuclear remnants confirms its cellular origin, for example, in arrested human embryos (Jurisicova *et al.*, 1996). Confocal microscopy of human blastocysts showed cells being engulfed by neighbouring cells (Hardy *et al.*, 1996) (Fig. 2m). Furthermore, autophagic vacuoles have been observed at the ultrastructural level in human (Mohr and Trounson, 1982) and rhesus monkey blastocysts (Enders and Schlafke, 1981). However, although it appears that many dead cells are cleared by phagocytosis within the blastocyst, other apparently arrested cells persist. Apoptotic cells formed in single-layered epithelia may be extruded into lumina and thus escape phagocytosis (Wyllie *et al.*, 1980; Harmon *et al.*, 1998). This process appears to occur in human blastocysts in which isolated cells have been observed in the blastocoel cavity (Fig. 2n) or between the TE and the zona pellucida (Lopata *et al.*, 1982; Hardy, 1993) (Fig. 2a,l). These cells and fragments are excluded from normal development. Isolated cells in the blastocoel cavity have been TUNEL labelled (S. Spanos and K. Hardy, unpublished; Fig. 2j), as have similar cells in mouse blastocysts (Brison and Schultz, 1997). In some cases, these cells are large (Fig. 2n), and this, coupled with the lack of intercellular junctions, poorly differentiated mitochondria and paucity of rough endoplasmic reticulum, indicates that these cells originated during early cleavage. It is possible that such cells lack cell surface markers that would promote their ingestion by neighbouring cells (Fig. 1), causing them to persist throughout preimplantation development.

Genetic regulation

A number of important genes have been identified that produce intracellular factors involved in the regulation of apoptosis (reviewed by Wyllie, 1995). The genetic analysis of specific cells that undergo apoptosis in the nematode worm *Caenorhabditis elegans* has been instrumental in the identification of many genes that regulate cell death. The gene *ced-3* is required for programmed cell death in nematodes and encodes a cysteine protease that is homologous to the ICE family of proteases (collectively termed caspases) found in mammals, which are involved in the execution of cell death (Wyllie, 1997). The protein encoded by *ced-9*, and its mammalian homologue *bcl-2*, acts as a survival factor and inhibits cell death, possibly by suppressing the activity of the *ced-3* gene product. Thus the caspase family and the Bcl-2 family are two major gene families involved in apoptosis.

Two members of the Bcl-2 family, BAX and BCL-2, have been examined in human embryos (Warner *et al.*, 1998). Bax promotes apoptosis, while Bcl-2 promotes survival; these proteins form homo- and heterodimers and the ratio of the two determines the fate of a cell (Wyllie, 1995). mRNA from 11 human embryos between the two- and 12-cell stages was analysed by RT–PCR, and both BAX and BCL-2 were expressed throughout these stages (Warner *et al.*, 1998). Preliminary data using immunohistochemistry with antibodies to BCL-2 and BAX showed high expression of BCL-2 at cleavage stages and the blastocyst stage, and low expression of BAX at cleavage stages and in blastocysts of good morphology. However, in a blastocyst with extensive fragmentation, BAX expression was increased (S. Rice and K. Hardy, unpublished; Fig. 4). These preliminary observations indicate that both BCL-2 and BAX are expressed in human embryos at preimplantation stages.

Possible causes and roles of apoptosis in preimplantation development

Suboptimal culture

Cell death in the preimplantation embryo is not an *in vitro* artefact. It is found in rodent, primate and human embryos and blastocysts that have been freshly removed from the reproductive tract (Hertig *et al.*, 1954; reviewed by Hardy, 1997). Hardy (1997) reported that over 80% of mouse blastocysts freshly flushed from the uterus on days 4 or 5 had one or more fragmented nuclei, and the mean percentage of fragmented nuclei ranged from 1 to 3% in the TE and from 10 to 20% in the ICM. However, studies in mice have shown that *in vitro* culture increases cell death (Brison and Schultz, 1997), and that the composition of the culture medium can affect the incidence of cell death in the embryo (Devreker and Hardy, 1997; Moley *et al.*, 1998).

Lack of growth or 'survival' factors

Growth factor deprivation appears to be involved in the initiation of apoptosis in many systems (Raff, 1992). In preimplantation embryos, there is increasing evidence that both maternally and embryonically derived growth factors play a key role in development (for review, see Kaye, 1997; Kane *et al.*, 1997). This evidence includes the observation that embryos cultured in small volumes of culture medium or in groups show improved development and that embryos express a variety of growth factor receptors and ligands. Furthermore, supplementation of culture medium with growth factors such

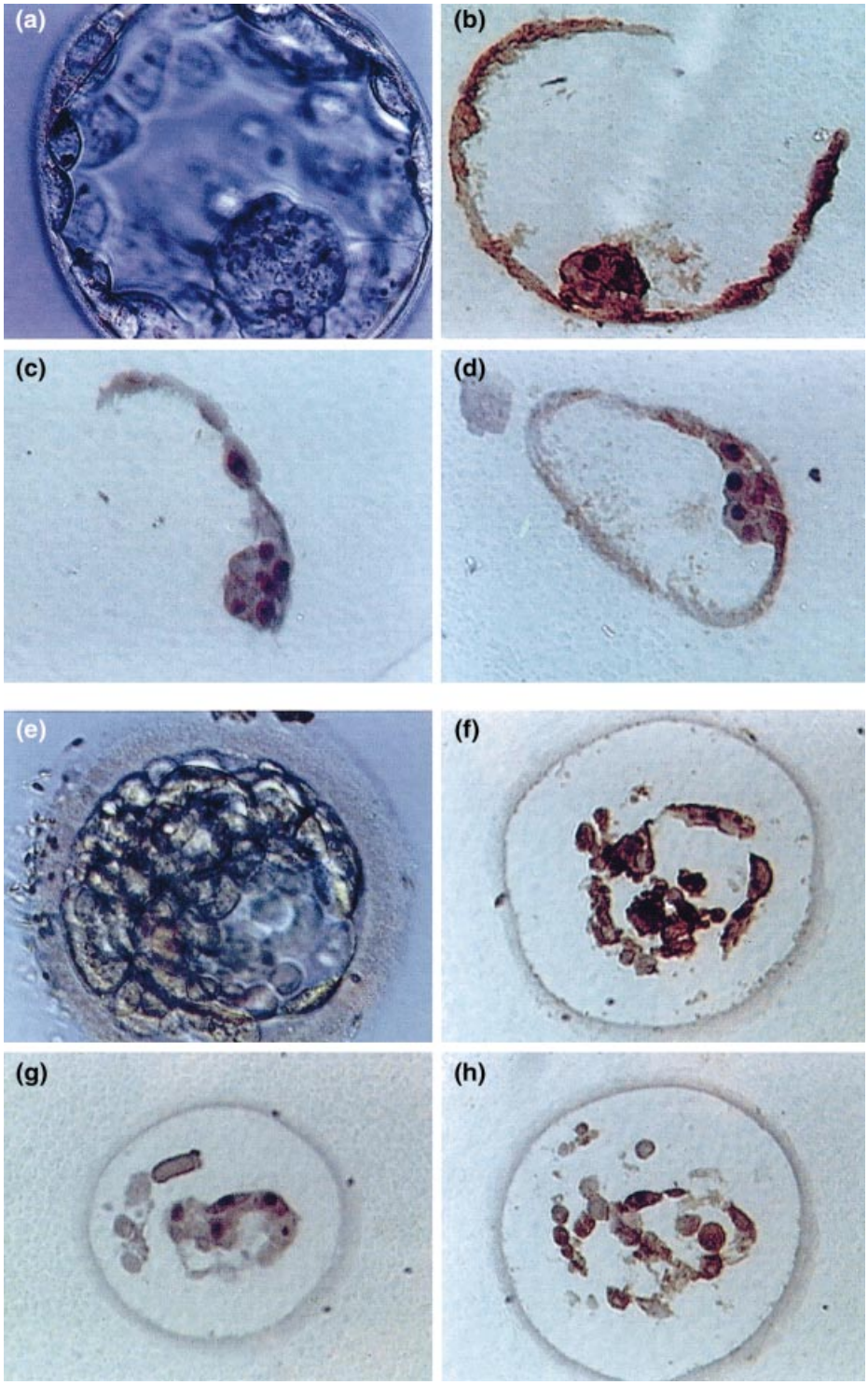


Fig. 4. Immunohistochemical localization of BAX and BCL-2 in a day 6 expanded human blastocyst of excellent morphology (a–d) and a day 5 blastocyst of poor morphology (e–h). Hoffman contrast light micrograph (a) and histological sections (b–d) of expanded day 6 blastocyst of good morphology with no excluded cells or fragmentation. Hoffman contrast light micrograph (e) and histological sections (f–h) of day 5 blastocyst of poor morphology with extensive fragmentation. (c,g) Control, no primary antibody. Immunohistochemical localization of BCL-2 (b,f) and BAX (d,h) (brown staining). Note the decreased staining of apoptosis-promoting BAX (d) versus BCL-2 (b) in the embryo of good morphology, compared with similar staining of BAX (h) and BCL-2 (f) in the fragmenting blastocyst. Courtesy of S. Rice.

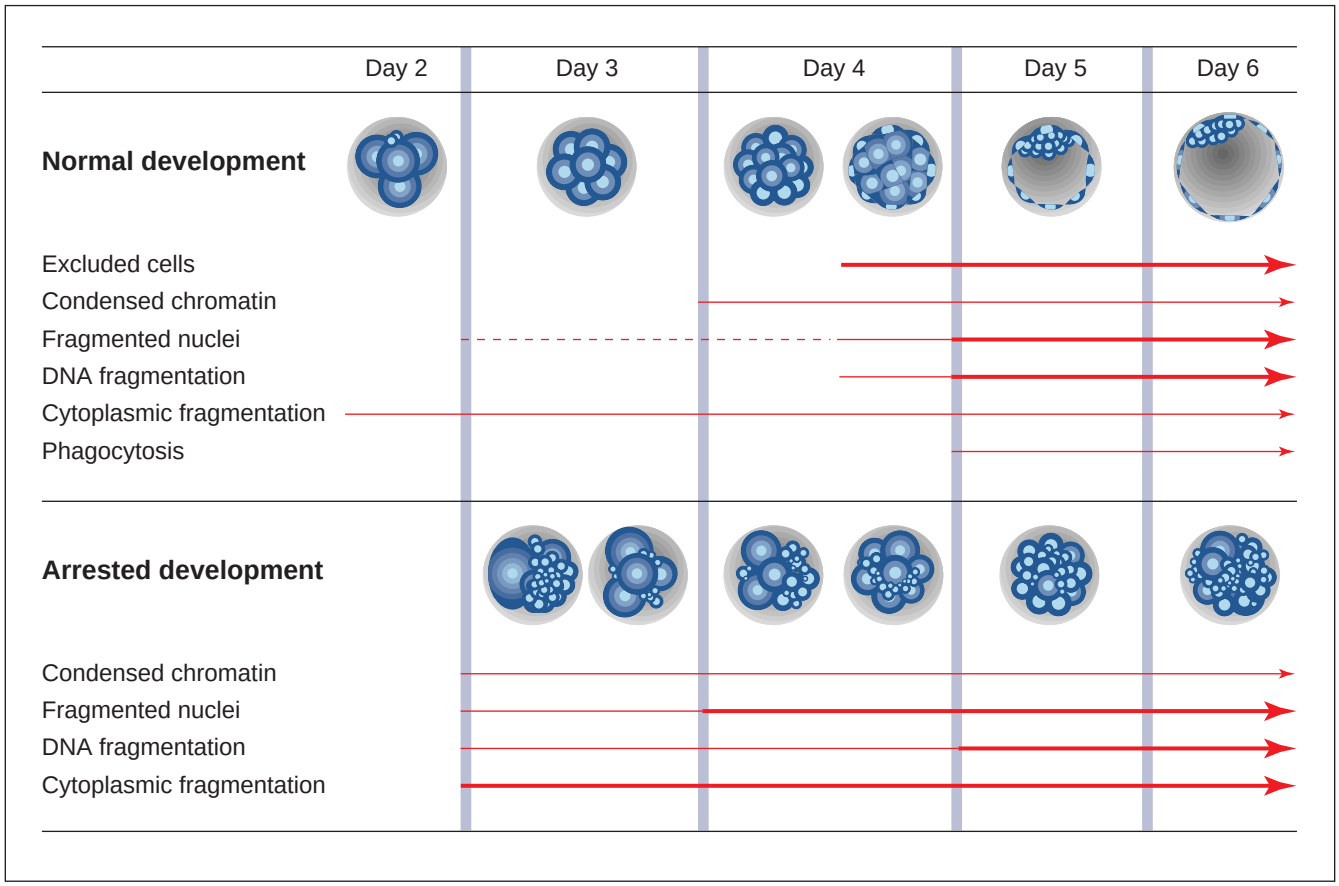


Fig. 5. Schematic representation of the appearance of morphological and biochemical features of apoptosis during normal and arrested human preimplantation development. During normal development, minor fragmentation can occur, but does not appear to be detrimental to further cell division and embryo viability (see Fig. 3). The other features of apoptosis generally do not appear before compaction and blastocyst formation. Embryonic arrest frequently occurs with, or is followed by, various degrees of cytoplasmic fragmentation. Nuclear and DNA fragmentation are also frequently observed after 24 h in embryos that have arrested at any stage.

as transforming growth factor α (TGF-α) (Brison and Schultz, 1997) and insulin-like growth factor I (IGF-I) (Lighten *et al.*, 1998) have a beneficial effect on preimplantation development, increasing the number of cells and the formation of blastocysts. In particular, studies with mouse embryos showed that culture of embryos in groups (Brison and Schultz, 1997; O'Neill, 1998), supplementation of culture medium with TGF-α (Brison and Schultz, 1997) and treatment of diabetic mice with insulin (Moley *et al.*, 1998) all reduce blastocyst cell death. In addition, IGF-I significantly reduces the percentage of apoptotic nuclei in human blastocysts (S. Spanos and K. Hardy, unpublished data). These studies indicate that growth factors play a key role in regulating apoptosis during preimplantation development.

Chromosomal and nuclear abnormalities

One possible role for apoptosis is the elimination of abnormal cells. Human embryos exhibit a high incidence of both nuclear (Hardy *et al.*, 1993) and chromosomal abnormalities (Jamieson *et al.*, 1994). Furthermore, a large proportion of human embryos are mosaic, that is, some cells within the same embryo are diploid whereas others have an abnormal chromosomal complement (Harper *et al.*, 1995). It is possible that apoptosis is the process by which these abnormal cells are eliminated.

Elimination of cells with inappropriate developmental potential

One of the functions of apoptosis during development is to eliminate cells that are no longer required. ICM cells from early blastocysts are able to regenerate TE cells, while those from older blastocysts are unable to do so. Handyside and Hunter (1986) proposed that cell death was a mechanism by which the blastocyst eliminates ICM cells that still have the potential to form trophectoderm. It was proposed that this may reduce the risk of inappropriate ectopic TE expression during germ layer differentiation (Pierce *et al.*, 1989).

Conclusion

This review outlines the increasing evidence that apoptosis occurs in preimplantation human embryos, including morphological evidence of nuclear and cytoplasmic fragmentation and biochemical and molecular evidence of membrane changes and DNA fragmentation (Fig. 5). Cytoplasmic fragmentation is a common feature of human embryos after IVF and culture,

although minor fragmentation does not appear to affect further cell division and viability (Fig. 3). As there is no evidence of cell loss by fragmentation in Grade II embryos, there is little to indicate that these fragments are apoptotic bodies. Although DNA fragmentation can occur after embryonic arrest and in more extensively fragmented cleavage stage embryos, there is little evidence of apoptosis in developing embryos of good morphology before compaction. At least some of these embryos would be expected to arrest if allowed to develop further, so the absence of DNA fragmentation indicates strongly that apoptosis is a consequence, rather than a cause, of cell cycle arrest. The persistence of cytoplasmic fragments that are not cleared by phagocytosis and the increasing amount of DNA fragmentation that occurs with prolonged arrest indicate that the ultimate fate of arrested embryos is secondary necrosis. However, at the morula and blastocyst stages there is increasing morphological, biochemical and molecular evidence that scattered cells die by apoptosis after compaction. Similar observations in blastocysts of other species *in vivo* indicate that apoptosis is playing a role in development at this stage, although the precise nature of this role remains unknown. It is becoming clear that the regulation of apoptosis involves a fine balance of external signals and endogenous gene products, but considerable work is still required to elucidate the causes, roles and control of apoptosis during early development.

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