Programmed Cell Death During Mammalian Preimplantation Embryo Development:
Genetic Regulation and Developmental Consequences

by

Andrea Jurisicova

A thesis submitted in conformity with the requirements for the degree
Doctor of Philosophy
Graduate Department of Zoology
University of Toronto

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Programmed Cell Death During Mammalian Preimplantation Embryo Development: Genetic Regulation and Developmental Consequences

Andrea Jurisicova, Doctor of Philosophy, 1998
Graduate Department of Zoology, University of Toronto, Toronto, Ontario, Canada

Abstract

The quality of embryos produced by In Vitro Fertilization (IVF) is variable. Many embryos contain unequal-sized blastomeres and multiple cellular fragments. The objective of my work was to determine if apoptosis could be observed in human preimplantation embryos and further explore genetic and molecular mechanisms regulating cell death during early embryo development. Electron microscopy as well as chromatin labelling confirmed the typical morphological features of apoptosis in fragmented human embryos. In a mouse model, maternal age beyond 40 weeks increased the rate of embryo fragmentation following IVF, but oocytes from females of all ages had a uniformly high rate of fragmentation when fertilized in vitro. Furthermore, in mice IVF significantly reduced the rate of blastocyst formation and decreased the mean cell number at the blastocyst stage in comparison with embryos produced in vivo. The temporal expression patterns of several cell death regulatory genes was assessed during murine and human embryo development, and showed that the expression of these genes is altered in mouse embryos undergoing fragmentation. This data are consistent with hypothesis that programmed cell death may occur by default in embryos which fail to execute essential developmental events during the first cell cycle and this process is actively regulated via expression of cell death regulatory genes.
Acknowledgement.

Foremost, I would like to thank my mentors, Professors Robert F. Casper and Susanah Varmuza, for their encouragement and guidance, but also for giving me “free hands” to let me explore all of the crazy ideas I ever had. However, their input, suggestions and support were worth gold.

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Last but not least, I would like to thank my husband Igor not only for support, understanding and accepting the fact that research requires long hours spent in laboratory, but also for his help and suggestions with photography work.
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% percent
°C degrees Celcius
AA amino acids
ANOVA analysis of Variance
Apaf-1 apoptosis factor 1
B6 C57BL/6J
Bad Bcl-xL/Bcl-2-associated death promotor homolog
Bag-1 Bcl-2 associated athanogene 1
Bak Bcl-2 homologous antagonist/killer
Bax Bcl-2 associated X protein
Bcl-2 B-cell leukemia/lymphoma 2
Bcl-x Bcl-2 like X protein
Bcl-w Bcl-2 like W protein
Bfl-1 Bcl-2 related gene from fetal liver
BH Bcl homology domain
Bid BH3 interacting domain death agonist
Bik Bcl-2 interactive killer
Bok Bcl-2-related ovarian killer
β-ME beta-mercaptoethanol
bp base pairs
BSA bovine serum albumin
Ced cell death defective
Caspase ced-3 like aspartate-specific cysteine protease
cDNA complementary DNA
CNS central nervous system
cpm counts per minute
CPP-32 cysteine protease-32
DCP-1 drosophila cysteine protease-1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EF-1</td>
<td>elongation factor 1 alpha</td>
</tr>
<tr>
<td>EGA</td>
<td>embryonic genome activation</td>
</tr>
<tr>
<td>ES cells</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>GVOOC</td>
<td>germinal vesicle oocyte</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>hid</td>
<td>head involution defective gene</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>Hrk</td>
<td>harakiri gene</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HTF</td>
<td>human tubal fluid medium</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin converting enzyme</td>
</tr>
<tr>
<td>ICSI</td>
<td>intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IL-1♦</td>
<td>interleukin 1-beta</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>IVF</td>
<td>in vitro fertilization</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>KSOM</td>
<td>synthetic oviductal medium enriched in potassium</td>
</tr>
<tr>
<td>mg</td>
<td>miligram</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
</tr>
<tr>
<td>mRNA</td>
<td>messanger ribinucleic acid</td>
</tr>
<tr>
<td>N</td>
<td>ploidy</td>
</tr>
<tr>
<td>PCD</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>Pp1cy</td>
<td>protein phosphatase 1 gamma gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNF-R</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>Ttg</td>
<td>tissue transglutaminase</td>
</tr>
</tbody>
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date: Jan 9th 1998
Chapter 1. Introduction.

For many couples, the failure to naturally achieve and maintain pregnancy is an ongoing problem. A solution for many of these couples is In Vitro Fertilization (IVF). When the first IVF baby was born, no one expected that this unconventional method of conception would become such a widely used treatment for infertility. In 1991, over 21,000 cycles of IVF were performed in the USA and Canada. Although 87.1% of these cycles led to a successful fertilization and embryo transfer, only 4,017 clinical pregnancies (19.1%) were achieved (Society for Assisted Reproductive Technology, 1993).

To increase the chances of favorable IVF outcome, superovulation of patients is used in order to produce more oocytes. A higher number of retrieved oocytes increases the probability of obtaining several good quality embryos. The best embryos, upon morphological evaluation, are subsequently transferred to the uterus. With improved protocols for ovarian stimulation, more defined culture conditions and transfer of embryos at day 3 rather then day 2, the implantation rate per embryo transferred after IVF slightly increased during the past 4 years - on average from 19% to approximately 23% (Dawson et al., 1995). Nonetheless, a significant proportion of transferred embryos (70% or more) fail to implant, and at most, 10-15% will give rise to a full-term infant (Winston and Handyside, 1993). Based on limited information available from the fertile human population, abnormal preimplantation embryo development was also observed in vivo with only a low proportion of fertilized oocytes reaching the blastocyst stage (Hertig et al., 1952, Buster et al., 1985). When embryos where flushed from uteri of donors at the blastocyst stage and immediately transferred into recipient patients, higher pregnancy rates (up to 50%) were reported (Formigli et al., 1990), suggesting that the low pregnancy rates observed during IVF could be caused in part by in vitro culture of human embryos. However, even in the general
population, approximately 25 to 40% of conceptions may be lost before they are clinically diagnosed as a pregnancy (Winston and Handyside, 1993.) Taken together, these observations suggest a high rate of inherent and/or acquired developmental abnormalities, occurring quite frequently, during the preimplantation period of embryo development in the human.

We hypothesize that one of the major abnormalities observed during preimplantation development, embryo fragmentation, is a consequence of activated programmed cell death (PCD) which leads to apoptosis. We will investigate the temporal expression patterns of several cell death regulatory genes during human and murine embryo development, and analyze whether or not the expression of these genes is altered in embryos undergoing fragmentation. Furthermore we will analyze and discuss whether or not certain factors (such as maternal age, conditions of fertilization, genetic background) might be involved in activation of PCD.

1.1. Mammalian preimplantation embryo.

1.1.1. Preimplantation embryo development.

In humans, the first visible sign of fertilization, the appearance of 2 pronuclei in the cytoplasm of the oocyte, can be observed 18-22 hours after insemination \textit{in vitro} (Balakier et al., 1993). From that point, the embryo starts to cleave approximately twice every 24 hours. At the 8-16 cell stage, mammalian embryos undergo a process called compaction, whereby the blastomeres flatten, maximizing cell-cell contact and minimizing intracellular spaces, to form a morula (Leese et al., 1993). At this developmental stage, cells acquire adhesion properties and undergo cellular polarization, and establish tight and gap junctions that are used for subsequent interblastomeric communications (Watson et al., 1992). The diameter of the embryo (approximately 120 µm) is still unchanged from that of the zygote because growth is restricted by
the zona pellucida. The first obvious embryonic differentiation event, the formation of the blastocyst, is observed 5 days after insemination in vitro, and full blastocoele expansion can be seen on day 6 to day 7 (Hardy et al., 1989, Dokras et al., 1991). Blastocoele formation is caused by increased transport of water and several ions such as Na\(^+\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\) and Cl\(^-\), through the tightly sealed outer blastomeres into the intercellular space (Watson, 1992). With more pressure inside the embryo, the zona pellucida decreases in thickness, allowing the embryo to expand in size. At this stage, the human embryo consists of approximately 100 cells (Hardy et al., 1989) and contains three different cell lineages: 1) polarized water transporting epithelial cells or trophectoderm which will give rise to placenta; 2) primitive endoderm; and 3) the inner cell mass (ICM) which will form the embryo proper. The ICM cells constitute about one third of the total cell number in the fully expanded human blastocyst (Hardy et al., 1989). The next stage of development for the blastocyst is to hatch out of the zona pellucida, allowing further expansion and growth of the embryo. This process is thought to be mediated by trypsin-like enzymes secreted by blastocyst-stage embryos (Yamazaki et al., 1994). Once the blastocyst has hatched, the number of cells in the primate embryo dramatically increases to a few hundred, and the blastocyst continues to expand to a diameter of 400 μm (Seshagiri and Hearn, 1993). The fully expanded, hatched blastocyst starts to interact with the uterine endometrium and initiates implantation. The implantation process in the human is thought to start in vivo on day 7 after ovulation and to be complete by about day 14 (Leese et al., 1993).

Progression of fertilized mammalian oocytes through cleavage, blastocyst formation and implantation is dependent on the successful implementation of the genetic and developmental program contained within the oocyte. In the human, as in other mammals, zygote development and the first two cleavage divisions depend upon, and are probably controlled by, maternal RNA
transcribed and accumulated during oogenesis. Major activation of the embryonic genome (EGA) is species specific and can be first observed between the 4- to 8-cell stage in human, swine, equine and cat embryos (Braude et al., 1988, Tesarik et al., 1988, Jarell et al., 1991, Brinsko et al., 1995, Hoffert et al., 1997), between 8-16 cell stage in cows and sheep (Telford et al., 1990) and at the 2-cell stage in the mice (Flach et al., 1982). The onset of EGA coincides with an increased rate of degradation of maternally-stored products (Piko and Clegg, 1982, Payton et al., 1988) and is accompanied by major changes in the profile of proteins synthesized, some of which are transcription dependent (Latham et al., 1991). Even though it is accepted that this trend is followed by the majority of genes expressed during oocyte and early embryo development, individual genes have their own "timing" of maternal product degradation or zygotic activation (Rambhatla et al., 1995, Mann et al., 1995, Kaneko et al., 1997). In the mouse, active transcription can first be detected during syngamy of the pronuclei, which corresponds to the S-phase of the first cell cycle (Matsumoto et al., 1994, Bouniol et al., 1995). Transcriptional activity during the late 1-cell stage reaches approximately 20% of transcription at the G2-phase of the cell cycle in the 2-cell stage embryo (Aoki et al., 1997). It is likely that different regulatory mechanisms are responsible for the initiation of transcription at the 1- and 2-cell stage, since 1-cell embryos are able to activate enhancer-responsive promoters even without enhancers, but 2-cell stage embryos require enhancers to prevent repression of weak promoters in order to maintain the same level of transcription (Majumder et al., 1993). Major EGA, occurring at the 2-cell stage, also seems to require active protein synthesis, and it is likely to be controlled by the recruitment of maternal transcripts encoding "genome-activating" transcription factors (Wang and Latham, 1997).

Another unique and evolutionarily conserved mechanism affecting the translational
availability of different transcripts in oocytes is poly- and de-adenylation of maternally stored products (Richter et al., 1990) coupled with packaging into ribonucleoprotein particles (informosomes) via binding to RNA-specific proteins (Bouvet and Wolfe, 1994). This sequestration has been proposed to maintain maternal mRNA transcripts in the inert state and thus facilitate translational repression or mRNA masking (Standart 1992). During oocyte maturation as well as during early embryonic stages, unmasking of maternal transcripts occurs partially due to stripping of RNA binding proteins (Meric et al., 1997) and increased cytoplasmic polyadenylation (Paynton and Bachvarova, 1994). Existence of specific regulatory AU-rich sequences, referred to as cytoplasmic polyadenylation elements, has been described (Fox et al., 1989) and these are usually located in the 3' untranslated region near the nuclear canonical poly-A sequence (McGrew et al., 1989, Verrotti and Strickland 1997). Both increased polyadenylation and mRNA unmasking can result in accumulation of translationally available poly-A mRNA, and thus potentiate expression of the protein product of a given gene. However, the exact signals involved in the regulation of translational availability for specific genes are currently unknown. Furthermore, gene expression analysis during mammalian oocyte and preimplantation embryo development is made more complex by differences in genetic background. It was previously shown that phenomena such as developmental potential in vivo (Renard and Babinet, 1986) or in vitro (Latham and Solter 1991, Suzuki et al., 1996), as well as endowment of oocytes with maternally deposited products (Rambhatla and Latham, 1995), are dependent on genetic background.

Very little is known about the regulatory mechanisms governing the pattern of early gene expression. Development of highly sensitive techniques for single cell RNA detection allowed identification of various groups of genes expressed during preimplantation embryo development.
These include housekeeping genes such as actin, elongation factor 1α, protein phosphatase 1γ and many others (Mann et al., 1995, Rambhatla et al., 1995, Wang and Latham, 1997); growth factors and their receptors (Rapolee et al., 1988, Chia et al., 1995), cytokines (Rothstein et al., 1992, Sharkey et al., 1995), surface recognition antigens (Warner et al., 1987, Juricicova et al., 1996a), hormones and their receptors (Bonduelle et al., 1988), transcription factors and proto-oncogenes (Kaneko et al., 1997, Pal et al., 1993, Hwang et al., 1997). It has also been shown that culture conditions can alter patterns of gene expression (Ho et al., 1994, 1995), introducing additional variables into the analysis of gene expression and affecting developmental potential of embryos cultured in vitro.

Using the powerful approach of targeted gene disruption, several genes important for preimplantation development have been identified. Embryos with mutations of the adhesion molecules α-E-catenin and its ligand E-cadherin are unable to form blastocysts due to the failure of blastocoele formation (Torres et al., 1997, Larue et al., 1994). Also, disruption of the prolactin receptor causes several reproductive abnormalities, one of which is defective preimplantation development (Ormandy et al., 1997). Interestingly, a gene responsible for DNA repair, Rad51, seems to play an important role in the 2-cell block in mice since in some, but not all, embryos with this disrupted gene, embryonic arrest was observed at this developmental stage (Tsuzuki et al., 1996). These and other mutations are listed in Table 1.

1.1.2. Abnormalities observed during human embryo development.

Early human embryos cultured in vitro display a remarkably high rate of spontaneous cleavage arrest (Bolton and Braude, 1987). Using various culture media in conjunction with very stringent quality-control procedures, it has been shown that only 30% to 40% of spare human
embryos cleave regularly to the 6-day-old blastocyst stage \textit{in vitro} (Hardy \textit{et al.}, 1989, Dokras \textit{et al.}, 1991, Bongso \textit{et al.}, 1994, Ben-Chetrit \textit{et al.}, 1996) despite the fact that 60\% to 70\% of fertilized zygotes develop to the four to six-cell stage (day 2) in most IVF centers.

Also, increased pregnancy rates were observed with transfer of embryos cultured \textit{in vitro} to the blastocyst stage in a subset of patients with previous repetitive implantation failure (Menezo \textit{et al.}, 1992, Olivennes \textit{et al.}, 1994). The quality of obtained blastocysts is also variable, since a significant proportion (more than 50\%) fail to express or secrete several trophectodermal markers such as $\beta$-hCG (Woodward \textit{et al.}, 1994), SP-1 (Dimitriadou \textit{et al.}, 1992) and HLA-G (Jurisicova \textit{et al.}, 1996a), suggesting that these blastocysts would not be able to initiate successful implantation and that they contribute to high rates of failed embryo transfers (Jurisicova \textit{et al.}, 1996b).

Table 1. Mutations affecting murine preimplantation embryo development (based on Copp 1995, and Rinkenberger \textit{et al.}, 1997).

<table>
<thead>
<tr>
<th>Gene/mutation</th>
<th>Protein function</th>
<th>Stage affected</th>
<th>Origin of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-mos</td>
<td>proto-oncogene</td>
<td>oocyte</td>
<td>Knock-out</td>
</tr>
<tr>
<td>$\alpha$-E-catenin</td>
<td>adhesion molecule</td>
<td>Morula/Blastocyst</td>
<td>Knock-out</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>adhesion molecule</td>
<td>Morula/Blastocyst</td>
<td>Knock-out</td>
</tr>
<tr>
<td>Prolactin receptor</td>
<td>hormone</td>
<td>Unknown</td>
<td>Knock-out</td>
</tr>
<tr>
<td>Rad 51*</td>
<td>DNA repair</td>
<td>2-cell - Blastocyst</td>
<td>Knock-out</td>
</tr>
<tr>
<td>DDK/Unknown*</td>
<td>Genomic repair</td>
<td>Morula-Blastocyst</td>
<td>Natural</td>
</tr>
<tr>
<td>$c^{35}$H/Pid locus</td>
<td>unknown</td>
<td>8 cell</td>
<td>Radiation-induced</td>
</tr>
<tr>
<td>t$^{12}$</td>
<td>unknown</td>
<td>Morula</td>
<td>Natural deletion</td>
</tr>
<tr>
<td>$A^{2}$/(Merc)*</td>
<td>RNA binding protein</td>
<td>8-cell - Blastocyst</td>
<td>Natural deletion</td>
</tr>
<tr>
<td>Mdn</td>
<td>unknown</td>
<td>Morula</td>
<td>Transgenic insertion</td>
</tr>
<tr>
<td>Om</td>
<td>unknown</td>
<td>Blastocyst</td>
<td>Transgenic insertion</td>
</tr>
</tbody>
</table>

* Phenotype is variable and is affected by genetic background

The highest incidence of developmental arrest in human embryos cultured \textit{in vitro} occurs between the four to eight cell stages, but it can also be seen after compaction of the morulae. In
several mammalian species, the stage of development when transcription-dependent proteins are first detected coincides with the peak incidence of cleavage arrest in vitro (Telford et al., 1990). The mechanisms responsible for cleavage arrest are unclear. Interestingly, maternal cytoplasmic components are likely to be involved in regulation of embryonic arrest, because the so called "2-cell block" in mice can be overcome by transplantation of ooplasm from zygotes of non-arresting strains into the zygotes of arresting strains (Muggleton-Harris et al., 1982). Moreover, optimizing culture medium by adding amino acids and antioxidants (such as EDTA) or by decreasing oxygen content and changing a combination of ions was shown to overcome embryonic arrest in several strains of mice (Mehta and Kissling et al., 1990, Gardner and Lane, 1996, Summers et al., 1994).

Three main causes of developmental arrest have been proposed:

1. Inadequate culture conditions (Menezo et al., 1990, Schultz and Heyner 1992)
2. Inherent or induced chromosomal abnormality (Macas et al., 1990, Pickering et al., 1990)
3. Failure of embryonic gene expression (Bolton and Braude, 1987)

With respect to the third possibility, Artley et al., (1992) examined the pattern of proteins from regularly cleaving and developing embryos and compared them with a protein profile of arrested embryos. Analysis of protein patterns of the cleavage-arrested embryos demonstrated that at least half of the embryos were capable of synthesizing some, but not always all, of the transcriptionally-dependent proteins which are present in the profile of regularly cleaving embryos. From these observations, the authors proposed that cleavage arrest is not always accompanied by a complete failure of EGA (Artley et al., 1992).

Human preimplantation embryos are affected by various kinds of cellular and
morphological abnormalities. The most frequently observed ones are alterations in nuclear and cytoplasmic ratios occurring at days 2 and 3 post-insemination. These alterations result in embryos with a high nuclear/cytoplasmic ratio - multinucleated blastomeres and blastomeres with fractionated nuclei, and embryos with low nuclear/cytoplasmic ratios - presence of anucleated blastomeres or fragments (Winston et al., 1991, Hardy et al., 1993). Less than 50% of embryos cleave regularly into equal-sized blastomeres without fragmentation. The remaining embryos often contain variably sized blastomeres with multiple cellular fragments. Subsequent in vitro and in vivo development of these fragmented embryos is impaired, often leading to cleavage arrest, embryo degeneration and lack of implantation (Bolton et al., 1989, Ziebe et al., 1997). In addition, the survival rate of fragmented embryos after cryopreservation is decreased, making these embryos unsuitable for storage for future transfers. Also, a wide range of chromosomal abnormalities, including premature chromosome condensation and a high incidence of aneuploidy, is frequently observed in human preimplantation embryos (Zenzes and Casper 1992). Embryos of poor quality with various degrees of fragmentation have a higher incidence of cytogenetic abnormalities than embryos of good morphology (Micheli et al., 1990, Pellestor et al., 1994). Monospermic fertilized, but cleavage-arrested, human embryos with extensive cellular fragmentation show a high incidence of mosaic polyploidy ranging from 3N up to 20N as assessed by FISH analysis (Munne et al., 1994). All of these morphological and genetic abnormalities interfere with the developmental potential of embryos and thus reflect on IVF outcome e.g., successful pregnancy (Erenus et al., 1991, Plachot and Mandelbaum 1980, Zenzes et al., 1992, Balakier and Cadesky 1997).

Our knowledge of the etiology and underlying mechanism of embryo fragmentation is limited. In mammals, embryo fragmentation can be observed in several species (Summers et al.,
1994) and seems to be a naturally-occurring phenomenon observed in embryos conceived in vivo as well as in vitro, from both stimulated and unstimulated cycles (Formigli et al., 1990). The morphological appearance of fragments in early embryos as well as previous reports describing nuclear abnormalities (Hardy et al., 1993) led us to hypothesize that fragmentation in these embryos is a consequence of programmed cell death (PCD).

1.2. Cell death

1.2.1. Programmed cell death

Programmed cell death is an intriguing biological phenomenon that appears to regulate homeostasis and morphogenesis of many multicellular organisms. The most common series of morphological changes which result in cell death is called apoptosis (Kerr et al., 1972). Upon induction by an appropriate trigger, the cell activates, or stops repression of, gene products responsible for the suicidal machinery, and cell death ensues.

During development, PCD results in a depletion of cells in a precise temporal and spatial manner (Schwartz and Osborne 1993) and appears to be an integral component of normal development in many phylogenetically diverse organisms. The following functions for cell death during embryonic development have been proposed (Jacobson et al., 1997).

1/ Sculpting structures – the best example of this is interdigital elimination of cells during limb formation (Jacobson et al., 1996) and formation of hollow structures such as the pre-amniotic cavity in gastrulating mouse embryos by elimination of core ectodermal cells (Coucouvanis and Martin, 1995).

2/ Deleting unneeded structures – this group is best represented by elimination of juvenile organs (gills and tail of tadpole, larval gut and head structures) not needed for further
development, allowing metamorphosis to occur in amphibians and insects (Abrams et al., 1993). In mammals, regression of primordial sex ducts not needed in the particular sex such as the Mullerian ducts in males or the Wolffian ducts in females are good examples (Haqq et al., 1994).

3/ Eliminating abnormal, misplaced, non-functional or harmful cells – quality control has to be ensured during development as well as throughout life and, therefore, cell death eliminates many harmful cells such as self-reactive T and B lymphocytes (Lynch, 1995) and cells with damaged DNA (Kastan et al., 1991). Similarly, when primordial germ cells end up in the wrong environment due to misguided migration, they undergo cell death (Buehr 1997)

4/ Controlling cell numbers – overproduction of both neurons and oligodendrocytes results in a two-fold increase in the numbers of these cells with respect to targets they innervate and, therefore, unneeded and misguided neurons are erased (Oppenheim 1991). Also, follicle atresia enables the selection and maturation of a restricted number of oocytes suitable for ovulation (Flemming 1887). Similarly, elimination of some diploid, constantly dividing, spermatogonia in adult testis prevents overpopulation of seminiferous tubules with undifferentiated cells only (Lee et al., 1997).

5/ Producing differentiated cells without organelles – this category is not bona fide cell death, since the cells eliminate only their nuclear structures, but they still actively metabolize nutrients. The best examples of these cells are mammalian red blood cells or differentiated lens cells. In addition, keratinocytes that form the superficial layer of mammalian skin lack nuclei.

It has been proposed that every nucleated somatic cell contains a PCD pathway in place, and its survival depends only on the proper activator/repressor balance (Raff et al., 1994). Many cell types, including terminally differentiated cells, will undergo cell death when the appropriate cell survival signal is removed or the proper death stimuli are provided. However, in the majority
of cases this should not be considered PCD, even though death in these cells is highly reproducible, stereotypical and is definitely a part of their developmental repertoire. Therefore, these cases should be referred to as examples of physiological cell death morphologically consistent with apoptosis.

1.2.2 Apoptosis.

Distinct morphological changes accompanying PCD in a majority of cases are used to define the phenomenon of apoptosis (Kerr et al., 1972). However, some cells dying via PCD display only one or a subset of the classical apoptotic features (Perreault and Lemieux 1993; Majno and Joris, 1994). Two major morphological characteristics are nuclear and cytoplasmic condensation. The nucleus undergoes chromatin condensation along the nuclear envelope with frequent remodeling to multiple round or irregular structures containing clumped chromatin. In addition, endogenous nucleases digest DNA into oligonucleosomal fragments (usually multiples of 180 base pairs) which give the appearance of a DNA ladder after electrophoresis (Arends and Wyllie 1991). In many cases, DNA fragmentation occurs early in apoptosis, often appearing several hours before the evidence of decreased cell viability. However in other cases, depending on cell type and apoptotic stimuli, cytoplasmic events occur simultaneously with nuclear ones.

The process of apoptosis is rapid (often detected 30 minutes after experimental administration of an appropriate trigger), making it difficult to detect and quantitate due to effective phagocytosis of cell debris. In a majority of studied cell types, measurable cell death assessed by the integrity of DNA, can be detected 12-24 hours after administration of the cell death trigger (Vinatier et al., 1996).

Degradation of chromatin is initiated by high molecular weight DNA cleavage, forming
50-300kb fragments (Roy et al., 1992), which seems to coincide with the first morphological signs of nuclear condensation (Cohen 1994). Later events of chromatin degradation involve the typical oligosomol DNA laddering. Apoptotic DNA fragmentation is thought to be mediated by Ca\(^{2+}\)/Mg\(^{2+}\) dependent endonuclease (Arends and Wyllie, 1991) and it is rapidly inhibited by Zn\(^{2+}\) ions (Treves et al., 1994). However, in some cell types, Ca\(^{2+}\) activation is not required (Treves et al., 1994; Vinatier et al., 1996). The best candidates identified so far that fit the requirements of an apoptotic endonuclease are Ca\(^{2+}\)/Mg\(^{2+}\) dependent DNaseI (Peitsch et al., 1993) and Ca\(^{2+}\)/Mg\(^{2+}\) independent DNaseII (Barry and Eastman, 1993). Also, RNA does not escape the process of degradation. Degradation of 28S RNA was observed in apoptotic cells as a result of the activity of a site specific ribonuclease (Houge et al., 1993).

The most pronounced features of the cytoplasmic changes accompanying apoptosis are the dilation of the endoplasmic reticulum and contraction of cytoplasmic volume, followed by extensive condensation of cytoplasm in remnants of the cell. Condensation of cytoplasm is most likely caused by polymerization and crosslinking of intracytoplasmic proteins due to the action of tissue transglutaminase (Fesus et al., 1987). The cell membrane convolutes and the cytoplasm is shifted into extracellular membrane covered processes. Subsequently, the cell breaks up into several membrane-bound buds of variable size. This process is often referred to as cellular budding (Joris and Majno 1994). Apoptotic bodies contain intact cytoplasmic organelles and occasionally pieces of chromatin. The cellular membrane, enclosing the apoptotic cytoplasm, appears to remain functional, since it efficiently excludes vital dyes. The number and size of apoptotic bodies is directly proportional to the size of the dying cell.

Apoptotic bodies appear to be potent stimulators of phagocytosis by neighboring cells ("amateur macrophages", cells that are not engaged in phagocytosis on a regular basis) and are
quickly removed without triggering an inflammatory reaction. This makes detection of apoptosis in an intact tissue even more difficult, because evidence of cell death - cell corpses - is recognized and cleaned up almost immediately after cell disintegration. This difficulty is further compounded by the fact that the triggers of PCD are not always known. The process of recognition of apoptotic remnants is thought to be triggered by specific antigens (apogens), expressed on the surface of apoptotic bodies (Rotello et al., 1994). In some cell types (e.g. in rodent thymocytes), the process of recognition of cell buds and their uptake by macrophages is mediated by unidentified carbohydrate-lectin interactions (Pradham et al., 1997). In other cell types, the vitronectin receptor seems to recognize a putative, so far unidentified, ligand on the surface of dying cells (Fadok et al., 1992; Cohen 1994). Moreover, certain asymmetry in membrane structure is created very early in apoptosis when phosphatidylserine performs a flip, exposing its cytoplasmic domain to the extracellular space (Fadok et al., 1992; Pradham et al., 1997; Schiratsuchi et al., 1997), and possibly mediating recognition of the apoptotic membrane by “professional and amateur macrophages”. However, in some cases, fragments are not phagocytosed and with time undergo secondary necrosis or "apoptotic necrosis" (Kerr 1987, Welsh 1993).

Some cells are not able to enter apoptosis after de novo RNA transcription and protein synthesis are inhibited (Wyllie et al., 1980). However, this phenomenon is not universal, since some cells without nuclei, which are unable to initiate de novo gene expression, will undergo apoptosis (Jacobson et al., 1994). This suggests that all components required for apoptosis are constitutively expressed in some mammalian somatic cells. It was proposed that cells exist in two different states with respect to apoptosis (Arends and Wyllie 1991). The first or primed state represents the situation in which cells possess all of the necessary effector molecules, and upon a
proper activation trigger, the apoptotic process begins. The second or unprimed state requires novel synthesis of effectors before cells are able to enter apoptosis. Alternatively, requirements for de novo gene expression may be associated with a specific cell death trigger rather than with a cell type. For example, the same cell may need to produce effector factors when activated by trigger A, but all the apoptotic components may be present when the cell is activated by trigger B.

1.2.3. Ischemic cell death - Necrosis

In contrast to PCD, ischemic cell death is often a consequence of cellular injury by metabolic toxins, changes in pH, or other noxious stimuli which compromise osmotic balance. Recently it was proposed that the term "ischemic cell death" should be used rather than "necrosis" since it more accurately describes cell death via failure of ionic pumps (Majno and Joris 1994). Following ischemic cell death, secondary changes in cell structure characterized by widespread disruption of all membranes and cellular swelling as a result of extensive ion and water influx leads to primary necrosis. Dilated lysosomes expel their hydrolases into the cytoplasm, which rapidly accelerates cell demise. DNA is not fragmented or is randomly degraded into a continuous spectrum of different size fragments that on gel electrophoresis form a continuous smear (Schwartzman and Cidlowski 1993). The final outcome is rapid disintegration and expulsion of cellular contents into the intercellular space.

Necrotic injury often results in a secondary inflammatory process affecting adjacent cells, which is always harmful to the organism. Ischemic cell death followed by necrosis does not seem to require activation of transcription (Lockshin and Zakeri 1992). However, in some cases, cell death consistent with necrosis occurs also as a part of normal tissue remodeling (e.g.
degeneration of epithelial cells of the endometrium during embryo implantation; for review see Welsh, 1993 and, therefore, it should be classified as a physiological event.

1.2.4. Triggers of PCD and apoptosis

Cells in a multicellular organism are exposed to a variety of intra- and extracellular signals. Exactly how these signals participate in PCD is unknown. One possibility is that cells are pre-programmed to die and require constant signals from other cells in order to survive (Raff 1992). On the other hand, a variety of signals, which on their own may not be lethal, may facilitate PCD in a responsive cell. These signals could be cell and tissue specific or could be a general mechanism locked within the cell/tissue to ensure the elimination of unwanted or impaired cells. It has been proposed that cells must reach a certain developmental stage in which effector mechanisms are coupled to the relevant signal transduction mechanisms to become responsive to suicidal triggers (Schwartz and Osborne 1993). Little is known about triggers and molecular pathways that lead to PCD during embryonic development. Good examples of developmental triggers are hormones such as ecdysone or thyroid hormone that initiate the process of metamorphosis. However, the majority of cell death triggers in a developmental context remain a mystery.

An increasing number of apoptotic triggers has been reported in connection with physiological cell death in mammalian tissues (for review see Table 2). One trigger of apoptosis in interphase cells may be premature induction of signals that are normally activated or involved in mitosis. These two very different cellular events, mitosis and apoptosis, have common morphological and biochemical profiles (condensation of DNA, shrinkage of cellular volume). This observation suggests the coupling of events associated with different checkpoints of the cell
cycle and decisions regarding cell division or cell death. PCD may be initiated as a protective mechanism to prevent carcinogenesis in cells that undergo aberrant mitosis leading to aneuploidy (Schimke at al. 1994). Moreover, cell injury that causes DNA single- or double-strand breaks should eventually lead to elimination of the cell via apoptosis (Kastan et al., 1991, Guiloff et al., 1995).

Another trigger for both PCD and apoptosis is the presence or absence of growth factors and hormones. Physiological regulation such as lack of, or overexposure to, growth factors, hormones and cytokines will trigger cell death. A few examples include ovarian follicle dependency on FSH and estrogen (Tilly and Tilly 1995; Billig et al., 1994), prostate dependency on androgen (Columbel and Buttyan 1995), and mammary gland regression after TGF-β1 exposure (Kordon et al., 1995). In a majority of cases, these triggers are tissue and/or cell type specific, which ensures that only targeted cells will respond. Often this pathway of cell death induction creates an environment permissive for activation of cell death via altering the cellular redox state (van den Dobbelsteen et al., 1996) or elevated ceramide (Kaipia et al., 1996) or cAMP production and Ca^{2+} signaling (Aharoni et al., 1995, Vinatier et al., 1996).

Reactive oxygen species (ROS) such as H$_2$O$_2$ generated during oxidative stress, have been suggested as one of several possible mediators of apoptosis and PCD (Ratan et al., 1994). Factors involved in destabilizing the optimal redox state of a cell such as an increase in ROS or a decrease in intracellular antioxidants will lead to a redox imbalance and cell death (Buttke and Sandstrom 1994). By adjusting culture conditions, the negative effects of an in vitro environment may be reduced or eliminated. For example, glutathione, or its precursors, β-mercaptoethanol and N-acetylcysteine, may prevent the induction of cell death triggered by ROS (Kurita and Namiki 1994, Ratan et al., 1994). Also, certain antioxidants can prevent cell
suicide caused by cytokine deprivation (Ferrari et al., 1995). Antioxidants such as vitamin C and E, estrogen and its metabolites, and iron chelating agents such as EDTA as well as enzymes involved in oxidative metabolism such as catalase and superoxide dismutase also partially protect cells from damage by ROS (Forrest et al., 1994, Tilly and Tilly 1995). However, although ROS generating agents are potent inducers of cell death in many situations, they are not common to all cases of PCD. For example, it has been shown that cells grown under anaerobic conditions will still die when induced with staurosporine, but are resistant to cell death induced by ROS-generating agents (Jacobson and Raff 1995).

Table 2. Table of agents that induce or inhibit apoptosis (based on Thompson 1995)

<table>
<thead>
<tr>
<th>Suppressors of apoptosis</th>
<th>Inducers of apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physiologic</strong></td>
<td><strong>Pharmacologic</strong></td>
</tr>
<tr>
<td>Growth factors</td>
<td>Caffeine</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>Caspase Inhibitors</td>
</tr>
<tr>
<td>Zinc</td>
<td>Tumor promoters</td>
</tr>
<tr>
<td>Hormones</td>
<td>Anti-oxidants</td>
</tr>
<tr>
<td>Neutral AA</td>
<td>Calpain Inhibitors</td>
</tr>
<tr>
<td>Viral genes</td>
<td>Inhibitors of sphingomyelinase</td>
</tr>
<tr>
<td>Cytokines</td>
<td>Glucose</td>
</tr>
<tr>
<td>Vitamins</td>
<td>Ceramide</td>
</tr>
</tbody>
</table>

Another potent inducer of apoptosis is ceramide. This universal second messenger is generated during stress by hydrolysis of sphingomyelin due to activation of sphingomyelinase enzyme (Kolesnik 1991). Factors known to operate via ceramide signaling include IL-1 receptor, FAS/Apo-1 (Cifone et al., 1993; Liu and Andreson 1995), and other receptors containing death domains (for more details see section 1.3.4.), as well as ionizing radiation (Haimovitz-Friedman
et al., 1994). In the context of ionizing radiation, which causes both membrane and DNA damage, it has been shown that ceramide and p53 pathways become activated and operate independently of each other during the induction of cell death (Santana et al., 1996).

Finally, calcium disturbances may be a trigger for PCD. Calcium is an important regulator of progression through the cell cycle and, therefore, may have a regulatory function in apoptosis. A sustained increase in intracellular Ca\(^{2+}\) is one of the first detectable events observed in cells undergoing apoptosis after exposure to a variety of different stimuli (Cohen and Duke 1984). However, similar to ROS, increased intracellular calcium does not seem to be a universal requirement for initiation of cell death (Treves et al., 1994). Alternatively, elevation of intracellular Ca\(^{2+}\) could be just a consequence of a second messenger effect triggered by a primary cell death inducer, such as hormone or growth factor.

1.3. Genetic Regulation of Cell Death.

1.3.1. PCD in C. elegans.

Several potential cell death genes involved in the suppression, initiation and progression of PCD were identified from extensive work in the field of nematode development. Caernohabditis elegans is a species of nematode, a small soil worm, composed of approximately 1000 cells at hatching. Development of this worm is highly stereotyped, and the lineages of all cells have been determined. Certain cells in C. elegans embryos routinely undergo PCD during development. By following the behavior of these cells, it is possible to analyze the impact of specific mutations on PCD. Genetic analysis revealed 14 genes associated with this stereotypical cell death. Two genes, Ced-3 and Ced-4, are important for initiation of PCD. Ced-3/Ced-4 mutants do not exhibit any obvious phenotype apart from lack of PCD and develop normally. In
these mutants, cells that are normally destined to die instead survive, differentiate and participate in further development (Ellis and Horvitz 1986). Recently, alternative splicing of the Ced-4 gene was reported. Based on cell death rates in transgenic worms, it was shown that Ced-4S protein stimulates cell death as reported previously, but its splicing sibling, Ced-4L, prevents cell death (Shaham and Horvitz, 1996). Interestingly, the Ced-4 gene is primarily expressed during embryogenesis with high transcript levels stored in eggs and with decreasing amounts in larval stages (Yuan and Horvitz 1992).

The Ced-3 gene bears highly significant homology to a novel family of aspartate-specific cysteine proteases, Caspases (Al nemri et al., 1996). All enzymes in this gene family contain a highly conserved recognition pentapeptide sequence QACXG (for details see section 3.3.2.). Furthermore, members of this gene family are able to substitute for each other in triggering cell death when transfected into cells of different species (Shigenaga et al., 1997).

The guardian of cell survival in C. elegans is the Ced-9 gene product, which acts to suppress the action of the Ced-3 and Ced-4 gene products. The progeny of animals lacking Ced-9 activity die during embryogenesis, indicating an important role for this gene in early embryonic development (Hengartner and Horvitz 1992). A genetic screen identified several mutant alleles in this gene with a strong maternal effect. Homozygote progeny of heterozygote females with a hypomorphic Ced-9 mutation develop to normal size even though they lack some cells present in the wild type animals. They suffer from an ovarian defect resulting in oocyte depletion and therefore display reduced fertility. A few oocytes survive and undergo successful fertilization; however, they arrest during early embryo development. Complete loss of function of the Ced-9 gene does not seem to cause typical apoptosis. Instead, embryos generally have swollen blastomeres with abnormal granularity of the cytoplasm and asynchronous, delayed cleavage. In
these embryos multinucleated blastomeres were observed, indicating abnormal cytokinesis. A different mutant allele of Ced-9 causes excessive activation of cell death in embryos containing approximately 200 cells. This is coincident with the time when PCD is normally activated in a few cells. Description of cellular abnormalities observed in Ced-9-/embryos is strikingly similar to the spectrum of morphologies observed among human embryos fertilized in vitro.

The Ced-9 protein has high sequence homology and functional similarities to the mammalian Bcl gene family. Bcl-2, one member of this gene family, is able to rescue Ced-9 defective mutants from PCD and restores almost normal development. (Hengartner and Horvitz 1994).

The other genes identified in the genetic screen of C. elegans are most likely involved in recognition and engulfment of dead cells with subsequent degradation of cellular debris (Ellis et al., 1991). Embryos with mutations of these genes are not able to recognize and/or initiate phagocytosis of cell corpses. However, lack of recognition of cell corpses does not seem to affect embryogenesis, or the pattern or timing of normal cell death during development. Interestingly, several of these genes also show maternal effects.

Current understanding of molecular mechanisms and their regulation with respect to cell death is still superficial. However, recent work has shed some light on the action of these genes. Ced-9 protein directly binds to and sequesters Ced-4 from the cytoplasm to the mitochondrial and perinuclear envelope (Wu et al., 1997). Ced-4 also binds to and stimulates processing of Ced-3, enhancing the ability of this enzyme to induce apoptosis (Seshagiri and Miller 1997). In addition, Ced-4 can simultaneously bind Ced-9 and Ced-3, which results in suppression of cell death (Chinnaiyan et al., 1997) thus suggesting that Ced-4 is a biochemical link between cell death suppressors such as Ced-9 and cell death executors such as Ced-3.
1.3.2. Genetic Regulation of Cell Death in Drosophila.

PCD has also been described during the development of Drosophila embryos. During development, cell death begins at stage 11 and subsequently affects many different cell types, especially the head, epidermis and central nervous system (Abrams et al., 1993). Unlike the situation in C. elegans, death in the developing Drosophila embryo is not completely stereotyped and pre-determined by cell lineage, even though the pattern of cell death is highly reproducible (Abrams et al., 1993; Steller et al., 1994).

Extensive analysis of the Drosophila genome has revealed clustering of several cell death genes in region 75C. Embryos homozygous for a deletion of this region have virtually no detectable cell death and as a result have an enlarged CNS (White et al., 1994; Zhou et al., 1997). So far there have been three cell death genes identified within this interval: reaper, hid and grim.

Reaper (White et al., 1994) is a rather small protein (65 amino acids) which appears to share homology to the cell death domains of FAS/APO 1 and tumor necrosis factor receptor (TNF-R; Goldstein et al., 1995). During Drosophila development, reaper expression is localized within cells destined to die and it appears to precede death by 1-2 hours (White et al., 1994). Unlike reaper, hid (Grether et al., 1995) is expressed not only in dying cells, but also in cells that survive. These two genes seem to cooperate in a very efficient induction of cell death, especially in the developing CNS (Zhou et al., 1997). Lastly, grim expression also coincides with developmental activation of PCD in Drosophila and its ability to kill does not require the presence of either reaper or hid (Chen et al., 1996). Interestingly, over-expression of grim was able to activate cell death earlier in development (stage 9). This does not occur in reaper transgenic experiments (Chen et al., 1996). Protein products of all three of these cell death genes
share sequence homology in the N-terminal region affecting 14 -amino acids, but only reaper contains the putative death domain also found in several mammalian death receptors (McCall and Steller, 1997).

Cell death in *Drosophila* is inhibited by caspase-inhibitors, implying evolutionary conservation of the Ced-3/caspase dependent degradation pathway. Moreover, another mechanism conserved among several species seems to be the induction of ceramide, which is also affected by caspase-inhibitors (Pronk *et al.*, 1996). Recently, two *Drosophila* caspases were cloned - DPC-1 and drICE. Both of these share homology with the *C. elegans* Ced-3 protease and their transcripts are very abundant during early embryonic development, possibly due to maternal storage (Song *et al.*, 1997; Fraser and Evan, 1997). A loss of function mutation in DPC-1 causes embryonic lethality and is responsible for the formation of melatonic tumors (Song *et al.*, 1997). The second caspase, drICE, sensitizes cells to apoptosis and its action is inhibited by the caspase inhibitor, baculovirus protein p35 (Fraser and Evan, 1997).

So far no genes bearing homology to Ced-9 or the Bcl-gene family have been identified in *Drosophila*.

1.3.3. Mammalian homologues of Ced genes.

1.3.3.1. Ced-9 and the Bcl-gene family.

Several vertebrate homologues of the nematode Ced-9 gene have been identified. However, some of these proteins seem to have a double identity and not all of these have cell death suppressor abilities. In contrast to Ced-9, some of them promote cell death. The family of Bcl-related proteins with cell survival function include Bcl-2 (Tsujimoto and Croce, 1986), Bcl-xL (Boise *et al.*, 1993), Bcl-w (Gibson *et al.*, 1996), A1/Bfl-1 (Choi *et al.*, 1996), Mcl-1 (Kozopas
et al., 1993) and several viral proteins such as BHRF1/EBV (Baer et al., 1984) and LMWS-HL/ASFV (Neilan et al., 1993). Most of these proteins contain a transmembrane region that enables their anchoring in the intracellular membranes of organelles such as mitochondria, the nuclear envelope and endoplasmic reticulum (Monagan et al., 1992, Gonzales-Garcia et al., 1995). A certain degree of cytoplasmic staining for Bcl-xL has also been reported (Hsu et al., 1997). The distribution of Bcl-2 protein within these sub-compartments in different cell types may reflect the requirement for protection of certain organelles due to the type of cell death triggers and pathways utilized.

Due to the localization of Bcl-family members in the mitochondrial membranes, it was originally proposed that these proteins function to prevent the ultimate and universal trigger of cell death - oxidative stress - by protecting intracellular membranes from lipid peroxidation (Hockenberry et al., 1993). Moreover, mitochondria in particular had been implicated as one of the essential components of the cell death pathway, and it was proposed that Bcl-2 alters mitochondrial function and thus protects cells from death. Even though it was later shown that neither mitochondria (Jacobson et al., 1993) nor oxidative damage are prerequisites for the activation of apoptosis (Shimizu et al., 1995), additional experiments provided strong evidence for the importance of the Bcl-family members in regulating mitochondrial function during cell death. It has been shown that Bcl-2 and Bcl-xL block the induced decrease in mitochondrial membrane potential (Shimizu et al., 1996) and that these molecules also inhibit the release of mitochondrial apoptotic proteases during the activation of cell death (Susin et al., 1996). These experiments culminated in identification of the primary inducer of the cell death machinery, cytochrome C, which is required for activation of caspases and DNA fragmentation (Martin et al., 1995). Both Bcl-2 and its cousin, Bcl-xL, inhibit the release of cytochrome C from
mitochondrial membranes, in turn preventing the activation of caspases and nucleases (Yang et al., 1997; Kharbanda et al., 1997). Furthermore, based on the three dimensional structure of Bcl-xL it was proposed that this molecule may be capable of forming a channel, since it shares similarity with pore forming domains of diphtheria toxin and bacterial colicins (Muchmore et al., 1996). Later, it was confirmed that Bcl-xL as well as Bcl-2, can form pH sensitive ion-conducting, cation-selective channels (Minn et al., 1997, Schendel et al., 1997). However, the physiological consequences of these observations, as well as the identity of molecules capable of utilizing these channels, remain unclear.

A second group of proteins belonging to this gene family is a rather diverse group of cell death inducers, such as Bax (Oltvai et al., 1993), Bcl-xS (Boise et al., 1993), Bad (Yang et al., 1995), Bik (Boyd et al., 1995), Bak (Farrow et al., 1995), Bid (Wang et al., 1996) and Hrk (Inohara et al., 1997). In contrast to cell death suppressors, inducers often reside in the cytoplasm (Wang et al., 1996; Inohara et al., 1997) except for Bcl-xS which is a splice variant of, and thus has a similar cellular distribution to, Bcl-xL (Minn et al., 1996). In addition, Bax also seems to localize to membranes upon induction of apoptosis (Hsu et al., 1997). Recently, a novel pro-apoptotic member with a pattern of expression restricted to reproductive tissues - Bok, was identified through its ability to interact solely with Mcl-1 and Bfl-1 (Hsu et al., 1997). Discovery of this protein further strengthens the hypothesis of the existence of tissue specific interactive partners involved in decisions of cell survival or death.

The overall amino acid homology among members of the Bcl-gene family is low (20-25%), but the majority of these proteins contain two highly conserved domains (BH1 and BH2). These are characteristic distinguishing features of all anti-apoptotic members such as Bcl-2, Bcl-xL, Mcl-1, BHFR 1 (Oltvai et al., 1993, Yin et al., 1994a) but can also be found in some pro-
apoptotic proteins (Kiefer et al., 1995; Inohara et al., 1997). Because it was shown that Bcl-family members have an ability to form hetero- and homodimers, it is not surprising to find that the BH1 and BH2 domains are involved in recognition, binding and function of Bcl- family members (Yin et al., 1994b). These domains are necessary, since deletion of either BH1 or BH2 disrupts the ability of Bcl-2 and Bcl-x to block cell death due to their inability to titrate Bax. However, these deletions do not effect Bcl-2 homodimerization (Yin et al., 1994a; Sedlak et al., 1995). Another two domains, BH3 and BH4, were recently discovered near the NH2 terminus (Zha et al., 1996). The BH4 domain can be found exclusively in cell death suppressers with the exception of Bcl-xS. The BH3 domain can be found in both protectors and inducers, and it was shown that mutations in this domain abolish ability of Bax to either homo or heterodimerize (Zha et al., 1996). Moreover, this domain is also required for heterodimerization of Bad with Bcl-xL and its disruption abolishes the ability of Bad to initiate cell death (Zha et al., 1997). Furthermore, peptides blocking the BH3 binding sites also inhibit the formation of Bcl-xL/Bcl-xL and Bcl-2/Bcl-2 homodimers, implicating the BH3 domain as an excellent candidate for the universal binding domain of Bcl-gene family members (Diaz et al., 1997). A schematic representation of the protein structure for Bcl-family members is shown in Figure 1.

From the extensive studies using the yeast two-hybrid system, as well as from immunoprecipitation experiments, it has been documented that Bcl-family members function through an intricate and balanced network of homo- and heterodimers. All studied members of this gene family dimerize with at least one other member of this family (see Table 3) and also with other, non-Bcl molecules (Vaux, 1997). Differences in the binding capabilities for different proteins, especially for Bcl-xS have been reported depending on the technique employed (Zhang et al., 1995; Minn et al., 1996). It has been proposed that cell death or survival decisions depend
on a "cell autonomous rheostat" regulated by ratios of these pro- and anti-apoptotic homo- and heterodimers (Oltvai et al., 1993; Yin et al., 1994b). When this rheostat is set more towards the formation of pro-apoptotic homodimers, the cell is committed to die. When the majority of pro-apoptotic proteins are kept in balance by interactions with anti-apoptotic molecules, the cell will survive. An interesting, and so far, the best understood mechanism of cell death evolved between Bad and Bax (both cell death promootors). Bad, having high binding affinity for Bcl-xL and Bcl-2, but not for other members of Bcl-gene family, displaces Bax from Bcl-x/Bax complexes and thus allows formation of the free Bax molecules that dimerize and lead to cell death (Yang et al., 1995). Bad itself is regulated by phosphorylation, which prevents its binding to Bcl-xL, and increases the affinity of Bad for 14-3-3 sequestering protein, inactivating the killing ability of Bad (Zha et al., 1996). Several kinase candidates mediating phosphorylation of Bad have been proposed but so far only one was identified - serine-threonine kinase - Akt (Datta et al., 1997).

Table 3. Formation of homo- and heterodimers by proteins of the Bcl-gene family (based on references provided above).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Bcl-2</th>
<th>Bcl-xL</th>
<th>Bcl-xS</th>
<th>Bax</th>
<th>Bad</th>
<th>Bak</th>
<th>Bik</th>
<th>Hrk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bcl-xS</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bax</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Bad</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Bak</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>?</td>
<td>-</td>
<td>-</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Hrk</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend: + strong binding; +/- weak binding; +/-! controversial results differing between reports; - no binding; ? not reported

Recently, yet another functional domain was identified in both the Bcl-xL and Bcl-2 proteins. Mutant forms of Bcl-xL and Bcl-2, lacking this 60 amino acid loop, showed enhanced
Bax, Bak, Bik, Hrk, Bad, Bid

Bcl-2, Bcl-xL, Bcl-w, Mcl-1

A1/Bfl-1

Bcl-xS

Bax, Bak

Bik, Hrk

Bad

Bid

based on Zha et al 1996
ability to suppress apoptosis without altering the binding affinity for Bax (Chang et al., 1997). Previously, phosphorylation of Bcl-2 was shown to affect the biological function of Bcl-2 by decreasing its ability to prevent cell death (reviewed in Gajewski and Thompson, 1996). The loop mutants of Bcl-2 protein displayed altered phosphorylation status, suggesting that this novel "negative regulatory domain" may be important for Bcl-2 posttranslational modifications (Chang et al., 1997).

1.3.3.2. Ced-3 and the Ice/Casp gene family.

The C. elegans cell death gene Ced-3 encodes a protein with high similarity to interleukin-1β converting enzyme (ICE, Cerreti et al., 1992). This similarity initiated efforts that led to the discovery of a novel and constantly growing family of aspartate-specific cysteine proteases. Caspases family membership for novel cloned ICE-like enzymes is based on the presence of highly conserved pentapeptide (QACXG) as well as by their ability to recognize and cleave aspartate specific sites. Excluding ICE, this enzyme family now includes at least 10 members, such as Nedd-2/Ich-1 (Kumar et al., 1994; Wang et al., 1994), CPP-32 (Fernandez-Alnemri et al., 1994), Ich2 (Kamens et al., 1995) and many others. Recently a new nomenclature for all members of the ICE enzyme family, Caspases, was proposed and is shown in Table 4. All of these enzymes are produced as inactive precursors that have to be properly processed by cleavage in order to become activated and to be able to initiate cell destruction (Thornberry, 1994; Tewari et al., 1995). In the majority of caspases, processing creates three polypeptides, a pro-domain important for dimerization of immature pro-enzymes, a small subunit (p10) and a large subunit (p20) containing the catalytic cysteine site. Processing of precursor molecules is mediated either by homo- or hetero-processing by other members of the caspase gene family.
(Srinivasula et al., 1996). Formation of mature subunits leads to assembly of an active tetrameric caspase with two p20 and two p10 subunits (Yamin et al., 1996).

Overexpression of all caspases activates cell death pathways and thus, these enzymes play a major role in apoptosis (Kumar and Harvey et al., 1995; Martin and Green, 1995). However, during transfection experiments, cells contain several times more caspase protein than is physiological.). It is believed that the major biological function of ICE is processing of pro-IL-1β, since mice lacking a functional ICE gene have no obvious abnormalities except for defective IL-1β processing and FAS-mediated apoptosis (Kuida et al., 1995). The only active ICE-like protease, which suppresses rather than induces apoptosis in mammalian cells, is the alternatively spliced form of Ich-1/caspase2, designated Ich-1S (Wang et al., 1994).

Table 4. Designation of human members of ICE/Caspase gene family .

<table>
<thead>
<tr>
<th>Proposed name</th>
<th>Gene/Protein name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-5</td>
<td>ICErel III</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>ICE</td>
</tr>
<tr>
<td>Caspase-4</td>
<td>TX, Ich-2, Ice rel II</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>Mch3, ICE-LAP3</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>CPP32, Yama, Apopain</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Mach, FLICE, Mch5</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Mch2</td>
</tr>
<tr>
<td>Caspase-10</td>
<td>Mch4</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>Ich-1</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>ICE-LAP6, Mch6</td>
</tr>
</tbody>
</table>

Existence of so many members of the same enzyme family able to cleave very similar substrates suggests functional redundancy or a requirement for a highly controlled cascade of events. In light of this, it has been proposed that pro-domains that are the most variable parts of caspases, allow specific recognition and activation of a given caspase at any given time (Wallach
et al., 1997). Thus, in a cascade model, a more upstream caspases would become processed upon induction by death stimuli and would initiate processing of the caspase next in order until the caspases at the end of the cascade become activated (Martin and Green, 1995; Fraser and Evan; 1996). More upstream caspases tend to have a larger pro-domain, suggesting that this regulatory region is important for selective processing. Consistent with this model are findings that Casp-2, 8 and 10 interact with Death Domain (DD) receptors, an early event in the initiation of cell death (Boldin et al., 1996, Muzio et al., 1996, Harvey et al., 1997). Downstream, execution caspases seem to be Casp-3 and Casp-6 which were found to be the two major activated caspases in many different cell types (Faleiro et al., 1997). Thus, caspases can easily amplify the cell death signals via the "net effect" of pro-enzyme processing plus cell specific substrate cleavage.

The main function of caspases is a degradation of cellular compartments, which enables disintegration of the cell. Reported caspase substrates, important for basic cell functions, are lamins, poly(ADP-ribose) polymerase, histone H1, topoisomerases, protein kinase C, U-1(70kD) riboprotein, actin and many other molecules (Kumar and Harvey, 1995). Even though these enzymes all recognize an aspartate specific cleavage site, certain enzyme specific preferences in substrate cleavage have been reported (Talanian et al., 1997). For example Casp-6, which itself is a substrate of Casp-3 (Srinivasula et al., 1996), preferentially degrades nuclear lamins (Orth et al., 1996). Recently, cell specific substrates such as MDM2 oncoprotein (Erhard et al., 1997), Alzheimer-Associated Presinilins (Kim et al., 1997), and huntingtin (Goldberg et al., 1996) have also been identified as substrates of the Casp-3 (executor) subfamily of caspases. Interestingly, Casp-3, which becomes activated in response to a release of cytochrome C from mitochondria, cleaves, and, thus induces activity of a novel heterodimeric protein, DFF, that is responsible for initiation of DNA fragmentation during apoptosis (Liu et al., 1997).
1.3.3.3. *Ced-4, Apaf-1, MRIT* and *Bag-1*.

Many laboratories have spent the past 4 years trying to identify a vertebrate homologue of Ced-4 without success. This molecule shares no significant homology to known proteins, except two domains with putative calcium-binding sites (Yuan and Horvitz, 1992). Since it has been shown that both Ced-9 and its mammalian counterpart, Bcl-xL, can interact and inhibit function of Ced-4 (Chinnaiyan *et al.*, 1997), it was obvious that a vertebrate homologue of Ced-4 must exist. Moreover, since Ced-4 can also bind Casp-8 (Chinnaiyan *et al.*, 1997), even the next step in the *C. elegans* pathway (Ced4→Ced3) seemed to fit cell death in vertebrate organisms.

Recently, a cytosolic 130 kd protein - Apaf-1, containing partial identity to both caspases, Ced-3 and Ced-4, was proposed to be a functional mammalian counterpart of Ced-4 (Zou *et al.*, 1997). This ubiquitously expressed protein seems to bind cytochrome C, possibly causing activation of caspases and thus fulfills the requirements for a linkage protein, uniting mitochondrial Bcl-2/Bcl-xL, cytochrome C and caspase activation in one direct pathway.

Recently, a novel molecule MRIT, capable of binding and activating caspases with large prodomain (Casp-3 and Casp-8), as well as the adaptor molecule FADD, was cloned (Han *et al.*, 1997). Moreover, this molecule also independently and simultaneously binds to Bcl-xL and thus it was proposed that MRIT may be another Ced-4 like link facilitating interaction between Bcl-xL and Casp-8 (Han *et al.*, 1997).

Another example of a Bcl-interactive, cell survival promoting factor is Bag-1. This protein was cloned from a murine day10 embryonic expression library during a search for novel Bcl-2 binding partners (Takayama *et al.*, 1995). It does not share any significant sequence homology to known proteins. Co-expression of Bag-1 with its interactive partner Bcl-2, induces
protection from cell death caused by a variety of stimuli such as staurosporine and FAS-activation (Takayama et al., 1995). In addition, its protein product becomes induced upon treatment with growth factors such as IL-2, IL-3, PDGF and HGF (Adachi et al., 1996, Clevenger et al., 1997, Bardelli et al., 1996). Direct interaction was shown between Bag-1 and Bcl-2, Bcl-xL, and Hsp70 (Takayama et al., 1995, Takayama et al., 1997), steroid receptors (Zehner and Gehring, 1995), and with tyrosine kinase growth factor receptors such as PDGF-R and HGF-R (Bardelli et al., 1996). This molecule is most likely a regulatory chaperone that unifies cellular signaling with cellular responses (Takayama et al., 1997).

1.3.4. Other genes involved in mammalian PCD

In mammalian systems, it is very difficult to generalize the results of specific gene expression patterns in various tissues during cell death, or even in different cell types. Different triggers of apoptosis may initialize disparate biochemical pathways in which various genes and their products are up- or downregulated in the same cell type. Unfortunately, very little is known about these pathways and the action of killer genes during mammalian embryo development.

One of the key players in cell cycle progression and differentiation is the proto-oncogene, c-myc. This transcription factor may induce a state during the cell cycle in which either mitosis or apoptosis predominates, depending on the microenvironment of the affected cell (Evan et al., 1992). More specifically, a high level of c-myc expression in fibroblasts or cells of the myeloid lineage causes growth arrest in G1, which in turn regulates differential expression of cyclins and a final decision of cell cycle progression or cell death (Hoang et al., 1994). This pathway of PCD, related to c-myc overexpression, can be abrogated by the overexpression of Bcl-2 (Bissonnette et al., 1992).
A second pathway that results either in cell proliferation, or arrest and apoptosis, involves the p53 tumor-suppressor protein whose expression is rapidly induced in response to DNA damage (Kastan et al., 1991, Clarke et al., 1993). Most cellular injuries involving double or single stranded DNA breaks trigger apoptosis through this pathway. Wild type p53 acts as a checkpoint in G1 and/or G2 phase, either allowing DNA damage to be repaired or committing cells to death (Guiloff et al., 1995, Stewart et al., 1995). Mutant forms of p53 have most likely lost the ability to commit impaired cells to death, and, therefore, cells with damaged DNA may progress through mitosis (Lane, 1992). It is possible that the appearance of malignant behavior in cells with mutant p53 alleles is a direct result of loss of this control mechanism. Many different kinds of cancers have a mutation in both p53 alleles, and loss of p53 function affects genomic stability in human cancers (Carder et al., 1993, Wyllie et al., 1994). A novel human homologue of p-53 was recently cloned. This protein, p73, has many structural and functional similarities to p53 and is able to cause either cell arrest by activating p21 (Kaghad et al., 1997) or to induce apoptosis, possibly via activation of genes with p53 responsive elements (Jost et al., 1997).

The molecular events leading to apoptosis via p53 activation in some cell types involves induction of transcription of variety of genes, many of which are involved in response to oxidative stress (Polyak et al., 1997). Moreover, p53 is a direct transcriptional activator of Bax and also has the ability to suppress Bcl-2 gene transcription (Miyashita and Reed, 1995), actively changing the ratio between pro- and anti-apoptotic proteins towards cell death. Conversely, negative regulation of cell death gene promoters is controlled by transcription factors such as Gfi-1 (Gilks et al., 1993). This DNA sequence-specific binding protein represses both Bax and Bak transcription, via binding to Gfi-1 recognition sites, some of which could be found in the p53 inducible Bax promoter (Grimes et al., 1996).
Figure 2. Schematic representation of molecular events occurring during cell death in mammalian cell. Several possible pathways are shown. One of them is induced by DNA damage, causing recruitment of transcription factor, p53, into the nucleus. p53 forms a tetramer and initiates transcription of Bax cell death inducer gene. Bax protein accumulates in cytoplasm, binds to and titrates Bcl-2 and Bcl-xL molecules. Eventually toxic Bax/Bax homodimers are formed, leading to release of Cytochrome C from mitochondrias into the cytoplasm. Cytoplasmic cytochrome C, in turn, activates downstream procaspases (procasp-3) into their enzymatically mature form. Similarly, de-phosphorylation of cell death inducer Bad causes replacement of Bax in Bcl-xL heterodimers which results in the formation of Bax/Bax homodimers. The phosphorylation of Bad increases its affinity to sequestering protein 14-3-3, preventing Bad from interacting with Bcl-xL. Another pathway involves activation of membrane receptor with death domain (FAS) with its ligand (FAS-li). Activated receptor binds adaptor molecule (FADD), causing recruitment of upstream procaspase (procasp-2) to the receptor. Binding of procaspase to the adaptor molecule induces cleavage of proenzyme into mature Casp-2. Afterwards Casp-2 cleaves and activates downstream procaspase (procasp-3), initiating active process of cell destruction.
Schematic representation of molecular events leading to cell death

- 14-3-3
- p53
- Bax
- Bad
- DD-Receptor
- FAS
- FAS-ligand
- Pro/Casp-2
- Pro/Casp-3
- Cytochrome C
- Linker - FADD
- Bcl-2/Bcl-xL

Mitochondria
Phosphorylation
Caspase Activation
Protein Degradation
DNA Damage
A third pathway, implicated in the elimination of many redundant (spermatogonia) or unwanted (T lymphocytes), cells involves a novel group of effector signaling proteins, the majority of which belong to TNF receptor gene family. These include FAS, TNF-R1 and R2, WSL-1, their signal transducing partners FADD, TRADD, RIP, TRAIDD and CRADD (for review see Wallach et al., 1997, Nagata et al., 1997). All these molecules contain a stretch of approximately 80 amino acids in their cytoplasmic region referred to as death domain (DD). This domain seems to be responsible for transducing the death signal and has a tendency of self-aggregation (for review see Nagata, 1997). APO1/FAS, the best known member of the TNF family of cell surface receptors, upon crosslinking with antibody or Fas-ligand, will activate apoptosis of FAS expressing cells (Schulze-Osthoff et al., 1994; Suda et al., 1993). Activation of apoptosis by these receptors is mediated by caspases since Crm A, a potent inhibitor of cysteine proteases, completely blocks cell death induced either by FAS or TNF (Tewari and Dixit, 1995). Recently, a number of novel proteins with affinity to an intracellular portion of the TNF receptor, containing DD, were identified. FADD is a signal transduction adapter protein that binds to activated FAS and TNF-R, which has been implicated in generation of ceramide and activation of the effector Casp-3 (Chinnayan et al., 1996). It was shown that FADD recruits Casp-8 and 10 that also contain DD to FAS and TNF receptors (Muzio et al., 1996). This translocation presumably causes proteolytic activation of these caspases. Inhibition of this recruitment can be caused either by I-FLICE or by FLAME-1 (other members of DED proteins) blocking the binding site on FADD and thus inactivating the FAS and TNF-R signaling pathways (Hu et al., 1997, Srinivasula et al., 1997).

In attempts to identify genes involved in apoptosis in different cell types, many novel genes with so far unknown or partially known function have been discovered. Several
transcription factors, especially Zn finger containing proteins such as Requiem (Gabig et al., 1994), brain specific Zac-1 protein (Spengler et al., 1997) and p53 inducible PAG608 (Israeli et al., 1997) were cloned due to their increased expression in cells undergoing apoptosis. Also, some genes such as MA-3 or RP-8 that are up-regulated during apoptosis, but whose over-expression is not sufficient to trigger cell death (Shibahara et al., 1995; Vaux and Hacker, 1995), have been reported.

1.4. Are genes associated with PCD in somatic cell types expressed during embryo development?

Very little is known about expression of genes involved in PCD during fetal and placental development. Term placenta and adult ovaries (which include oocytes) express many genes associated with cell death, including Bcl-2, Bcl-x, p53, Bak, Bax (Kiefer et al., 1995, Tilly et al., 1995, Tilly et al., 1995) and the ICE subfamily (Kamens et al., 1995, Wang et al., 1994, Flaws et al., 1995). The transcripts of several genes associated with apoptosis in somatic cells, have been detected in mouse oocytes and early embryos. C-myc, a key player in cell cycle checkpoint control, is expressed in low levels in both oocytes and early embryos, with a slow increase of expression towards the blastocyst stage (Pal et al., 1993). Recently, transcripts for two members of the tumor necrosis factor family, TNFα (Chen et al., 1993) and APO-1/FAS antigen (Guo et al., 1994) were both detected in oocytes and eggs. Moreover, FAS-ligand, but not FAS receptor protein was detected in oocyte lysates by Western blotting, implicating FAS/FAS-ligand in follicle atresia (Hakuno et al., 1996). Interestingly, no detectable FAS-ligand transcripts were observed in oocytes by in-situ hybridization (Suda et al., 1993). This controversy became even more complicated by a recent presentation of Zaninovic et al. (1997), reporting the presence of
FAS-receptor, but lack of FAS-ligand, on the surface of human oocytes and preimplantation embryos.

Both Bcl-2 and Bcl-xL are expressed during murine post-implantation embryo development (Weis-Novack and Korsmeyer, 1994; Gonzales-Garcia et al., 1994). Unfortunately, nothing is known about their earlier pattern of expression or overlap/restriction of expression in different tissues. The pattern of expression of Bcl-2 and p53 has been studied in the human placenta during different stages of gestation (Sakuragi et al., 1994). Both proteins are localized to syncytiotrophoblast with increased expression towards term. Only very weak expression is observed in cytotrophoblast. This pattern of expression suggests a connection between terminal differentiation and susceptibility to apoptosis.

Since Ced-9 mRNA is stored in oocytes and mutations in this protector molecule cause very early embryonic death, it is likely that a similar molecule will be found to be involved in the protection of early mammalian embryos.

In order to determine which genes may be involved in preimplantation embryo death, we have initiated an analysis of cell death gene expression in embryonic stem (ES) cells. ES cells, derived from the inner cell mass of murine blastocysts, represent a powerful system that allows us to study the cellular events that occur during early embryogenesis. Furthermore, these cells die via apoptosis after a reducing component, β-mercaptoethanol (β-ME), is removed from the culture medium (Castro-Obregon and Covarrubias, 1996), mimicking oxidative stress, a trigger of PCD at the blastocyst stage (Pierce et al., 1991). Using Northern blot analysis, we determined the expression of 13 cell death genes in murine ES cells. Relatively high expression of the cell death suppressor genes such as Bcl-xL and Bag-1, with lower expression of the cell death inducer p53 and cell death executioners Nedd-2/Ich-1 and ICE was observed in both healthy and dying
cells. No detectable expression of MA-3, Bcl-2, Bad, Bax, CPP-32, c-myc, FAS or FAS-ligand was observed using this approach. Tissue transglutaminase (TTG), involved in cellular fragmentation associated with apoptosis, was also constitutively expressed in ES cells. This series of preliminary experiments led us to further investigate expression patterns of genes involved in cell death during preimplantation embryo development. Here we will demonstrate that embryo fragmentation is a result of PCD and that it can occur during the 1-cell stage of mouse embryogenesis and 2- to 8-cell stage in human. We will also demonstrate for the first time the temporal expression patterns of several cell death regulatory genes, and show that the expression of these genes is altered in murine embryos undergoing fragmentation. We propose that PCD may occur by default in embryos that fail to execute essential developmental events during preimplantation embryo development and is increased in embryos that are exposed to in vitro culture conditions.
Chapter 2.

Programmed cell death and human embryo fragmentation

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2.1. Introduction

The quality of human embryos produced by IVF is variable. Less than 50% of embryos cleave regularly resulting in equal-sized blastomeres without fragmentation. The remaining embryos often contain variable-sized blastomeres with multiple cellular fragments enclosed within the zona pellucida. Subsequent in vitro development of these fragmented embryos is impaired, often leading to cleavage arrest and embryo degeneration. Upon transfer, fragmented embryos have limited developmental potential and rarely result in pregnancy (Plachot and Mandelbaum 1990; Erenus et al., 1991). Interestingly, cytogenetic observations of spare human embryos confirmed the presence of a spectrum of nuclear anomalies, e.g. multinucleated or anucleated blastomeres, flocculent and fragmented nuclei, (Winston et al., 1991; Hardy et al., 1989; Hardy et al., 1993). A wide range of chromosomal abnormalities including premature chromosome condensation and a high degree of aneuploidy and polyploidy has been reported in spare human embryos (Pappadopoulos et al., 1989; Zenzes and Casper 1992). Compared to embryos with good morphology, a higher incidence of these cytogenetic abnormalities was found in embryos with fragmentation (Michaeli et al. 1990, Pellestor et al., 1994, Munne, et al., 1994).

Our knowledge of embryo fragmentation following IVF in different mammalian species is very limited. The morphologic appearance of fragments in early embryos and previous reports describing nuclear abnormalities (Hardy et al., 1993) led us to hypothesize that fragmentation in these embryos is the consequence of programmed cell death (PCD) with typical features of apoptosis.

PCD refers to physiological cell death, which is a normal part of development. True PCD can be precisely predicted in time and space in developing organisms (Cohen, 1994). Because this cell death is genetically programmed it requires activation of specific genes involved in the
execution of cell death. Often, but not always, this leads to a common series of morphological changes termed apoptosis (Kerr et al., 1972). Cells dying via apoptosis have very distinct cellular morphology easily recognizable from ischaemic cell death followed by necrosis. Typical characteristics of apoptosis, previously described in various tissues, include nuclear chromatin condensation with subsequent DNA degradation into oligonucleosomal fragments (demonstrable by the appearance of DNA "laddering" on gel electrophoresis) and abnormal nuclear shape. Cytoplasmic changes include cellular shrinkage as a result of extensive budding and the appearance of multiple cellular fragments which are directly proportional to the size of the dying cell. Cellular fragments contain cytoplasm, intact cytoplasmic organelles, and occasionally, pieces of condensed chromatin. Because the cellular membrane remains intact, the fragments often exclude vital dyes.

The objective of the present study was to determine if the morphological features of apoptosis are observed in fragmented human preimplantation embryos, supporting the possible involvement of PCD in early human embryo arrest and demise.

2.2. Materials and Methods

In Vitro Fertilization and embryo culture

Spare human preimplantation embryos were obtained from the IVF Program, Division of Reproductive Sciences, Department of Obstetrics and Gynaecology, at the University of Toronto. Patients who chose not to freeze their spare embryos for future transfers were asked to donate them for research and informed consent was obtained. This research was approved by the human ethics committee of the Toronto Hospital.

Ovarian stimulation was carried out using a gonadotropin releasing hormone (GnRH)
agonist (Lupron, Abbott Pharmaceuticals, Montreal) in a long protocol, and human menopausal
gonadotropin (hMG; Pergonal, Serono Canada, Oakville, Ontario or Humegon, Organon
Canada, Scarborough, Ontario). Both IVF and embryo transfer were performed using standard
techniques as previously described (Segal and Casper, 1992). Briefly, ovum retrieval was carried
out 36 hours following injection of 10,000 IU human chorionic gonadotropin (Profasi, Serono).
After retrieval, oocytes were cultured for 3 h in human tubal fluid medium (HTF) supplemented
with 10% human serum albumin at 37°C in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂.
Fertilization resulted from insemination with 50,000 to 100,000 washed sperm per dish
containing one to three ova. Eighteen to 22 h after insemination, oocytes were examined for the
presence of two pronuclei and 43-45 hours after insemination up to 3 of the most rapidly and
evenly dividing embryos were transferred (at the 2 to 6 cell stage) to the patient's uterus.

Spare embryos of variable quality that appeared to arise from normally fertilized oocytes
with 2 pronuclei were used for subsequent analysis. Embryos were cultured in HAM's F10
medium (Gibco BRL, Burlington, Canada) supplemented with 10% human serum at 37°C in 5%
CO₂ and 95% air. Assessment of embryo quality and developmental stage was recorded daily
until embryos showed cleavage arrest with no further progress compared to the previous 24
hours.

Scanning (SEM) and transmission (TEM) electron microscopy.

For SEM, embryos were washed in phosphate buffered saline (PBS) and fixed for 1 hour
in 2.5% glutaraldehyde diluted in Sorrensen's phosphate buffer, post fixed in 1% osmium
tetraoxide, and dehydrated in serial dilutions of ethanol followed by propylene oxide. The final
dehydration was done in hexamethyldisilizane (Polysciences, Warrington, PA). Samples were
coated with gold particles and analyzed using a Hitachi 2500 scanning electron microscope.

Specimens for TEM were fixed similarly as for SEM, dehydrated through an alcohol series, embedded in Spurr and serially sectioned. Semi-thin sections (1μm) were cut and stained with 1% toluidine blue, while ultrathin sections (70 nm) were stained with alcoholic uranyl acetate followed by Reynold's lead citrate, and were examined by a Hitachi 7000 electron microscope.

Combined nuclear and fragmented DNA labeling.

To analyze the status of chromatin in arrested, fragmented embryos, we used a combined technique for simultaneous nuclear and terminal transferase-mediated DNA end labelling (TUNEL). Briefly, embryos were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) at a concentration of 0.02 mg/ml for 1 hour and washed in medium for 10 min. The zonae pellucida were removed using acid Tyrodes, and embryos were immediately fixed for 10 min at room temperature on microscope slides in 4% paraformaldehyde diluted in PBS. After air drying, slides were stored at -20°C until further use.

TUNEL was performed according to White et al., (1994) with a few modifications. After thawing, slides were washed in PBS. Specimens were preincubated in One-Phor-All Buffer (Pharmacia, Quebec, Canada) consisting of 10 mM Tris-acetate, 10 mM magnesium acetate and 50 mM potassium acetate supplemented with 0.1% Triton X. Afterwards, the embryos were overlayed with reaction cocktail, which contained One-Phor-All Buffer with 0.1% Triton X, 0.25 U/ml terminal transferase (Pharmacia, Quebec, Canada), 6 μM dATP and 3 μM Bio-dUTP (Sigma, St. Louis, MO). Reactions were carried out in a humidified chamber for 1 hour at 37°C. After washing slides twice for 10 minutes in PBS, incorporated biotinylated nucleotides were detected with streptavidin-Texas red conjugate (Calbiochem, San Diego, CA).
diluted 1:150 in PBS plus 0.1% Triton X. To decrease nonspecific binding, conjugate incubation was performed at 4°C for 30 minutes. After three washes in cold PBS, slides were examined and photographed using a Leica fluorescent microscope with appropriate filters.

2.3. Results.

Our combined approach allowed us to distinguish between chromatin status and DNA fragmentation associated with apoptosis as opposed to necrosis. When live, healthy cells are exposed to DAPI, staining is restricted to chromatin. When staining is observed in the cytoplasm, this suggests a lack of integrity of the cellular membrane and is a sign of necrosis. The status of the chromatin condensation can be assessed by the intensity of DAPI labelling and imparts the shape of the chromatin. Normal, uncondensed chromatin has pale uniform DAPI staining and an oval shape of the nucleus. Condensed chromatin stains brightly with DAPI, and is often irregular in shape. These features were identified as apoptosis positive. In comparison with DAPI staining, TUNEL labelling is based on an enzyme reaction and reflects the integrity of the DNA. Negative labelling is equivalent with background, while positive labelling is very bright (see Fig 1.). Necrotic cells can be distinguished from apoptotic ones by distribution of TUNEL labelling, which is cytoplasmic and uniform in necrotic blastomeres and punctuate in apoptotic cells.

Nuclear analysis of arrested embryos.

A total of 229 human embryos, arrested at different stages of development ranging from the 2-cell stage to uncompacted morulae, were studied using combined DAPI and TUNEL analysis. Out of these, 203 showed various degree of fragmentation. Within this population, we observed several different categories of staining:

1. No nuclear or DNA abnormalities (DAPI and TUNEL negative - 12%)
2. Nuclear staining only, with condensed chromatin and intact DNA (DAPI positive, TUNEL negative - 30%)

3. Nuclear staining only, with condensed chromatin and fragmented DNA (DAPI and TUNEL positive signals - 21%)

4. Normal, uncondensed chromatin and DNA fragmentation (DAPI negative, TUNEL positive - 1.5%)

5. Diffuse cytoplasmic staining corresponding to necrotic cells (DAPI positive with or without TUNEL signal - 5.5% and 5.5% respectively).

6. Fragmented embryos displaying evidence of both apoptosis and necrosis in at least two, but often in more blastomeres of the same embryo (24.5%, see Table 1).

Table I. Nuclear status in arrested and fragmented human embryos

<table>
<thead>
<tr>
<th>Embryo morphology</th>
<th>Nuclear morphology</th>
<th>DAPI b</th>
<th>TUNEL b</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>26</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Apoptotic</td>
<td>+</td>
<td>-</td>
<td>61</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Apoptotic</td>
<td>+</td>
<td>+</td>
<td>42</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Apoptotic &amp; Necrotic</td>
<td>+</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Apoptotic &amp; Necrotic</td>
<td>+</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Necrotic</td>
<td>+</td>
<td>+</td>
<td>11</td>
</tr>
<tr>
<td>Fragmented</td>
<td>Necrotic</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
</tbody>
</table>

a - Condensed chromatin was scored as apoptotic while diffuse staining extending into the cytoplasm was scored as necrotic. b - DAPI and TUNEL staining were scored (+) if signal was strong and (-) if staining was weak. See Figure 1 for example.

An additional twenty-six apparently normal embryos, with no visible cellular fragmentation, had normal looking nuclei as judged by both a diffuse DAPI staining pattern and
negative TUNEL labelling. In 3 of these embryos, we observed multinucleated blastomeres. In contrast, 153 of 203 fragmented embryos (75.4%) displayed hallmarks of apoptosis with or without some normal nuclei.

_Ultrastuctural analysis of fragmented embryos._

Evidence of apoptosis was confirmed by light, transmission, and scanning electron microscopy (Figure 2 and 3). 17 fragmented human embryos which arrested at various cleavage stages were serially sectioned. In 15, we observed several undegraded cell corpses with dense cytoplasm, multiple cellular fragments which contained normal appearing cytoplasmic organelles, and dense masses resembling condensed chromatin. Despite condensed cytoplasm in these cell corpses, intact mitochondria and several other organelles were visible. Corpses were not phagocytosed and were always found in the intercellular space within the zona pellucida (Figure 3a).

Some of the cellular fragments showed secondary necrotic changes with disrupted cellular membranes and swollen cytoplasmic organelles (Figure 3a). These necrotic changes were also evident in some non-apoptotic blastomeres (disrupted cell membrane, swollen organelles and dilated lysosomes). These observations were consistent with the generalized DAPI/TUNEL signal characteristic of necrotic cells observed in some embryos.

While embryos varied in their degree of fragmentation, each embryo also contained a few apparently normal cells with regular, round nuclei and no extensive chromatin condensation. In addition, several blastomeres also contained two or three nuclei of normal size and shape (Fig 3b).
2.4. Discussion.

Programmed cell death through apoptosis is characterized by a number of well defined morphological features including chromatin condensation and cellular shrinkage. Another of these features, DNA oligonucleosomal fragmentation, can be visualized by the appearance of a DNA ladder pattern on agarose gel electrophoresis. The small number of cells found in preimplantation embryos makes gel electrophoresis impractical. However, an in situ technique utilizing terminal transferase mediated end labelling of DNA - TUNEL- has been developed to demonstrate the lack of DNA integrity (Gavrieli et al., 1991). This technique has allowed investigators to trace the events that follow the triggering of apoptosis and has revealed a rapid progression from blebbing to phagocytosis, occurring over a period of a few hours. In some cells, secondary necrosis rather than phagocytosis is the culmination of apoptosis (Kerr et al., 1987; Welsh 1993).

Our experiments provide clear evidence of apoptosis in human preimplantation embryos, using the combined techniques of DAPI/TUNEL, TEM, SEM and stereomicroscopic observations. The present study concentrated on early cleavage stage embryos that arrest and fail to develop to the blastocyst stage in vitro. The distinguishing feature of these embryos was excessive blastomere fragmentation, which was easily visible under a dissection microscope. Embryos were processed 24 hours after the last cleavage and had reached variable stages of development or arrest. Therefore, embryos with asynchronously dying blastomeres were sampled at different times with respect to the first apoptotic process that produced the original fragments. This may account for the different categories of embryos observed in our population (Table I). We hypothesize that embryos with a few fragments but no condensed DAPI/TUNEL signals may represent an earlier stage of apoptosis than those with fragments and both condensed
DAPI/TUNEL signals. Embryos showing evidence of necrotic changes may be more advanced still, although we cannot rule out the possibility that some cells/embryos die through arrest-mediated necrosis rather than apoptosis. We do not know whether the preponderance of DAPI signal over co-incident DAPVTUNEL represents an experimental limitation or an effective means of distinguishing cells at different stages in the apoptotic pathway.

Blastomeres probably have no phagocytic capability since we found cell corpses in the intercellular space, but none which were engulfed by other cells. Alternatively, blastomere corpses may not promote phagocytosis, possibly through an inability to express cell surface molecules - apogens, responsible for recognition of apoptotic cells (Ellis et al., 1991, Rotello et al., 1994). Thus, within developing preimplantation embryos, cell corpses and fragments which are not phagocytosed effectively may undergo secondary necrosis, which in turn may trigger arrest and subsequent necrosis of surrounding blastomeres. We did not find phagocytosed cell corpses in the cytoplasm of surrounding blastomeres. However our population of studied embryos represented those that fail to reach the blastocyst stage. Fragmented embryos that managed to reach the blastocyst stage may have the ability to deal with cell corpses more effectively than their arrested counterparts. Trophoblast and trophoectoderm, the first differentiated cell types that arise in the embryo during blastocyst formation have been reported to possess highly effective phagocytic activity (Drake and Rodger 1987).

These observations support the hypothesis that PCD and the resultant apoptosis is responsible for a significant proportion of fragmented human embryos, and reinforces the original morphologic description of Hardy et al., (1989;1993). However, extremely fragmented embryos were excluded from their study, which may be one reason that the morphological indicators of apoptosis (chromatin condensation and fragmentation) were observed in a much
smaller number of embryos. Interestingly, a higher number of anucleated cells was found in embryos of poor morphology (Hardy et al., 1993). These cells/fragments could have been mistaken for large apoptotic bodies that do not contain pieces of condensed chromatin.

Several articles have described the morphologic appearance of dying cells in mammalian embryos at the blastocyst stage. Analysis of ultrathin sections of blastocysts of various mammalian species revealed the presence of cells with clumped, condensed chromatin, swelling of the endoplasmic reticulum, multiple cellular fragments and several cellular corpses (El-Shershaby and Hinchcliffe 1974; Mohr and Trounson 1982). Occasionally, parts of cell corpses were found undigested in the cytoplasm of surrounding cells. The presence of cell death has been observed in blastocysts obtained in vivo, as well as in embryos cultured in vitro following IVF. The morphological descriptions, as well as the published photographs, show typical signs of apoptosis. It has been proposed that the blastocyst is the first stage during mammalian embryogenesis in which PCD can be observed (Parchment, 1993). Apoptosis at this stage was coupled with elimination of redundant ICM cells with trophectodermal potential. Our results indicate that PCD can occur at even earlier stages of embryo development, with detrimental effects leading to embryo demise in some cases.

In summary, our results demonstrate the morphological appearance of apoptosis in fragmented human embryos. These findings strongly suggest that PCD is triggered in human embryos at a stage prior to blastocyst formation. In view of the fact that cellular fragmentation is one of the consequences of apoptosis in somatic cells and that fragmented embryos display many morphological hallmarks of apoptosis (chromatin condensation, DNA fragmentation and presence of cell corpses), our observations suggest that embryo fragmentation is a result of activated PCD in some blastomeres. However, this hypothesis cannot be directly tested at the
present time because we do not know the triggers of cell death in early embryos. Further efforts in our laboratory are now aimed at identifying genes whose activation or suppression results in the initiation of PCD, and identifying the triggers for such gene expression in human embryos. We believe this research will prove valuable in the development of strategies to prevent, or reduce, embryo fragmentation and death.
2.5. Legends to Figures.

Figure 1. Combined nuclear DAPI/TUNEL analysis.(magnification 400 x).

Fig 1a. Fragmented human embryo arrested with four normal appearing blastomeres and several cellular fragments. As assessed by DAPI, staining of chromatin within a few cellular fragments is heavily condensed and misshapen. In contrast, the nucleus in normal blastomeres is lightly stained (large opened arrowhead).

Fig 1b. DNA fragmentation in condensed chromatin is extensive as observed by the intense TUNEL signal. However, as indicated by the arrow, some condensed chromatin did not display fragmented DNA. No labelling can be seen within blastomeres with normal nuclei.
Figure 2. SEM micrographs of human embryos at day 3 after insemination (magnification 1,200 x).

Fig 2a. Eight-cell stage human embryo with blastomeres of regular size and almost no fragmentation. The smaller cells with smooth surfaces are polar bodies. Bar represents 25 μm.

Fig 2b. Human embryo of the same age and at the same developmental stage with a few normal blastomeres and excessive cellular fragmentation. Fragments are comparably smaller in size than normal blastomeres and fill approximately 1/4 of the total volume of embryo within the zona pellucida.

Figure 3. TEM sections of fragmented human embryos.

Fig 3a. Fragmented human embryo that arrested in development at day 4 after insemination with approximately six normal blastomeres and excessive cellular fragmentation. Arrow indicates remnants of apoptotic blastomere with several apoptotic bodies, some of which are undergoing secondary necrosis. The remaining part of the cell (cell corpse) has heavily condensed cytoplasm (magnification 1,000 x).

Fig 3b. Unphagocytosed cell corpse found in compacting 14 cell embryo with low amount of fragmentation. The corpse had several intact mitochondria present in condensed cytoplasm. Note also slight budding and pinching of the cytoplasm. Most of the cells within this embryo appeared normal. However, a multinucleated blastomere similar to those found in several embryos can be seen (magnification 5,000 x).
Chapter 3.

Effect of conditions of fertilization and maternal age on programmed cell death during murine preimplantation embryo development.

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Running Title: PCD during murine preimplantation embryo development.

Key words: embryo fragmentation, programmed cell death, apoptosis, IVF, maternal age

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3.1. Introduction.

Mammalian preimplantation embryonic development involves a very simple series of cleavage divisions that result in a critical cell mass, allowing the embryo to undergo the first differentiation event - formation of the blastocyst. In comparison with early embryos of other vertebrates, mammalian embryos activate their genomes relatively early, within the first few cleavage divisions. Thus, they appear unique in their ability to regulate very early developmental decisions. However, many mammalian embryos are unable to proceed through preimplantation development, often fragmenting, arresting and dying within a few days post fertilization.

Human embryos cultured in vitro display a high rate of cellular abnormalities (Hardy et al., 1993). Despite the fact that 70% of inseminated oocytes result in 4 to 6 cell stage embryos, only 30%-40% of human embryos cleave regularly to the blastocyst stage in vitro (Plachot and Mandelbaum 1980). The remaining embryos often contain variable-sized blastomeres with multiple cellular fragments. Fragmented embryos have limited developmental potential and rarely result in viable pregnancies (Bolton and Braude 1987; Erenus et al., 1991).

Embryo fragmentation has been observed in several mammalian species and occurs in embryos conceived in vivo as well as in vitro, from both stimulated and unstimulated cycles (Formigli et al., 1990). The morphology of fragmented human embryos together with previously described nuclear abnormalities (Hardy et al., 1993) led us to hypothesize and later confirm (Jurisicova et al., 1995b, 1996) that fragmentation in these embryos is the consequence of programmed cell death (PCD). Often, but not always, this leads to a series of cellular changes termed apoptosis (Kerr et al., 1972). Typical characteristics of apoptosis include nuclear chromatin condensation with subsequent DNA degradation, cellular shrinkage, and the
appearance of multiple cellular fragments which are directly proportional to the size of the dying cell.

PCD results in the elimination of redundant cells as part of normal development in mammalian embryos at the blastocyst stage. The presence of cell death has been observed in blastocysts from different mammalian species obtained in vivo, as well as in embryos produced in vitro (El-Shershaby and Hinchliffe 1974, Mohr and Trounson 1982, Enders and Schlafke, 1981). Although not specifically mentioned in these reports, the morphological descriptions, as well as the published photographs, show typical hallmarks of apoptosis. Cell death at the blastocyst stage mostly affects inner cell mass (ICM) cells, but can be seen occasionally in trophodermal lineage (Hardy et al., 1989, Handyside and Hunter, 1986).

A significant proportion of human embryos fragment and die through apoptosis, suggesting a natural "pre-programmed" response to external stimuli or internal defects. Are these stimuli/defects caused by the IVF process itself or does the high rate of fragmentation reflect a natural predisposition to embryo wastage? If IVF increases the rate of embryo fragmentation, can the triggers responsible for stimulation of PCD be identified and modified? One potential trigger for mammalian embryo fragmentation could be the clinical and laboratory procedures leading to fertilization and cleavage during IVF. Since the majority of women undergoing IVF treatment are middle aged, we examined whether maternal age in the mouse may be a possible contributing factor to embryo fragmentation. The reproductive life span for female mice is strain specific, but it usually lasts up to 12-14 months (Rugh, 1990). We selected our oldest age group (>40 weeks) based on the observation that retired breeders (10-12 months) bear smaller litters and have a decreased average number of implantation sites when compared to younger females (Rugh, 1990). While murine maternal age beyond 40 weeks increased the frequency of early embryo
fragmentation when fertilized in vivo, oocytes from females of all ages had a uniformly high rate of fragmentation when fertilized in vitro. Furthermore, both advanced maternal age and conditions of fertilization adversely affected embryo development to the blastocyst stage. An increase in cell death and a decrease in cell number was confirmed in blastocyst stage embryos conceived during IVF. Our data indicate that factors specific to IVF and independent of maternal age contribute significantly to the high rate of programmed cell death during preimplantation embryo development observed during IVF.

3.2. Materials and Methods.

In vitro Fertilization, embryo retrieval and culture.

Female mice of the outbred CD-1 strain were split into three different groups and aged until they reached 10-12 weeks (n=44), 22-24 weeks (n=15) and 42-44 weeks (n=24) in groups of 4-5 per cage. We used 83 females of which 48 were naturally mated and 35 were subjected to IVF. After reaching the appropriate age, females were stimulated with 5 IU of PMSG (Sigma) followed 48 hours later by 5 IU of hCG (Sigma) and split into two sub-groups. The first group was caged with CD-1 males of proven fertility. Mating was assessed by the presence of a vaginal plug on the following morning (considered as day 0.5). Embryos were recovered by flushing the oviducts at day 1 post mating. The numbers of normal embryos at the 2-cell stage, unfertilized oocytes and fragmented embryos were recorded. Of 821 2-cell stage embryos retrieved from females of all age groups, 279 were subsequently cultured in groups of 10-15 in 1ml of KSOM medium (Erbach et al., 1994) until the embryos reached the fully expanded blastocyst stage (day 4.5). The remaining 2-cell stage embryos were used for other experiments.

In order to compare cell death rates in blastocysts cultured in vitro with naturally occurring cell death rates in blastocysts fertilized and maintained in vivo, 3 young females (10-12
weeks) were hormonally stimulated and mated as described above. Females were then sacrificed at day 3.5 and blastocysts were obtained by flushing uterine horns approximately 92 hours post hCG. These were processed for DAPI/TUNEL analysis (see following section).

The second group of females at each age group was sacrificed at 13 hours post HCG injection and ovulated oocytes were recovered from the oviducts. Spermatozoa from cauda epididimus from CD-1 males of proven fertility were collected and capacitated in modified T6 medium for 1 hour at 37°C. In vitro fertilization was performed as previously described in modified KSOM medium (Summers et al., 1995). One hour post insemination, oocytes were viewed and the number of fragmented and normal appearing oocytes was recorded. Six hours post insemination, oocytes were washed in KSOM, and the frequency of oocyte fragmentation was recorded. Normal appearing oocytes were placed in KSOM and checked for cleavage 24 hours post insemination. Again, the number of 2 cell stage embryos, unfertilized oocytes and fragmented embryos was recorded. The total rate of embryo fragmentation was calculated by summing the number of fragmented embryos observed immediately, 1 hour, 6 hours and 24 hours post insemination. Subsequently, 199 2-cell stage embryos were cultured in groups of 10-15 in 1ml of KSOM medium until they reached the blastocyst stage (day 4.5).

Combined nuclear and fragmented DNA labeling.

To analyze the status of chromatin in fragmented embryos and blastocysts, we used a combined technique for simultaneous nuclear staining and terminal transferase-mediated DNA end labeling (TUNEL) as previously described (Jurisicova et al., 1996). Briefly, embryos were fixed in 4% paraformaldehyde diluted in PBS on microscope slides. After air drying, slides were stored at -70°C until further use. After thawing, slides were washed in PBS and samples were preincubated in 1x One-Phor-All Buffer (Pharmacia, Quebec, Canada) supplemented with 0.1%
Triton X. Afterwards, the samples were overlaid with reaction cocktail which contained One-Phor-All Buffer with 0.1% Triton X, 0.25 U/ml terminal transferase (Pharmacia, Quebec, Canada), 6 • M dATP and 3 • M Bio-dUTP (Sigma, St. Louis, MO). Reactions were carried out in a humidified chamber for 1 hour at 37°C. After washing slides in PBS, incorporated biotinylated nucleotides were detected with streptavidin-Texas red conjugate (Calbiochem, San Diego, CA). To decrease nonspecific binding, conjugate incubation was performed at 4°C for 30 minutes. After three washes in cold PBS, slides were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) at a concentration of 0.2 • g/ml for 10 min and slides were examined and photographed using a Leica fluorescence microscope with appropriate filters.

Assessment of cell death was recorded based on DNA condensation, fragmentation and nuclear morphology as previously described (Jurisicova et al., 1996). The cell death index (CDI) was calculated as the percent of total cells which exhibited intense DAPI staining due to condensation of chromatin, which in mammalian embryos is seen to proceed to DNA fragmentation detected by TUNEL (Jurisicova et al., 1996).

In order to assess whether global DNA damage in the oocytes is a possible trigger of embryo fragmentation, we examined oocytes harvested from 10 week and 44 week old mice 14 hours post hCG. TUNEL was performed as described above, except that the oocytes were pre-incubated in proteinase K (20 • g/ml for 5 min at room temperature) before applying the reaction cocktail.

Statistical Analysis.

Differences between the rate of embryo fragmentation depending on conditions of fertilization or age as well as differences between proportions of embryos developing to blastocyst were compared by chi-square analysis with Yates’ correction. The effect of age and
conditions of fertilization on total cell number and cell death index were analyzed by Two Way
Analysis of Variance and by Kruskal-Wallis One Way Analysis of Variance on Ranks using the
SigmaStat statistical package (Version 1.0, Jandel Corp., Corte Madera, CA)

3.3. Results.

Do conditions of fertilization affect the rate of murine embryo fragmentation?

Many IVF laboratories use mouse embryos as an indicator of laboratory quality and
predictor of embryo culture medium purity. In the majority of cases, 2-cell stage mouse embryos
from non-arresting strains are used. However, these mice do not provide a good model, since
many of them will reach the blastocyst stage even in media of inferior quality. Furthermore, use
of 2-cell stage in vivo fertilized embryos obviates all the problems of early embryo arrest and pre-
selects for embryos of normal morphology. In order to address these concerns, we compared the
frequency of embryo fragmentation during in vivo and in vitro fertilization in all age groups.

Assessment of overall results of embryo morphology at day 1.5 post coitus suggests that
in mouse, as well as in humans, embryo fragmentation is one of the pathways an early embryo
can follow. During normal in vivo embryo development, the majority (79.9%) of fertilized
oocytes successfully cleaved into 2-cell stage embryos (821/1028) and only 13.5% of embryos
were observed to be fragmented (139/1028). However, we noted a significant increase
(P<0.0001) in the frequency of embryo fragmentation during IVF to 32.4% (186/574) with the
proportion of normally cleaving embryos decreasing to 40.1% (230/574). Results are summarized
in Table I.

We also examined the time course of fragmentation during murine IVF. Four percent of
oocytes were fragmented at the time of ovulation and were not used for insemination. These most
likely represent spontaneously activated oocytes. Within one hour post insemination, 35/544
inseminated oocytes (7%) fragmented. The peak level of fragmentation (15%) was observed 6 hours post insemination (e.g. 20 hours post hCG). Subsequently, an additional 11% of embryos fragmented between 6 and 24 hours post insemination (20-38 hours post hCG), when mouse embryos are usually scored for cleavage (see Graph 1.). No additional fragmentation was observed during development from the 2-cell stage onwards. Thus, fragmentation in mouse embryos occurs during the first cell cycle (e.g. between activation of the oocyte and the 2-cell stage). None of the fragmented embryos proceeded in development because the whole embryo was compromised.

Table I. Effect of maternal age and conditions of fertilization on early murine embryo fragmentation.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>In vivo Fertilization</th>
<th>In vitro Fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 cell (%)</td>
<td>Fragmented (%)</td>
</tr>
<tr>
<td>10-12</td>
<td>599 (82)</td>
<td>86 (12)*#</td>
</tr>
<tr>
<td>22-24</td>
<td>153 (78)</td>
<td>25 (13)</td>
</tr>
<tr>
<td>42-44</td>
<td>69 (71)</td>
<td>28 (29)#</td>
</tr>
</tbody>
</table>

Chi Square test: *P<0.0001 for comparison of embryo fragmentation frequency depending on conditions of fertilization and #P=0.001 for comparison of embryo fragmentation frequency depending on maternal age.

We observed that in mouse zygotes cytoplasmic fragmentation occurs prior to DNA disintegration, since we observed 4 zygotes with two intact pronuclei one of the cellular fragments (Fig 1.A.) and 2 zygotes with two distinct chromosomal sets in which cytoplasmic fragmentation had occurred before formation of pronuclei. However, in the majority (52/58) of fragmented embryos abnormal DNA morphology and subsequent destruction of nuclear material, consistent with apoptosis was observed. Subsequently, the DNA completely disintegrated and nuclear material remained scattered within the cytoplasm of the largest fragment (Fig 1.B.). This
observation corroborated our previous findings in fragmented human embryos (Jurisicova et al., 1996).

**Does age contribute to the increased rates of embryo fragmentation in vivo?**

To assess the effect of maternal age on murine embryo fragmentation and further development to the blastocyst stage, we decided to use three different age groups. Young females (10-12w) each ovulated an average of 23 oocytes, of which 81.5% (599/735) cleaved into 2-cell stage embryos after natural mating. The baseline level of embryo fragmentation at day 1.5 (36-38 hours post hCG) in this age group was 11.7% (86/735), confirming that embryo fragmentation does happen naturally in vivo. Middle aged mice (22-24 weeks) ovulated fewer oocytes (an average of 18 per female), but the rate of embryo cleavage to 2-cell stage and the rate of fragmentation in vivo remained the same as observed in young females (78% and 13% respectively). Interestingly, the oldest group of females (42-44 weeks) showed a decrease in the number of ovulated oocytes (average of 15 per female) accompanied by a significant increase (P=0.001) in the frequency of embryo fragmentation to 29% (28/97).

In order to assess whether global DNA damage could be responsible for increased frequency of cellular fragmentation in aged females in vivo, we performed TUNEL on oocytes retrieved from two 10 week and two 44 week old females 14 hours post hCG. No labeling could be observed on the chromosomal plate of oocytes arrested in MII from either age group (data not shown). Frequently (in 35/58 oocytes), the first polar body labeled positively for TUNEL, confirming that this cellular structure undergoes apoptosis within two hours post ovulation. Thus, it appears that maternal age may be responsible for inferior embryo quality, independent of oocyte DNA fragmentation prior to fertilization.

**Does maternal age and IVF affect PCD during murine blastocyst formation?**
Subsets of 2-cell stage embryos obtained by IVF or by \textit{in vivo} fertilization were subsequently cultured in KSOM medium until they reached the fully expanded blastocyst stage, which occurred at day 4.5 of \textit{in vitro} embryo development. Because there was no difference in the rate of embryo fragmentation in 10-12 and 22-24 weeks old mothers (young and middle aged females, respectively), the data for these groups were pooled together for statistical analysis. The overall rate of blastocyst formation was unaffected by maternal age (See Table II.).

\textbf{Table II.} The frequency of blastocyst formation at day 4.5.

<table>
<thead>
<tr>
<th>Maternal age (weeks)</th>
<th>In vivo Fertilization</th>
<th>2-cell embryos cultured</th>
<th>Blastocysts</th>
<th>(%) *</th>
<th>Maternal age (weeks)</th>
<th>In vitro Fertilization</th>
<th>2-cell embryos cultured</th>
<th>Blastocysts</th>
<th>(%) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-24</td>
<td>210</td>
<td>184</td>
<td>88</td>
<td></td>
<td>10-12</td>
<td>135</td>
<td>85</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>42-44</td>
<td>69</td>
<td>59</td>
<td>86</td>
<td></td>
<td>42-44</td>
<td>64</td>
<td>43</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

*Chi square test with \(P<0.0001\)

IVF significantly reduced the rate of blastocyst formation when compared with the ability of \textit{in vivo} conceived embryos to form blastocysts \textit{in vitro} (65\% versus 87\% respectively, \(P<0.0001\)). Furthermore, the mean cell number at day 4.5 in the \textit{in vivo} conceived embryos was significantly higher in both age groups, approximately 84 cells per blastocyst, in comparison with the IVF produced embryos, which had approximately 50 cells per blastocyst (See Table III).

The cell death index for both \textit{in vivo} and \textit{in vitro}-conceived embryos, as determined by the proportion of apoptotic cells shown by DAPI/TUNEL positive staining, was significantly affected by maternal age, with more apoptotic cells found in blastocysts originating from oocytes from older mothers (\(P=0.005\)). Furthermore, embryos conceived \textit{in vitro} had a significantly elevated cell death index - to about 10.5 \% for \textit{in vitro} conceived embryos (all age groups) as
compared to 4.5% for *in vivo* conceived embryos, P<0.0001. Cell death appeared random in most of the embryos (see Fig 2. A/B) with dying cells scattered in both the inner cell mass (ICM) and trophectoderm. Thus, conditions of fertilization and maternal age affect both cell number and susceptibility of cells within the blastocyst to undergo PCD. We also determined cell death rates in 25 blastocysts obtained from normal mating at day 3.5 and compared them with *in vivo* fertilized, but *in vitro* cultured blastocysts. No statistical difference was detected in mean cell number, though the cell death index was slightly elevated in blastocysts cultured *in vitro*, almost reaching statistical significance (Mann-Whitney Rank Sum test P=0.06).

**Table III.** The cell number and cell death index in murine blastocysts.

<table>
<thead>
<tr>
<th>Age / weeks</th>
<th>Embryos analyzed</th>
<th>Fertil.</th>
<th><em>In vitro</em> Culture</th>
<th>Day</th>
<th>Cells per blastocyst</th>
<th>Cells with conden. DNA</th>
<th>Cells with Fragm. DNA</th>
<th>CDI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-12</td>
<td>25</td>
<td><em>in vivo</em></td>
<td>No</td>
<td>3.5</td>
<td>65±3</td>
<td>2.1±0.5</td>
<td>1.6±0.5</td>
<td>3±0.8</td>
</tr>
<tr>
<td>10-12</td>
<td>11</td>
<td><em>in vivo</em></td>
<td>Yes</td>
<td>3.5</td>
<td>60±2</td>
<td>3.4±0.8</td>
<td>2.1±0.4</td>
<td>5.4±1.2</td>
</tr>
<tr>
<td>10-24</td>
<td>34</td>
<td><em>in vivo</em></td>
<td>Yes</td>
<td>4.5</td>
<td>85±2</td>
<td>2.6±0.3</td>
<td>2.6±0.4</td>
<td>3.2±0.5</td>
</tr>
<tr>
<td>10-24</td>
<td>19</td>
<td>IVF</td>
<td>Yes</td>
<td>4.5</td>
<td>52±3</td>
<td>4.6±0.6</td>
<td>2.8±0.6</td>
<td>9.1±1.1</td>
</tr>
<tr>
<td>42-44</td>
<td>15</td>
<td><em>in vivo</em></td>
<td>Yes</td>
<td>4.5</td>
<td>83±7</td>
<td>4.5±0.8</td>
<td>3.1±0.6</td>
<td>6.2±1.5</td>
</tr>
<tr>
<td>42-44</td>
<td>17</td>
<td>IVF</td>
<td>Yes</td>
<td>4.5</td>
<td>49±6</td>
<td>5.5±0.9</td>
<td>4.8±0.8</td>
<td>12.5±1.7</td>
</tr>
</tbody>
</table>

Cell #, DAPI, TUNEL and CDI are shown as mean ± standard error. CDI is significantly increased with maternal age (P=0.005) and during IVF (P<0.0001).

### 3.4. Discussion.

An underlying purpose behind the experiments described above was to find a mouse model that mimics the degree of cellular fragmentation observed in humans. Even though murine IVF is one of the most widely used techniques to study early events during and after fertilization, very little information is available on the rate of *in vivo* and *in vitro* embryo fragmentation. We have previously shown that extensively fragmented human embryos displayed several hallmarks of apoptosis (Jurisicova *et al.* 1996). In this report we confirm that the same mechanism exists in
murine embryos.

There are two known triggers of rodent oocyte/early embryo fragmentation published in the literature so far. The first is lack of maternal c-Mos, resulting from targeted disruption of the c-mos gene. Oocytes produced by c-mos -/- mothers fail to arrest at MII, activate spontaneously and subsequently undergo fragmentation within 32 hours post hCG (Colledge et al., 1994). The second trigger is fertilization by males treated with 5-aza-cytidine, which interferes with proper DNA methylation. Treated male rats sired embryos that fragmented within 24 hours post fertilization (Doerksen and Trasler, 1996). These observations and those reported here indicate that rodent embryos undergo fragmentation between activation of the oocyte and the early 2-cell stage. This timing is slightly different from that of fragmentation in human embryos, which occurs between the 2- to 8-cell stage (A. Jurisicova and J. Meriano, unpublished observation). However, rodent embryos activate their genomes at the 2-cell stage (Flach et al., 1982), while human embryos activate the embryonic genome at the 4-8 cell stage (Braude et al., 1988). Therefore, in both rodents and humans, PCD and the resulting fragmentation may slightly precede the major activation of the embryonic genome.

Controlled ovarian stimulation, used to obtain more oocytes, may lead to an abnormal follicular response to administered gonadotropin, resulting in abnormal "biochemical maturity" of the oocytes. Embryos arising from such oocytes may not be able to proceed through development if, for example, their PCD "machinery" (e.g. accumulation of or deregulated expression of products belonging to Bcl-2 and ICE gene families) is unbalanced due to abnormal deposition of maternally stored products. This phenomenon could be especially pronounced with increased maternal age, where "cytoplasmic aging" rather than global DNA damage could explain higher rates of embryo fragmentation. We observed an increased frequency of embryo
fragmentation resulting from \textit{in vivo} mating of aged females that could not be attributed to global DNA damage, since unfertilized oocytes were negative for TUNEL staining. This observation contradicts the findings of Fujino \textit{et al.}, (1996), who reported increased rates of DNA fragmentation in oocytes obtained from aged females. However, since these authors did not assess the rate of oocyte cytoplasmic fragmentation in old mice, it is difficult to establish whether or not the DNA fragmentation observed in their study was related to apoptosis.

We observed a 2-fold higher frequency of fragmentation in IVF embryos compared with \textit{in vivo} fertilized embryos in samples from young and "middle-aged" females. One possible explanation for increased frequency of murine embryo fragmentation is the artificial environment for fertilization and cleavage utilized during IVF. Sub-optimal culture conditions as well as prolonged exposure to high concentrations of sperm may both contribute to excessive activation of PCD. Previous work has suggested that both the number of sperm and the time of exposure of the oocyte to sperm \textit{in vitro} may have a negative effect on embryo development and subsequent pregnancy rates in humans (Dumoulin \textit{et al.}, 1992). We hypothesize that prolonged exposure to a large number of sperm may expose the oocyte to oxidative stress generated by the sperm suspension. Mouse oocytes and embryos on their own are able to generate small but measurable amounts of H$_2$O$_2$ which increase during the transition from the 2 to 4 cell stage (Nasr-Eshafani \textit{et al.}, 1990). However, the elevated levels of hydrogen peroxide were detected in 2-4 cell stage embryos, and murine embryo fragmentation occurs in the late 1 cell stage, it is unlikely that this factor is a trigger of early embryo fragmentation.

PCD is a part of normal mammalian embryo development at the blastocyst stage including human (Hardy \textit{et al.}, 1989). Most reports have found that cell death in blastocysts from different species is most predominant in ICM cells and could be seen occasionally in the
trophectodermal lineage (Hardy et al., 1989, Brison and Schultz, 1997). Based on our observation of condensed and fragmented nuclei in trophoderm cells (not just the presence of cell corpses phagocytosed by trophoderm), apoptosis occurs frequently in the trophoderm lineage in mouse. Our results on cell death in murine blastocysts are comparable to those reported by Handyside and Hunter (1986), who assessed blastocysts obtained from in vivo mating. These authors reported a peak level of apoptosis in blastocysts 97 hours post coitus (e.g. 109 hours post hCG injection), with cell death restricted predominantly to ICM cells. In agreement with the findings of Handyside and Hunter, we could detect no apoptosis in earlier developmental stages (4 cell - morula). Recently, Brison and Schultz (1997) reported an increased cell death index in blastocysts cultured separately, compared to blastocysts cultured in groups. Furthermore, cell death could be decreased by the addition of TGF-α to the culture medium, suggesting that this growth factor may be one of several survival signals required by the developing embryo. It would be of interest to know whether TGF-α can also suppress cell death in blastocysts from older mothers.

It is well known that increased maternal age has a negative impact on fertility. The responsible factor seems to be oocyte aging, since oocyte donation to older patients results in very similar implantation rates to those in younger patients (Pellicer et al., 1995). Recently, Lim and Tsakok (1997) reported a highly significant increase in the rate of sister chromatid separation in oocyte chromosomes in older patients. It has been proposed that an increased rate of chromosomal and/or spindle abnormalities, possibly triggered by follicular hypoxia (Gaulden, 1992; Van Blerkom et al., 1997), may be responsible for the decreased developmental potential of oocytes from women over 40. We would like to extend this hypothesis further by suggesting that with increased age, granulosa/cumulus cells are not able to supply growing oocytes with
appropriate quantities of maternally stored products (e.g. proteins and mRNAs), which subsequently leads to increased PCD in both embryos and blastocysts.
3.5. Legend to Figures.

**Graph 1.** Time course of murine embryo fragmentation during *in vitro* fertilization.
Figure 1. Fragmented murine embryo stained with DAPI. DAPI/TUNEL staining on the majority of fragmented murine embryos revealed abnormal DNA morphology and subsequent destruction of nuclear material, consistent with apoptosis. Fig.1/A illustrates a fragmented zygote with two intact overlapping pronuclei marked by arrows. The presence of normal pronuclei in embryos with complete cytoplasmic fragmentation suggests that cytoplasmic fragmentation occurs prior to DNA digestion. Condensed DNA was still visible in the remaining polar body. Subsequently, the DNA completely disintegrated and nuclear material remained scattered within the cytoplasm of the largest fragment indicated by arrow (Fig 1B).

Figure 2. Expanded mouse blastocyst conceived by in vitro fertilization of an oocyte obtained from a 44 week old female, cultured in vitro till day 4.5 and analyzed for apoptotic cells by assessing chromatin status and fragmentation of DNA. 2.A/ The embryo was stained with the nuclear dye diamidino-phenolindol (DAPI). Normal nuclei have pale blue staining in contrast to nuclei with condensed chromatin showing bright white staining (for example see arrows) 2.B/. The same embryo after Terminal transferase-mediated DNA end labelling (TUNEL) using biotynilated nucleotides and streptavidin - Texas Red conjugate. Many cells that showed an intense DAPI signal are also labeled with TUNEL as indicated by the bright red fluorescence. Cell death appeared random in most of the murine blastocysts with dying cells scattered throughout both the inner cell mass (ICM) and trophectoderm.
Chapter 4.

Expression and regulation of genes, associated with cell death, during murine preimplantation embryo development.

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Running title: Apoptosis and preimplantation embryo development

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4.1. Introduction.

Progression of fertilized mammalian oocytes through early cleavage stages, blastocyst formation, and implantation depends on successful implementation of genetic and developmental programs, and on successful interaction of the pre-implantation embryo with its environment. Zygote survival depends heavily on maternal mRNAs and proteins deposited and stored in the oocyte prior to its ovulation. Most of these products will be used within the first few cleavage divisions at which point the preimplantation embryo must support its own development by transcriptional activation of its genome. The timing of embryonic genome activation (EGA) varies slightly among different mammalian species. In the mouse, a major activation event occurs during the 2-cell stage (Flach et al., 1982), while in humans, swine, equine and cats, a major activation event occurs between the 4 and 8 cell stages (Braude et al., 1988, Jarell et al. 1991, Brinsko et al., 1995, Hoffert et al., 1997) and between 8-16 cells stage in cattle and sheep (Telford et al., 1990). In all species, however, some transcription occurs well in advance of the major activation event, and this is observed even during the 1-cell stage in mouse (Clegg and Piko 1982; Latham et al., 1991; Matsumoto et al., 1994; Cao et al., 1995; Buinol et al., 1995). A recent report by Aoki et al., (1997) indicated that transcription in late 1-cell stage reaches approximately 20% of total transcription at the late 2-cell stage. The purpose of this early phase of genome activation has not been established, but may serve essential functions, such as promoting the broader transcriptional activation of the genome or promoting early embryo survival.

One possible function that may be served by the early phase of genome activation could be the prevention of programmed cell death. The ovulated egg, if not fertilized, is destined to undergo cell death. Egg activation following fertilization and the concomitant progression of early developmental events leading up to EGA may collectively lead to the production of
molecules that inhibit cell death. Evidence in support of this hypothesis has been obtained in both human and mouse embryos. We observed previously that embryos of the outbred mouse CD-1 strain undergo fragmentation and apoptosis, and that the rate of embryo fragmentation changes with maternal age and fertilization conditions (Jurisicova et al., 1998). Murine embryos undergo PCD during the blastocyst stage as a normal part of development (Mohr and Trounson 1982; Handyside and Hunter 1986; Brison and Schultz, 1997; Jurisicova et al., 1998). PCD at the blastocyst stage probably serves to eliminate inner cell mass cells that retain trophectodermal potential (Pierce et al., 1989). A majority of human preimplantation embryos exhibit extensive cellular fragmentation occurring most frequently between 2- to 8-cell stage. The morphology of fragmented human embryos together with previously described nuclear abnormalities (Hardy et al., 1993), led us to hypothesize (Jurisicova et al., 1995b) and later confirm (Jurisicova et al., 1996) that fragmentation in these embryos is the consequence of programmed cell death (PCD). We now report that murine embryos may also undergo fragmentation during the period between egg activation and the early 2-cell stage. Thus, in both rodents and humans, PCD and the resulting fragmentation may occur during the period immediately preceding the major activation of the embryonic genome. We have examined the patterns of expression of cell death protector and/or killer genes during murine preimplantation development. With one exception, all of the investigated genes could easily be detected throughout preimplantation development, with stage-specific changes in expression. We found that the expression of some of these genes is altered in embryos undergoing PCD during the 1-cell stage. We propose that PCD is the default outcome for early embryogenesis unless the embryo is of adequate constitution to prevent PCD through the expression of an appropriate array of protector functions. PCD would provide an important mechanism for the early elimination of unfit embryos, thus conserving maternal
resources.

4.2. Materials and methods.

Frequency of early embryo fragmentation.

The frequency of early embryo fragmentation for different genetic backgrounds was determined by isolating embryos from superovulated (5 IU each of PMSG and hCG administered 48 hours apart) females of CD-1, [C57BL/6 and (B6D2)]F1 mouse strains. Females were mated with males of CD-1, C57BL/6 or (B6D2)F1 strain. Embryos were retrieved by flushing the oviducts at 22h post-hCG and their morphology was characterized by light microscopy as either normal (one cell with one or two polar bodies) or fragmented. Immature oocytes and unfertilized oocytes were excluded from the analysis.

Expression of cell death regulatory genes during preimplantation development.

Germinal vesicle-intact oocytes, ovulated eggs, and fertilized embryos from the 1-cell through blastocyst stages were analyzed for expression of nine genes associated with apoptosis in somatic cells. Oocytes, eggs, and embryos were obtained from adult superovulated (B6D2)F1 female mice, and for embryos these were mated to (B6D2)F1 males as previously described (Rambhatla et al., 1995). Samples of isolated inner cell mass cells, obtained by immunosurgery (Solter and Knowles, 1975) were also included. Between three and six samples, each containing 8-10 oocytes or embryos, were obtained and analyzed for each stage.

The patterns of expression of the nine genes were determined by a quantitative PCR-based assay involving the amplification of the 3' termini of the entire mRNA population followed by quantitative dot blot hybridization (Rambhatla et al., 1995). Estimates of mRNA copy number were calculated as described (Rambhatla et al., 1995). The cDNA probes used for analysis contained 3' untranslated region sequences. cDNA clones were donated by S. Korsmeyer.
(Bcl-2, Bad), S. Cory (Bcl-w) and K. Shibahara (MA-3) or were cloned from an oligo dT primed extra embryonic ectoderm cDNA library from d7.5 murine embryos (Mann et al., 1995) using probes recognizing the coding regions of Bcl-x, Bax, p53, and Ttg. The identities of cDNA clones were confirmed by DNA sequencing. The caspase-1 probe was obtained by RT-PCR of RNA isolated from embryonic stem cells using primer sequences based on the murine ICE cDNA sequence, corresponding to 516-537bp and 1244-1268bp respectively (Cerretti et al., 1992; Acc.#: L03799): 5' primer ttcaacatctttctccgagg, 3' primer cttcttatggcagattctagc. The Casp-1 primers span part of the coding region and the 3' untranslated region. The identity of the Casp-1 cDNA was also confirmed by sequencing.

Alternative Splicing of Bcl-x.

In order to determine which form of Bcl-x is expressed during murine early preimplantation development we performed RT-PCR with specific primers spanning the alternative splice site of the Bcl-x gene, as previously described (Boise et al., 1993). Briefly, five mature oocytes or normal zygotes were lysed in 50μl of G1TC solution (Brady and Iscove 1993). Total nucleic acid was recovered by ethanol precipitation using glycogen as a carrier. The reverse transcriptase reaction was performed with an oligo dT primer using Superscript II (Gibco BRL) according to the manufacturers protocol. One fifth of the cDNA product was amplified using Taq polymerase cocktail (Gibco BRL, Grand Island, NY). Primers used for amplification were designed according to the murine Bcl-xL sequence: 5' tgggacaatggcactgttga, and 3'gtgagttgagctcagttg (Gonzales-Garcia et al., Acc. #: L35049). Each PCR cycle consisted of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for 1.5 minutes. The expected sizes for the amplified products were 760 bp for Bcl-xL and 550 bp for Bcl-xS. To confirm the identity of amplified products, Southern blot analysis was performed.
with a radiolabelled cDNA probe recognizing the shared coding region of both \textit{Bcl-xL} and \textit{Bcl-xS}. This experiment was repeated several times with seven sets of samples obtained on different occasions.

4.3. Results.

\textit{Genetic background and the frequency of early embryo fragmentation.}

Previous studies indicated that PCD in the preimplantation mouse embryo was limited to the blastocyst stage. The observation of PCD in early human embryos prompted us to examine 1-cell stage mouse embryos of different mouse strains to ascertain whether PCD might occur during the 1-cell stage in embryos from mothers of different genetic backgrounds. (B6D2)F1 intercrosses produced only approximately 5\% fragmented embryos (Table 1). In contrast, about 10\% of zygotes obtained from females of the outbred CD-1 strain showed distinct signs of cellular fragmentation. This frequency of fragmentation is consistent with the frequency of embryo fragmentation previously observed in our laboratory on this genetic background (Jurisicova et al., 1998).

Table 1. Frequency of early embryo fragmentation.

<table>
<thead>
<tr>
<th>Strain FxM</th>
<th>Normal zygotes</th>
<th>Fragmented</th>
<th>(%) Fragmented</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6D2xB6D2 (n=3)</td>
<td>125</td>
<td>7</td>
<td>5.3</td>
</tr>
<tr>
<td>CD-1xCD-1 (n=6)</td>
<td>150</td>
<td>17</td>
<td>10.2</td>
</tr>
<tr>
<td>C57BL6xC57BL6 (n=5)</td>
<td>108</td>
<td>15</td>
<td>12.2</td>
</tr>
<tr>
<td>C57BL6xCD-1 (n=7)</td>
<td>77</td>
<td>25</td>
<td>24.5</td>
</tr>
</tbody>
</table>

\(n = \text{number of females plugged}\)

Embryos from females of the inbred C57BL6 strain mated with C57BL6 males exhibited
a similar frequency of fragmentation (12.2%). Interestingly, when C57BL6 females were mated to CD1 males, the frequency of fragmentation doubled to about 24%. Thus, embryo fragmentation can occur at a significant frequency among mouse embryos, and that frequency is affected by the genotype of both the mother and father or interaction between maternal and paternal genotypes.

Expression of genes associated with PCD during normal murine embryo development.

Our finding that mouse embryos of certain genetic compositions may undergo fragmentation reminiscent of PCD during the 1-cell stage indicated that this might be a normal part of early murine development. If so, the mouse provides a valuable model with which to study the molecular and genetic control of PCD in preimplantation embryos of other species, including humans. Previous studies indicated that apoptosis in mouse embryos at the blastocyst stage allows the mouse to serve as a model for investigating the control of PCD during inner cell mass formation (Pierce et al., 1989, Brison and Schultz, 1997). It was therefore of interest to learn which of the genes that are known to regulate cell death are expressed during preimplantation development. To meet this objective, we examined the temporal expression patterns of nine cell death regulatory genes, divided among three groups: cell death repressors, cell death inducers, and executors of cell death.

Cell Death Suppressors: Bcl-2, Bcl-w and Bcl-xL.

All three of the cell death suppressors analyzed in this study were expressed at all stages of development from germinal vesicle stage and unfertilized oocytes to later preimplantation stages (Fig. 1) in B6D2 mice. Bcl-2 and Bcl-w (Gibson et al., 1996) mRNAs were far less abundant than the Bcl-x mRNA, especially during early embryonic stages, making Bcl-x the most
likely candidate for inhibiting early embryonic cell death. There was an apparent increase in the abundance of the \textit{Bcl-x} mRNA at 29 hours post-hCG. Treatment of embryos with \( \alpha \)-amanitin resulted in only slightly lower \textit{Bcl-x} mRNA abundance (19\% reduced; Table II). This indicates that a small amount of \textit{Bcl-x} mRNA may be transcribed by the embryonic genome during the 1-cell stage but most of the \textit{Bcl-x} mRNA detected is of maternal origin. The apparent increase in abundance could result from either preferential stabilization of the \textit{Bcl-x} mRNA on a background of overall maternal mRNA degradation or possibly enhanced polyadenylation as suggested for other genes (Rambhatla \textit{et al.}, 1995).

In addition to a small amount of \textit{Bcl-x} gene transcription during the 1-cell stage, a small amount of \textit{Bcl-2} transcription also occurred as evidenced by the slight reduction of expression in \( \alpha \)-amanitin treated embryos during the 2-cell stage (Table II). De novo transcription of \textit{Bcl-2} could be detected by the late 2-cell stage, where \( \alpha \)-amanitin treatment reduced expression. In the blastocyst, \textit{Bcl-2} mRNA was predominantly expressed in the ICM. Maternal \textit{Bcl-w} mRNA persisted through the late 2-cell stage. The \textit{Bcl-w} gene was expressed at a comparatively constant, low abundance (as a fraction of the total mRNA content) throughout the remainder of preimplantation development.

The experiments described above for \textit{Bcl-x} reveal total \textit{Bcl-x} polyA mRNA expression based on hybridization to 3’ untranslated region probes, and therefore fail to distinguish between different splicing products resulting from alternative exon/intron splicing within the coding region of the \textit{Bcl-x} gene. Two alternatively spliced forms of the \textit{Bcl-x} mRNA exist. One form, \textit{Bcl-xS}, makes the cell more susceptible to apoptotic signals, while the other, \textit{Bcl-xL} functions as a protector against apoptosis (Boise \textit{et al.}, 1994). In order to determine the relative contributions of \textit{Bcl-xL} and \textit{Bcl-xS} to expression in the early embryo, we analyzed mature oocytes, zygotes at
22h and 29h, and late 2-cell stage embryos 42 hours post hCG by RT-PCR using the above diagnostic primers. In all samples of normal oocytes and embryos only Bcl-xL mRNA could be detected, represented by amplification of a 760 bp fragment (Fig. 3).

Expression of Cell Death Inducers: Bax and Bad

Because cell death in many somatic systems is based on the balance of cell protectors and killers, we also analyzed the expression patterns of Bax and Bad mRNAs. Both mRNAs were expressed as maternal transcripts, most abundantly in GV stage oocytes and less so in mature oocytes (Fig 3). Transcription from the embryonic genome for Bax occurred at the late 2-cell stage, being reduced by α-amanitin treatment (Table II). Bax expression then showed a slow, but steady, increase through the blastocyst stage. Bad mRNA expression mimicked that of Bax, except that the upregulation from the embryonic genome began at the 8-cell stage, again reaching a maximum at the blastocyst stage (Fig. 3). Interestingly, Bad expression in ICM cells was one-fifth than that observed in whole blastocysts, suggesting that trophectodermal cells are the principal contributors of Bad mRNA in blastocysts.


The last group of genes analyzed included the DNA-binding protein p53, MA-3 (function unknown), Caspase-1 (ICE), and Tissue transglutaminase (Ttg), which is involved in crosslinking of cytoplasmic proteins during cellular budding associated with apoptosis (for review see Fesus et al., 1991). Caspase 1 (ICE) was not detected during any developmental stage. This was the only cell death regulatory mRNA that was neither actively transcribed by early embryos nor present as a maternal transcript. The Ttg mRNA was expressed first as a maternal mRNA, which declined in abundance during the 2-cell stage. The Ttg mRNA increased in abundance from the 8-cell stage onwards, reaching a maximum at the blastocyst stage (Fig. 4). P53, a well known
inducer of cell cycle arrest and/or apoptosis in cells with damaged DNA (Kastan et al., 1991, Guiloff et al., 1995), was expressed abundantly in mature oocytes, and throughout the one cell stage (Fig. 4). Interestingly, p53 expression was α-amanitin sensitive and wholly attributable to embryonic transcription at 49h post hCG (i.e., late 2-cell stage), indicating transcription of the p53 gene by this time (Table II). p53 expression in the blastocyst was 2.5 fold higher in ICM relative to the whole blastocyst. The MA-3 gene, a novel murine gene of unknown function but commonly upregulated upon induction of cell death (Shibahara et al. 1995), was expressed abundantly in oocytes and in embryos through the two cell stage. Maternal MA-3 mRNA apparently persisted beyond the two cell stage, given its insensitivity to α-amanitin treatment. Embryonic MA-3 expression appeared to be induced at the 8-cell stage, reaching a maximum at the blastocyst stage (Fig 4.).

Table II. Effect of α-amanitin on expression of cell death regulatory genes in murine embryos at late 1-cell and 2-cell stage.

<table>
<thead>
<tr>
<th>Time</th>
<th>29 h/post hCG</th>
<th>32h/post hCG</th>
<th>39h/post hCG</th>
<th>49h/post hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Normal treated</td>
<td>Normal treated</td>
<td>normal treated</td>
<td>Normal treated</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>19±2 12±2 11±2 12±1 7±2 12±3 19±2 2±2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-w</td>
<td>38±3 28±3 25±2 26±6 27±5 22±5 27±3 21±2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-x</td>
<td>303±24 247±9 275±13 195±19 182±72 236±25 74±6 172±86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>18±3 21±8 12±2 5±1 20±9 16±6 20±10 2±2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bad</td>
<td>12±2 7±1 8±2 11±1 6±1 7±1 13±1 10±1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>396±96 516±72 334±65 140±95 55±44 96±52 132±65 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA-3</td>
<td>464±151 388±62 568±32 597±74 511±185 528±92 164±43 166±48</td>
<td></td>
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<tr>
<td>Ttg</td>
<td>41±3 41±3 46±6 32±8 31±4 30±6 37±4 22±5</td>
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Values in Table are shown as mean Cpm bound ± SEM.

Expression of selected cell death genes in fragmented murine embryos.

The expression data described above indicate that the newly fertilized embryo expresses a number of mRNAs encoding proteins that promote cell death. Maternal transcripts of cell
protector genes were also expressed, and some evidence for de novo transcription of at least two genes and possibly increased transcript utilization mediated by increased polyadenylation was also obtained. These results suggest that the balance of expression of these two classes of mRNAs may regulate cell death during the 1-cell stage, and that the embryo fragmentation we observe in certain strains of mice may be attributable to a failure to achieve a balance of expression that favors embryo survival (for results see Fig.5.). To test this possibility, we examined expression of these cell death regulatory genes in embryos undergoing fragmentation. For these studies, we used embryos from C57BL/6 mothers mated to C57BL/6 males, and assayed expression of both fragmenting and unfragmented embryos at 22 and 29 hours post-hCG. Each sample contained 5-10 embryos, and each developmental stage was represented by two samples.

For most of the genes, the pattern of expression in C57BL/6 embryos was very similar to that in the developmental panel derived from (B6D2)x(B6D2) embryos (see above). Fragmented embryos at both 22 and 29 hours post hCG, however, often failed to yield good quality cDNA (15/17 samples), as judged by the strength of hybridization to the constitutively expressed EF-1α mRNA, probably due to degradation of mRNA during cell death. The two samples that produced cDNA that hybridized strongly with the EF-1α cDNA (both from 29 hours post hCG) were used for further analysis and compared with oocytes, and unfragmented mid-, and late 1-cell stage zygotes (Fig. 5). No detectable Bcl-2 expression was observed in unfragmented C57BL/6 oocytes and zygotes at 22h post hCG. Both unfragmented samples at 29 hours exhibited low, but readily detectable Bcl-2 expression, while fragmented embryo samples had barely detectable amounts of Bcl-2 mRNA, much lower than normal zygotes at 29h post.

One transcript more highly expressed in fragmented embryos was MA-3. p53 mRNA
abundance was also slightly elevated in one of the two samples of fragmented embryos when compared to normal embryos at 22 and 29 hours post hCG. The fragmented embryo sample that showed elevated expression of p53 mRNA also showed elevated expression of Bax mRNA (Fig. 5). Bad mRNA was consistently elevated in both fragmented embryo samples (Fig. 5).

Fragmented embryos also showed altered expression of Bcl-x mRNA when diagnostic PCR primers to detect Bcl-xL and Bcl-xS transcripts were used (Fig. 3, lane 2). For these studies, a separate set of samples from those used above was analyzed. In two out of seven samples of fragmented embryos harvested at 22 hours post hCG, an additional band of approximately 550 bp was observed. Amplified 550 bp fragment hybridized with the Bcl-x cDNA probe and its size was indicative of Bcl-xS mRNA. This contrasted with the expression of Bcl-xL alone in unfragmented embryos (Fig. 3). In the remaining pools of fragmented embryos, three had only Bcl-xL and two produced no Bcl-x fragment. With the latter two samples, attempts to amplify mRNA for protein phosphatase 1γ, which is known to be expressed in the early embryo (Mann et al., 1995, Jurisicova unpublished) also failed (data not shown) indicating that the mRNA in these samples was most likely degraded.

4.4. Discussion.

We have demonstrated that programmed cell death can occur during the 1-cell stage of mouse embryogenesis, and that this is dependent upon the genetic background. This provides a valuable model for examining the molecular and genetic control of PCD in human embryos, which occurs at a very high frequency (Erenus et al., 1991, Jurisicova et al., 1996). We have also demonstrated for the first time the temporal expression patterns of nine cell death regulatory
genes, and shown that the expression of these genes is altered in embryos undergoing fragmentation, so that expression of genes involved in cell death (MA-3, p53, Bad, Bax, and Bcl-xS) is elevated and expression of genes involved in cell survival (Bcl-2) is reduced. By contrast, normal embryos exhibit a detectable level of Bcl-2 transcription, which augments maternally derived stores of Bcl-2, Bcl-xL, and Bcl-w mRNAs, thus promoting survival. Moreover, normal embryos do not express the Bcl-xS transcript.

We propose that PCD may occur by default in embryos that fail to execute essential developmental events during the first cell cycle. Such events could obviously include a long list of possibilities, but among the more interesting ones would be completion of S phase, proper recruitment of maternal mRNAs, and onset of the early phase of gene transcription, all of which could be mechanistically coupled. The array of macromolecules with which a given oocyte is endowed initially could obviously affect any of these processes, so that less fit oocytes would tend to be eliminated, thus avoiding wastage of maternal resources in promoting the continued growth of embryos that may be developmentally compromised. The intensive studies in recent years directed toward the ways to identify human oocytes that possess the greatest developmental potential (Van Blerkom et al., 1995) and the numerous studies in mice revealing genetic differences in oocyte quality and sensitivity to in vitro culture conditions (Suzuki et al., 1996) collectively support the view that a great deal of variability in oocyte quality may exist under normal circumstances, so that the existence of a mechanism to promote the early elimination of embryos derived from such oocytes could be advantageous.

Data from studies undertaken in other systems support a model in which PCD must be actively prevented in the early embryo. Recently, a similar hypothesis was published by Stack and Newport (1997) regarding cell death during Xenopus development. Based on their
observations, treatment of early cleaving *Xenopus* embryos with hydroxyurea and cycloheximide, prior to but not after initiation of zygotic transcription, resulted in widespread apoptosis. These authors also showed that apoptosis is actively suppressed by some maternal inhibitors that are degraded during the maternal-zygotic transitional period, and that must then be re-expressed from the zygotic genome (Stack and Newport, 1997). Similar observations were reported recently by Sible *et al.*, (1997). These authors proposed the existence of a maternally controlled developmental checkpoint that can trigger an apoptotic response when *Xenopus* embryos are unable to initiate zygotic transcription. Interestingly, treatment of *Xenopus* embryos after initiation of zygotic transcription leads to arrest, not apoptosis. Our unpublished findings in murine embryos are consistent with this observation (A. Jurisicova, unpublished). Thus, a likely scenario in mammalian oocytes includes an inactive cell death pathway that is kept in balance by the presence of cell death protectors and killers. During oocyte activation, which is accompanied by intracellular Ca$^+$ release, the balance may shift toward PCD due to synthesis of PCD inducers. Balance in favor of survival may be restored by a signal originating from the newly formed zygote, possibly via activation of the embryonic genome or translational recruitment of maternal mRNAs encoding cell protectors. If the cell death program is not balanced in the oocyte due to inadequate storage of maternal products or if the zygote fails to send a signal, PCD ensues. The recent findings of Faure *et al.*, (1997), who reported that cytoplasmic extracts from metaphase-II arrested *Xenopus* oocytes are less sensitive to apoptotic signals than those from activated oocytes also support the hypothesis that egg activation initially shifts the ooplasmic state in the direction of PCD.

There are only two reports relating to possible triggers of embryo fragmentation. The first is a consequence of spontaneous activation of mature oocytes due to targeted disruption of the c-
mos gene (Colledge et al., 1994). The other possible trigger was recently revealed through treatment of male rats with 5-aza-cytidine (5-AZAC), which interferes with proper DNA methylation during spermiogenesis (Doerksen and Trasler, 1996). Treatment with 5-AZAC may affect the pattern of gene expression from the paternal genome in early embryos, thereby preventing the shift toward survival. Both of these triggers affect early zygotic stages, suggesting that there may be active regulation of the cell death cascade during this period. In mouse, frequencies of embryo fragmentation are affected by maternal age, fertilization conditions (Jurisicova et al., 1998) and by genetic background (current study), all of which may affect the ability of the zygote to suppress PCD.

A model for how PCD may be regulated in the early embryo is given in Fig. 6. Several potential promoters and mediators of apoptosis are expressed as maternal transcripts, including p53, MA-3, Bax, and Bad, and several of these genes also appear to be transcribed at some level from the embryonic genome during the first two cell cycles. The maternal expression of these genes can thus provide an inherent bias toward PCD in the oocyte (Kastan et al., 1991; Oltavi et al., 1993; Yang et al., 1995). Mice with a targeted disruption of the Bax gene show increased resistance to follicular atresia (Knudson et al., 1995). The primordial follicle pool in Bax -/- mice is markedly increased when compared to wild type littermates (Knudson et al., 1996). Both of these observations implicate Bax as an important factor in the regulation of follicular atresia. Furthermore, oocytes obtained from Bax -/- females are more resistant to apoptosis induced by chemotherapy agents (Perez et al., 1997). We failed to detect an increase in Bax mRNA in fragmented embryos, implying that if Bax is involved in cell death in early embryos, it is posttranslationally regulated, perhaps by interactions with its dimerization partners (e.g., Bcl-xL or Bcl-2) and their regulators (Bad and Bag-1). mRNAs encoding Bag-1 interactive partners, Bcl-2
and Bcl-xL (Takayama et al., 1995) are also expressed abundantly during murine and human preimplantation development and these mRNAs are downregulated in fragmenting embryos (Jurisicova, in preparation). PCD could also be favored when fragmenting embryos augment their maternal stores of factors that may promote PCD (e.g., the Bcl-xS transcript, MA-3, Bad, and p53 mRNAs). PCD would also be favored by the reduced expression of PCD suppressers, which we observed. Mice carrying a null allele of Bcl-2 are viable and fertile, but females have reduced number of primordial follicles and many of these lack oocytes (Ratts et al., 1995). This observation supports the view that reduced expression of PCD suppressers promotes apoptosis in the oocyte and early embryo. In embryos that are destined to develop, the increases in the expression of cell killer genes would be inhibited while cell protector expression (e.g., Bcl-xL, Bcl-2) would be increased, augmenting maternal stores (e.g., Bcl-xL, Bcl-2, Bcl-w).

It is also interesting to note that the differential expression of the Bcl-xL and Bcl-xS forms of the Bcl-x transcript between embryos that survive and some of those that undergo apoptosis may reflect a molecular switch, in which part of the signal to undergo apoptosis involves a decision as how to splice the Bcl-x transcripts newly synthesized from the embryonic genome. If this is true, then this would indicate that, not only must the embryonic genome be activated at the correct time during early development, but that other signals that convey information about the health of the cell to the nucleus must permit appropriate post-transcriptional events to allow embryo survival. We also observed differential expression between the inner cell mass and trophoderm portions of the blastocyst for some of the genes assayed. ICM cells expressed a higher abundance (as a fraction of total mRNA population) of the Bcl-2 mRNA, but a lower abundance of the Bad mRNA. This result suggests that the expression of these two genes may play a role in controlling apoptosis within the inner cell mass, thus providing for a part of the
regulative ability of the mammalian embryo.

Last, it should be appreciated that the effect of paternal genotype on apoptosis in 1-cell mouse embryos provides additional evidence that embryonic genes are transcribed during the 1-cell stage. Most importantly, our results provide the first indication of a specific function that may be served by this early phase of embryonic genome activation, namely the suppression of apoptosis. Further studies of the genetic and molecular factors that regulate apoptosis in 1-cell mouse embryos should prove valuable to our understanding of how apoptosis in human embryos may be caused, and how it may be avoided through the optimization of embryo culture conditions that will provide for improved embryo health and allow the in vitro cultured embryos to suppress apoptosis.
4.5. Legends to figures.

Figure 1. Normal 2-cell stage (A) and fragmented (B) embryos, isolated from oviducts of mated females at 42 hours post hCG. Embryos were stained with the nuclear fluorochrome DAPI and photographed under fluorescence microscopy. Chromatin staining revealed the presence of normal nuclei and a polar body in the normal embryo, and condensed, degraded DNA, scattered within apoptotic bodies of the fragmented embryo. Morphological features of this embryo are consistent with apoptosis.
Figure 2. Expression of cell death suppressors *Bcl-2, Bcl-w, Bcl-x* (A) and cell death inducers *Bax* and *Bad* (B) in murine oocytes and normal embryos. The panels at the left show the data expressed in units of bound cpm. The panels at the right show the same data converted to estimated copies per embryo. Embryo stage is shown above each graph. No estimate of copy number is made for the isolated inner cell mass (ICM). Bars denote the mean ±SEM of expression. Between 4 and 6 samples of approximately 10 oocytes or embryos were obtained for each stage or time. GVOC = germinal vesicle intact oocyte, B=blastocyst.
**Figure 3.** RT-PCR analysis of *Bcl*-x gene expression.

A. Ethidium bromide stained gel with amplified fragments. B is an autoradiogram of a southern blot of the same gel upon hybridization with a *Bcl*-x cDNA probe. Each lane represents amplified product equivalent to that derived from a single oocyte or zygote. Samples contained pools of 5 unfertilised oocytes -13h/post hCG (lane 1), fragmented zygotes/22h post hCG (lane 2), normal mid-1-cell zygotes/22h post hCG (lane 3), normal late-1-cell zygotes/29h post hCG (lane 4), normal 2-cell embryos/42h post hCG (lane 5) and 1 kb DNA marker (lane 6). One fifth of each pool was amplified for this experiment. All samples of normal oocytes and embryos resulted in amplification of a 760 bp fragment expected of *Bcl*-xL. Interestingly, a subset of fragmented embryos also contained a second DNA fragment of approximately 550bp, which is the expected size of *Bcl*-xS. All amplified fragments hybridized strongly with *Bcl*-x cDNA probe.
Figure 4. Expression of p53, Ma-3, and Ttg, genes associated with cell death in murine oocytes and normal embryos. Graphs are as described in Figure 2.
Figure 5. Expression of PCD associated genes in pre-EGA stages. *Bcl-2, p53, Ma-3, Bax* and *Bad* expression was analyzed in unfertilised oocytes (MII-13h/post hCG), mid 1-cell stage zygotes (22 hCG), late 1-cell stage zygotes (29h/post hCG) and in fragmented zygotes (29h/post hCG). Bars indicate the average of bound cpm produced by two pools of analyzed oocytes and zygotes. The points, produced by each of the two samples, are also shown.
**Figure 6.** Schematic representation of PCD regulation during mammalian early embryo development. A delicate balance between cell death suppressors and inducers is established during oogenesis. Healthy oocytes contain a passive cell death pathway that becomes activated upon fertilization. In order for the early embryo to survive, it must suppress the activated cell death machinery by a signal such as posttranslational modification of existing molecules or transcription and/or synthesis of cell death suppressors. If this signal is not sent or if other developmental abnormalities occur, cell death ensues. Alternatively, the oocyte may contain higher levels of cell death inducers, therefore disturbing the balance of inducers and protectors such that even the appropriate signal emanating from the zygote is not able to override the activated cell death pathway. Both of the last two scenarios would lead to embryo fragmentation. However, at the present time, the existence of oocytes with an early bias towards PCD is purely hypothetical.
Normal development

Abnormal development

Healthy oocyte, no bias toward PCD

Abnormal oocyte, early bias toward PCD

Active Cell Death Suppressor

Inactive Cell Death Suppressor

Active Cell Death Inducers

Inactive Cell Death Inducers

Normal Cleavage

Apoptosis
Chapter 5.

Expression of genes associated with cell death during human preimplantation embryo development.

5.1. Introduction.

With improved protocols for hormonal stimulation, more defined culture conditions and transfer of embryos at day 3 rather than day 2, implantation rates in humans after IVF have increased slightly in the past 4 years – from 19% to approximately 23% (Dawson et al., 1995). However, the overwhelming majority of embryos still fail to establish viable pregnancies. Based on developmental potential of human embryos during in vitro culture, less than 45% of embryos fertilized in vitro will be able to reach the fully expanded blastocyst stage (for review see Bongso et al., 1994). Many embryos arrest earlier, most frequently between the 4 and 8 cell stages (Dokras et al., 1993).

Human preimplantation embryos can be affected by various kinds of cellular and morphological abnormalities. The most frequently observed defects are alterations in nuclear/cytoplasmic ratios beginning at days 2 and 3 post insemination. These alterations result in embryos with a high nuclear/cytoplasmic ratio - multinucleated blastomeres and blastomeres with fractionated nuclei, and embryos with low nuclear/cytoplasmic ratios - anucleate blastomeres/fragments (Tesarik et al., 1987, Winston et al., 1991, Hardy et al., 1993). Such abnormalities interfere with the developmental potential of embryos and thus reflect on IVF outcome (Erenus et al., 1991, Balakier and Cadesky, 1997). Recently, it was reported that embryos with regular morphology and embryos with less than 20% fragmentation have very similar implantation potential (21% and 26% respectively) which is four to five fold higher than
that reported for embryos with a substantial amount (20-50%) of cellular fragmentation (Ziebe et al., 1997). Moreover, these authors also reported 3 times higher levels of biochemical pregnancy rates with subsequent embryonic loss in the group of extensively fragmented embryos, suggesting that they are unable to proceed through peri- and post-implantation development.

One of the most commonly observed morphological abnormalities in human preimplantation embryos is extensive cellular fragmentation, occurring between the 2 and the 8 cell stages. Our knowledge of the etiology and underlying mechanisms of embryo fragmentation is limited. Cellular fragmentation has been observed in early embryos of several mammalian species and has also been described in embryos conceived in vivo as well as by IVF, from both stimulated and unstimulated cycles (Summers et al., 1995, Formigli et al., 1991). The morphological appearance of these embryos as well as several descriptions in the literature led us to perform a series of experiments with the underlying hypothesis that embryo fragmentation is a result of programmed cell death. Results of these experiments revealed that a high incidence of cellular abnormalities associated with apoptosis can be found in extensively fragmented arrested human embryos (Jurisicova et al., 1996c). These findings have now been confirmed by several other investigators (Guerin et al., 1997, Rimarachin et al., 1997).

Programmed cell death (PCD) via apoptosis occurs as an integral part of preimplantation embryo development at the blastocyst stage. Cell death, especially in the inner cell mass lineage, occurs in blastocysts of many different mammalian species (El-Shershaby and Hinchliffe 1974, Mohr and Trounson 1982, Papaioannou and Ebert 1988). Primate blastocysts, including those of humans, eliminate approximately 18% of cells at day 7 post insemination (Hardy et al., 1989) with cell death occurring not only in the ICM but often in the trophectodermal lineage as well (Hardy, 1997). Thus, there are at least two periods during human preimplantation embryo
development when embryos display the ability to undergo PCD, the 2-8 cell stage and blastocysts.

Because PCD is under genetic regulation and is tightly governed by a set of "cell death suppressors and inducers" (for review see Jacobson 1997, Kumar and Harvey, 1995), it is important to examine the temporal expression pattern of these genes during human embryo development. At the present time there are at least 50 different genes whose expression affects cell survival. However, we do not know which of these genes are involved in early embryo death, nor do we know the expression pattern of these genes during normal development. Taking into consideration that cell death occurs at very defined time points during preimplantation embryo development, one should be able to detect changes in expression of genes associated with apoptosis in dying embryos. Thus, we decided to establish a developmental profile of a subset of cell death suppressors (e.g. Bcl-x, Mcl-1, Bcl-w, Bfl-1/A1, Bag), cell death inducers (Bax, Hrk) and the cell death executioners (Caspases-1, 2 and 3) during normal human preimplantation embryo development. Here we report that human oocytes as well as preimplantation embryos abundantly express the cell death suppressors Mcl-1, Bcl-x and Bag, and the cell death inducer Bax and Caspase-2. Lower, but detectable expression of Bfl-1/A1, Hrk and Caspase-3 was detected during all developmental stages. The first detectable embryonic expression of Bcl-w was observed in morula and persisted to the blastocyst stage. Caspase-1 was the only gene in our group showing no detectable expression.

5.2. Materials and Method.

*In vitro fertilization and embryo culture*

Spare human preimplantation embryos were obtained from the IVF Program, Division of
Reproductive Sciences at the Toronto Hospital or from Toronto Center for Advanced Reproductive Technologies. Patients who chose not to freeze their spare embryos for future transfers were asked to donate them for research and informed consent was obtained. This research was approved by the human ethics committee of the Toronto Hospital and by the University of Toronto.

Ovarian stimulation was carried out using a gonadotropin releasing hormone (GnRH) agonist (Lupron, Abbott Pharmaceuticals, Montreal) in a long protocol, and human menopausal gonadotropin (hMG; Pergonal, Serono Canada, Oakville, Ontario or Humegon, Organon Canada, Scarborough, Ontario). Both IVF and Intra Cytoplasmic Sperm Injection (ICSI) were performed using standard techniques (Segal and Casper, 1992, Greenblatt et al., 1995). Eighteen to 22 h post insemination, oocytes were examined for the presence of two pronuclei and 43-45 hours after insemination up to 3 of the most rapidly and evenly dividing embryos were transferred (at the 2 to 6 cell stage) to the patient's uterus.

Spare embryos of variable quality that appeared to arise from normally fertilized oocytes with 2 pronuclei were used for subsequent analysis. Embryos were cultured in HTF medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% Synthetic Serum Substitute (Irvine Scientific) at 37°C in 5% CO2 and 95% air. Assessment of embryo quality and developmental stage was recorded daily. Immature oocytes prior to germinal vesicle breakdown (GV) as well as uninseminated spare mature oocytes (MII) were denuded of cumulus cells with hyaluronidase and used in subsequent studies.

*Expression of cell death genes during preimplantation embryo development.*

The expression of cell death genes was determined by a quantitative reverse transcription-
polymerase chain reaction (RT-PCR) based assay (Rambhatla et al., 1995). Between 6-10 samples of cDNA of each developmental stage, each derived from a single oocyte or embryo, were amplified as described (Brady and Iscove 1993). The amplified material was then analyzed by hybridization of dot blots with random prime radiolabelled cDNA probes, followed by quantitation of signals on a phosphorimager as described in Rambhatla et al., (1995). The cDNA probes used for analysis recognized the 3' untranslated regions of target genes and were either donated for this study (Bcl-w S. Cory, Hrk-l G. Nunez, Caspase-3 I. Alnemri, Bfl-1 H. S. Shin) or were cloned from oligo dT primed human full term placenta RNA (ATCC, Rockville,USA) or teratocarcinoma cDNA (obtained from J. Skowronksi) libraries using probes recognizing coding regions of target genes (Bcl-x, Bag, Caspase-1 and -2). The identity of cDNA clones was confirmed by sequence analysis using a Sequenase version 2.0 DNA kit (USB). Bax and Mcl-1 probes were obtained by RT-PCR of RNA isolated from human ovary, using primers from published sequencs: Mcl-1 5' primer gcccatctcgagccataagg and 3' primer agttgtctcaacctctcctgg (Kozopas et al., 1993, Acc.#: L08246); human Bax 5' primer: tgcagaggatgattgccgcc (Oltvai et al., 1993, Acc.#: L22473) and 3'primer:agatgccgaagtgtccc (Miyashita and Reed, 1995; Acc # U17194). Identity of the 600 bp and 500 bp long DNA fragments amplified respectively, was confirmed by sequencing using a dsDNA Cycle Sequencing Kit (Gibco BRL).

Estimates of mRNA copy number were calculated as described (Rambhatla et al., 1995) with some modification regarding size differences between human (120 µm) and mouse (80 µm) oocytes as well as differences in mRNA turnover due to the delayed activation of zygotic genome in humans.

The following equation was used:

\[
\text{copy number} = C \times \text{bound cpm} \times \text{SA actin/SA gene} \times Y \times \frac{N}{3.4 \times 10^7}
\]
where $C$ is number of copies of actin in blastocyst divided by number of bound cpm obtained after hybridization with $\beta$-actin probe, $SA$ is the specific activity of the labeled actin or gene ($Y$) probe in cpm/ng, $N$ is the number of mRNA templates per embryo at a given developmental time. For all stages the mRNA content for mouse embryos calculated by Rambhatla et al., (1995) was used, but all values were multiplied by 1.5 in order to correct for the larger size of human embryos. The copy number of $\beta$–actin at the blastocyst stage was calculated from mouse blastocysts according to Rambhatla et al., (1995) $- 2.3 \times 10^5$ and multiplied by 1.5 (as above) = $3.45 \times 10^5$. Also, since the major activation of the embryonic genome occurs at the 2-cell stage in the mouse and at the 4-cell stage in humans, the $N$ value for late 1-cell mouse zygotes was used for 2-cell stage human embryos, the $N$ value for 2-cell mouse embryos was used for 4-cell stage human embryos, and the $N$ value for 4-cell stage mouse was used for 8-cell human embryos. GV, MII, morula and blastocyst values remained the same.

5.3. Results.

Work with human preimplantation embryos is often very difficult, because of the paucity of available study material. We have obtained 45 samples of uninseminated human oocytes and 101 samples of spare preimplantation embryos with normal morphology at different stages of development. These were processed as described and pre-screened for quality of amplified cDNA by hybridization with elongation factor 1α ($EF-1\alpha$). Only a subset of these (n=99) showed good hybridization signals with this house keeping gene. We used 6-10 embryos of each developmental stage for further analysis.

5.3.1. Expression of Cell Death Suppressors: Bfl-1/A1, Bcl-w, Bcl-x, Mcl-1 and Bag-1.
Of five cell death suppressors analyzed, all were detected in the germinal vesicle stage and unfertilized oocytes, and later throughout preimplantation development. Oocytes express a low level of Bcl-w that diminishes and becomes undetectable at the 2-cell stage. The first embryonic stage that had a detectable level of Bcl-w transcript was the morula, and this expression persisted to the blastocyst stage, although at low copy numbers (data not shown). Bfl-1 (Fig 1.A) and Bcl-x (Fig 1.B) were easily detected during early embryonic stages, but their level of expression was about 5-10 fold lower than that of Mcl-1 (Fig.1.C) and Bag-1 (Fig.1.D). All of the genes analysed had a tendency to increase from the 8-cell stage on, reaching maximum levels at the blastocyst stage.

Interestingly, both Mcl-1 and Bag-1 displayed a transient increase at the two-cell stage, followed by a decline at the 4-cell stage, and up-regulation at the 8-cell stage which continued up to the blastocyst stage. Both of these mRNAs reached maximum levels of accumulation at the blastocyst stage with approximately 146,000 and 530,000 copies per embryo, respectively. Thus, Mcl-1 and Bag-1 are the most abundantly expressed cell death protectors detected during early human embryonic development, making them two likely gene candidates for early embryonic cell survival decisions.

5.3.2. Expression of genes associated with induction and execution of cell death.

Bax and Harakiri (Hrk) are known interactive partners of several Bcl-family members that alter cell survival. Transcripts for both these cell death inducers were detected in both immature and mature oocytes and were persistently expressed during all developmental stages. Bax mRNA (Fig.2.A) appeared to be much more abundant than Hrk (Fig.2.B) in oocytes. Intriguingly, an increase of Hrk mRNA was detected at the 2-cell stage, when many human
embryos undergo fragmentation. Also, both *Hrk* and *Bax* displayed a dramatic increase in their transcript levels beginning at the morula stage, and culminating in maximum levels at the blastocyst stage reaching 6,500 and 40,000 copies per embryo respectively.

Caspases are proteases involved in the cellular destruction during apoptosis. Analysis of caspase expression during preimplantation development confirmed high levels of *Casp-2* and *-3* mRNA, but no detectable level of transcription of *Casp-1*. *Casp-2* mRNA (Fig. 3.A) showed increased accumulation from immature oocytes towards the blastocyst stage, reaching maximum levels of 40,000 copies. A transient increase in mRNA copy number for *Casp-2* was seen at the 2-cell stage. In contrast to *Casp-2*, *Casp-3* (Fig. 3.B) was most abundant in GV stage oocytes, reaching 5,000 mRNA copies/oocyte. The expression of this gene could be detected at all developmental stages, but was present at one tenth the copy number of *Casp-2* during early cleavage stages.

These results confirm the presence of several cell death suppressors and inducers during human preimplantation embryo development and suggest the presence of a functional pathway for the execution of apoptosis in very early embryonic stages.

5.4. Discussion.

Identification of PCD as an underlying mechanism of embryo fragmentation (Jurisicova *et al.*, 1996c) opened up a new avenue of investigation of early embryonic decisions. The impact of PCD lies in active regulation of cell survival and therefore embryonic developmental potential may be partially predicted by establishing levels of expression of cell death genes. However, before we can assess the predictive power of these molecular parameters, we have to establish the “normal” pattern of expression of these genes during early development, keeping in mind that it
is unlikely that the survival or death decision will be determined by the presence or absence of a single gene product that will turn an "ON-OFF" switch, because most of the cell death genes work in a complex network of homo- and heterodimers.

In order to explore physiological and genetic pre-dispositions involved in mammalian embryo fragmentation, we have previously used a murine model. In the CD-1 outbred strain, the rate of embryo fragmentation is significantly increased during IVF and is also affected by maternal age and by both maternal and paternal genetic background (Jurisicova et al., 1998). Analysis of the pattern of gene expression during mouse preimplantation embryo development revealed that oocytes and early embryos contain mRNA for many cell death genes. Interestingly, embryos that underwent apoptosis not only contained low levels of mRNA for cell death suppressors, but they also appeared to have augmented maternal stores of some factors that may promote PCD (Jurisicova et al., 1998b). We have previously proposed a model for regulation and execution of cell death or survival during mammalian preimplantation embryo development (Jurisicova et al., 1997, 1998b). Similar observations, consistent with this model, were reported recently in *Xenopus* oocytes and embryos (Stack and Newport 1997, Sible et al., 1997, Faure et al., 1997). The experiments described in this report confirm a very similar profile of expression for several cell death genes in human and mouse embryos, suggesting that there may be evolutionary conservation of this mechanism for ensuring embryo quality.

The most widely studied genes associated with PCD are members of the *Bcl-2* gene family of cell death suppressors and inducers (for review see Jacobson 1997). Many of these proteins interact with each other establishing an intricate network of homo- and hetero-dimers. Moreover, some of these proteins can bind to other, non Bcl-like, partners that subsequently alter susceptibility of the cell to apoptosis. Two cell death protectors showing very high levels of
expression in human embryos are Mcl-1 and Bag-1. It was reported previously that Bag-1 can bind to Bcl-2 and Bcl-x (Takayama et al., 1995; 1997) and, via this interaction, is able to promote cell survival (Clevenger et al., 1997). At the present time it is not known whether Bag-1 can also bind to Mcl-1, but since these are co-expressed in the same cell/embryo at comparable levels, it is tempting to speculate that interaction of these two proteins may be responsible for increased survival in normal embryos. Interestingly, fragmented mouse embryos have dramatically reduced level of Bag-1 gene expression (Jurisicova et al., in preparation). The preliminary results indicate variability of expression of the Bag-1 gene during early cleavage stages (2-8 cell) in human embryos and we are currently analyzing levels of Bag-1 expression in more depth in both normal and fragmented human embryos.

Bcl-x, a known interactive partner of Bag-1, is also expressed during preimplantation development. However, its transcript levels are much lower than those of Bag, especially at the blastocyst stage. Furthermore, Bcl-x mRNA undergoes alternative splicing, creating two proteins, Bcl-xS which lacks two typical Bcl-domains BH1 and BH2 and Bcl-xL which retains these domains (Boise et al., 1995). Bcl-xL is a very potent cell death suppressor, while Bcl-xS renders cells more susceptible to apoptotic signals even during concomitant expression of Bcl-xL (Minn et al., 1996; Heermeier et al., 1996). Unfortunately, our approach of cDNA analysis could not distinguish between different splicing products resulting from alternative exon/intron splicing within the coding region of the Bcl-x gene. Bcl-x expression in mouse embryos is entirely attributable to the protective Bcl-xL form of the transcript in embryos successfully proceeding through the one-cell stage Jurisicova et al., 1998b). We detected Bcl-xS transcripts in a subset of fragmented murine embryos by RT-PCR with specific primers (Jurisicova et al., 1998) and experiments on human fragmented embryos are currently underway in our laboratory to
determine whether alteration of the \( Bcl-xL/S \) ratio in some embryos could be responsible in part for cell death in some blastomeres.

Human oocytes and embryos also express high levels of the cell death promotors \( Bax \) and \( Harakiri \) (Fig.2). Both of these proteins bind \( Bcl-xL \) with high affinity, but not \( Bcl-xS \) (Tao et al., 1997, Inohara et al., 1997). If the embryo does not produce enough \( Bcl-xL \) and possibly \( Mcl-1 \) protector molecules and concomitantly expresses high levels of \( Bax \) and/or \( Hrk \), these killers may titrate the few available cell death inhibitor molecules, thus leading to activation of the cell death pathway. Thus, the decision of cell survival or death is based on the balance between cell protectors and cell killers. Once the balance of interacting partners is disturbed due to down regulation or lack of free interactive partners and/or their inactivation by post-translational modifications (reviewed in Jacobson, 1997), cell executioners (caspases) become activated (for review see Kumar and Harvey, 1995, Salvensen and Dixit, 1997).

According to Alnemri et al., (1996) there are currently three known groups of caspases: ICE-like (Casp-1), CED-3 like (Casp-3) and Ich-1 like (Casp-2). These enzymes have distinct preferences for different cellular substrates (Talanian et al., 1997). The cascade model of caspase activation via a chain reaction cleavage of inactive precursors of downstream caspases by upstream, activated caspases was proposed by Fraser and Evan (1996). Casp-2 activation occurs very early upon induction of apoptosis and therefore this enzyme most likely lies up-stream in the chain reaction (Harvey et al., 1997). On the other hand Casp-3 seems to be the end product of the caspase cascade and is considered to be the major active caspase involved in cellular degradation during apoptosis (Faleiro et al., 1997). Both Casp-2 and -3 transcripts are expressed during all cleavage stages of human development, again confirming the presence of a functional PCD pathway during preimplantation embryo development. Interestingly, neither murine nor human
embryos express Caspase-1/ICE mRNA. Even though this enzyme is involved apoptosis in some
cell types (Kuida et al., 1995), its major function is in processing of the inactive precursor for IL-
1b to its active form (Black et al., 1989).

As we have shown above, preimplantation embryos express genes associated with cell
death. The obvious next step is a detailed comparison of expression of many cell death genes in
normal and fragmented embryos. However, there are several problems associated with this
approach. First, it is important to analyze the same oocyte/blastomere for expression of several
genes and quantitatively determine the level of mRNA or protein expression for as many genes as
possible. This is a very difficult task and is further complicated in humans, because the same
embryo may contain both normal and dying blastomeres, which may skew results. Fragmented
blastomeres degrade their nuclear and cytoplasmic compartments and therefore may not
contribute to the pool of mRNA. Thus, the extracted mRNA pool might only reflect surviving
blastomeres in the embryo, even though the embryo may have been assessed as apoptotic. On the
other hand, PCD is an active cellular process. If one uses an embryo that appears normal at the
time of analysis, some blastomeres may already have been pre-destined to die via an altered
pattern of gene expression. Thus, results on cell death gene expression in normal and fragmented
human embryos should be interpreted carefully and with caution.
5.5. Figure Legends.

Fig 1. A-D Expression of cell death suppressors Bfl-1, Bcl-x, Mcl-1 and Bag-1 in human oocytes and normal embryos. The panels at the left show the data expressed in units of bound cpm. The panels at the right show the same data converted to estimated copies per embryo. Embryo stage is shown above each graph. Bars denote the mean (SEM of expression obtained from 6 to 8 single embryo samples at each developmental stage. GVOC = germinal vesicle intact oocyte, MII=mature oocyte, M= morula, B= blastocyst.
Data shown are mean levels +/- st. error.
**Figure 2.** A-B Expression of cell death inducers *Bax* and *Hrk* in human oocytes and normal embryos. The panels at the left show the data expressed in units of bound cpm. The panels at the right show the same data converted to estimated copies per embryo. Embryo stage is shown above each graph. Bars denote the mean (SEM of expression obtained from 6 to 8 single embryo samples at each developmental stage. GVOC = germinal vesicle intact oocyte, MII=mature oocyte, M=morula, B=blastocyst.

**Fig 3.** A-B Expression of cell death executioners *Caspase-2* and *Caspase-3* in human oocytes and normal embryos. Graphs are as described in Figure 2.
Figure 2.

A

**Bax**

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>GV</th>
<th>MII</th>
<th>2c</th>
<th>4c</th>
<th>8c</th>
<th>M</th>
<th>B</th>
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<tr>
<td>Cpm bound</td>
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<td>4000</td>
<td>3000</td>
<td>2000</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>Estimated copy number/embryo</td>
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<td>8000</td>
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B

**Hrk**

<table>
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<th>4c</th>
<th>8c</th>
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<th>B</th>
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</thead>
<tbody>
<tr>
<td>Cpm bound</td>
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<td>200</td>
<td>150</td>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Estimated copy number/embryo</td>
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<td>6000</td>
<td>5000</td>
<td>4000</td>
<td>3000</td>
<td>2000</td>
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</tbody>
</table>

Figure 3.

A

**Caspase-2**

<table>
<thead>
<tr>
<th>Developmental stage</th>
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<th>MII</th>
<th>2c</th>
<th>4c</th>
<th>8c</th>
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<th>B</th>
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<tbody>
<tr>
<td>Cpm bound</td>
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<td>400</td>
<td>200</td>
<td>100</td>
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<td>10000</td>
<td>4000</td>
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</table>

B

**Caspase-3**

<table>
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<tr>
<th>Developmental stage</th>
<th>GV</th>
<th>MII</th>
<th>2c</th>
<th>4c</th>
<th>8c</th>
<th>M</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpm bound</td>
<td>300</td>
<td>250</td>
<td>200</td>
<td>150</td>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Estimated copy number/embryo</td>
<td>6000</td>
<td>5000</td>
<td>4000</td>
<td>3000</td>
<td>2000</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Data shown are mean levels +/- st. error
Chapter 6.

PCD during human blastocyst formation.

6.1. Introduction.

Successful mammalian preimplantation embryo development culminates in a series of events leading to the first obvious cellular differentiation - formation of trophectoderm (TE) and the inner cell mass (ICM) during blastocyst formation. Trophectoderm, a fluid-transporting epithelium, is the cellular precursor of the majority of extraembryonic tissues, including the embryonic portion of the placenta. ICM cells, comprising approximately 1/3 of the total cell count in the human expanded blastocyst (Hardy et al., 1989), contribute to the formation of the embryo proper. Our current knowledge of the cellular and molecular events leading to blastocyst formation is very limited.

The role of programmed cell death (PCD) in mammalian embryo development has received very little attention so far. PCD during the blastocyst stage has been observed in several species (El-Shershaby and Hinchliffe 1974, Mohr and Trounson 1982, Papaioannou and Ebert, 1988) including primates (Hardy et al., 1989). Based on the results of transplantation experiments performed with embryonic carcinoma cells injected into the blastocoel cavity, it was proposed that PCD in blastocysts regularly eliminates redundant ICM cells with trophectodermal potential (Pierce et al., 1989). Extracellular hydrogen peroxide generated via polyamine oxidation was suggested as a mediator of PCD in the blastocyst (Pierce et al., 1991).

The mouse is the best studied animal with respect to mammalian development. Several groups have analyzed the extent of cell death in murine expanded blastocysts developed in vivo or in vitro. The peak level of apoptosis in mouse blastocysts maintained in vivo was observed at 97 hours post coitus, with cell death predominantly restricted to ICM cells.
(Handyside and Hunter, 1986). Recently, Brison and Schultz (1997) reported an increased cell death index in blastocysts cultured separately, in comparison to blastocysts cultured in groups. They also reported that cell death could be decreased by the addition of TGFα to the culture medium, suggesting that this growth factor may be one of several survival signals required by the developing embryo. Interestingly, the cell death rate in mouse blastocysts is also affected by conditions of fertilization and by maternal age (Jurisicova et al., 1998) as well as by components of the culture medium (Devreker and Hardy, 1997).

The rate of human blastocyst formation in vitro does not exceed 50% (for review see Bongso et al., 1994). Upon transfer, only a subset of these blastocysts, about 40%, are able to implant (Olivennes et al., 1994, for review see Bavister and Boatman, 1997). Cell number and cell death in human blastocysts was previously studied by nuclear staining (Hardy et al., 1989). Their results suggested that at day 7 post insemination during IVF, cell death eliminates approximately 18% of cells in human expanded blastocysts. The cell death index increased dramatically, to 27% and 38% respectively, among morphologically abnormal blastocysts or blastocysts originating from zygotes with single or triple pronuclei (Hardy et al., 1989).

In this study we further explore cell death in human blastocysts through analysis of chromatin status, by nuclear staining combined with terminal transferase-mediated DNA end labeling (TUNEL), in order to define how many cells die during blastocyst formation in vitro and how this may affect the future development of the embryo. We compared cell number and the cell death rate in blastocysts cultured in two different media, as well as in abnormal blastocysts developed from 1PN and 3PN zygotes. Furthermore, we analyzed the expression pattern of nine genes associated with cell death using quantitative RT-PCR and compared the levels of expression of these genes with the extent of cell death.

Embryo Culture

Spare human preimplantation embryos were obtained from the IVF Program, Division of Reproductive Sciences, at the Toronto Hospital or from the Toronto Center for Advanced Reproductive Technologies. Patients who elected not to freeze their spare embryos for future transfers either by choice or because the embryos were not suitable for cryopreservation due to extensive fragmentation or multinucleation of blastomeres, were asked to donate these embryos for research. This project was approved by the human ethics committee of the Toronto Hospital and by the University of Toronto.

Spare embryos of variable quality that appeared to arise from normally fertilized oocytes with 2 pronuclei were used in this study. IVF and Intra Cytoplasmic Sperm Injection (ICSI) were performed according to standard techniques as previously described (Segal and Casper 1992, Greenblatt et al., 1995). At day 2 (48 hours post insemination), single embryos were placed in culture in 0.5 ml of either HTF medium (Irvine Scientific, CA) or KSOM (Erbach et al., 1994) with 0.5 x non-essential amino acids (Gibco BRL) supplemented with 10% SSS (Irvine Scientific, CA) at 37°C in 5% CO₂ and 95% air. Assessment of embryo quality and developmental stage were recorded daily until the embryos reached the expanded blastocyst stage.

Analysis of cell number and cell death.

To analyze the status of chromatin in these blastocysts, we used a combined technique for simultaneous nuclear staining with the fluorochrome 4,6-diamidino-2-phenylindole (DAPI) and, in a subset of embryos, terminal transferase-mediated DNA end labeling (TUNEL). Briefly,
blastocysts were fixed in 4% paraformaldehyde diluted in PBS on microscope slides. After air drying, slides were stored at -70°C until further use. After thawing, slides were washed in PBS, and TUNEL was performed as previously described (Jurisicova et al., 1996, 1998). After TUNEL, the slides were stained with DAPI (Sigma, St. Louis, MO) at a concentration of 0.2 μg/ml for 10 min. The slides were examined and photographed using a Leica fluorescence microscope with appropriate filters. Further assessment of cell death was based on DNA condensation, fragmentation and nuclear morphology, as well as TUNEL labeling (Jurisicova et al., 1998a). The cell death index (CDI) was calculated as the percent of total cells which exhibited intense DAPI staining due to condensation of chromatin, which in mammalian blastocysts is known to precede DNA fragmentation detected by TUNEL.

Analysis of gene expression in human blastocysts.

A subset of human blastocysts (n=37) was stained alive by adding DAPI to the culture medium. After 30 minutes incubation, the zona pellucida was removed and embryos were viewed briefly under the fluorescence microscope. The numbers of normal cells and cells with condensed DNA were recorded and single embryos were used for analysis of gene expression. Mcl-1, Bcl-x, Bcl-w, Bag, Hrk, Bax, Caspase 2 and Caspase 3 expression were determined by a quantitative reverse transcription-polymerase chain reaction (RT-PCR) based assay (Rambhatla et al., 1995). With this procedure, the 3' ends of the entire polyA+ mRNA population are reverse transcribed and then amplified by PCR without altering the relative abundance of individual sequences (Brady and Iscove 1993). The amplified material was then analyzed by hybridization of dot blots with radiolabelled cDNA probes, followed by quantitation of signals on a phosphorimager as described in Rambhatla et al., (1995). The cDNA probes used for analysis recognized the 3' untranslated region of the genes, and were either donated for this study or were cloned in our
laboratory as previously described in Chapter 5. cDNA products of single embryos were pre-
screened for the quality of cDNA by hybridization with elongation factor 1α (EF-1α). Using this
control, we determined that 5/37 samples had failed to produce good quality hybridization
signals, and these cDNA samples were excluded from further analysis.

Statistical analysis.

Differences between the proportions of embryos developing to the blastocyst stage were
compared by chi-square analysis with Yates’ correction. The effect of culture conditions,
abnormal nuclear morphology and ploidy on total cell number and the cell death index were
analyzed by the Mann Whitney Rank Sum Test and by Kruskal-Wallis One Way Analysis of
Variance on Ranks, respectively, using the SigmaStat statistical package (Version 1.0). Analysis
of cell death and the level of expression of different genes was performed by Multiple Regression
Analysis and by Spearman Rank Correlation using the SPSS Software package (Version 5.0).
Analysis of the distribution of Bcl-x expression was performed using the Kolmogorov-Smirnoff
test.

6.3. Results.

6.3.1. Embryo culture.

We obtained 1040 spare human preimplantation embryos, after either IVF or ICSI, at day
2 post insemination. From these, 611 developed from zygotes with 2 pronuclei (2PN) and the
majority of these embryos (approximately 80%) contained at least 30% cellular fragments. Of
these 611 embryos at day 2, 122 appeared to have multinucleated blastomeres and 16 contained
blastomeres with fractured nuclei. All the remaining 429 embryos were fertilized by ICSI. One
hundred and seventy of them appeared to have only a single pronucleus (1PN) with two polar
bodies while 259 had 3 visible pronuclei (3PN).

The type of culture medium used had a significant effect on the rate of blastocyst formation of 2PN embryos (Table I). Twenty two percent of embryos developed to blastocyst stage in KSOM medium, which was significantly higher (P=0.0002) than 12 % blastocyst development in HTF medium. In contrast, no significant effect of medium on the rate of blastocyst formation was observed in 1PN (7.8% versus 7.4%) or 3PN (14.8% versus 11.2%) embryos, suggesting that these embryos were developmentally compromised by abnormal fertilization, and that improved culture medium, such as KSOM, was unable to rescue development to the blastocyst stage (see Table I).

Table I. Developmental potential of human embryos cultured in HTF or KSOM medium in vitro.

<table>
<thead>
<tr>
<th>Medium</th>
<th>HTF</th>
<th>KSOM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cultured</td>
<td>Blastocyst (%)</td>
</tr>
<tr>
<td># Pronuclei</td>
<td>2</td>
<td>475</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>178</td>
</tr>
</tbody>
</table>

* differences are statistically significant (P=0.0002)

The mean (±SEM) number of cells per blastocyst on day 6 was 54 ± 3 with only a few cells (6±1) displaying signs of apoptosis for 2PN embryos (Table II.) By day 7, after further development to the fully expanded or hatching blastocyst stage, the mean cell number had increased significantly to 83 ± 7 (P=0.0012). Interestingly, the number of cells with typical signs of apoptosis in this later stage had doubled to 14 ± 2 (P=0.02). Occasionally we noticed necrotic cells (0.8± 0.6 per blastocyst) which most likely represented arrested blastomeres from earlier cleavage divisions.
Some differences in cell number and cell death index were observed among blastocysts developed from normally (2PN) and abnormally (1PN or 3PN) fertilized embryos. However, due to our small sample size, these differences did not reach statistical significance (see Table II).

Table II. Comparison of cell number and cell death in the blastocysts developed from normally fertilized (2PN) and abnormally fertilized (1PN and 3PN) embryos.

<table>
<thead>
<tr>
<th># of pronuclei</th>
<th>Day</th>
<th>Total Cell #</th>
<th>DAPI Apoptotic</th>
<th>CDI (%)</th>
<th>Depletion of ICM (%)</th>
<th>Elevated CDI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n=7)</td>
<td>6</td>
<td>73±15</td>
<td>11±4</td>
<td>16±5</td>
<td>0</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>2 (n=48)</td>
<td>6</td>
<td>54±3</td>
<td>6±1</td>
<td>13±1</td>
<td>4 (8.3)</td>
<td>10 (21)</td>
</tr>
<tr>
<td>3 (n=24)</td>
<td>6</td>
<td>48±3</td>
<td>4±1</td>
<td>9±1</td>
<td>2 (8.3)</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td>1 (n=5)</td>
<td>7</td>
<td>91±18</td>
<td>18±6</td>
<td>25±11</td>
<td>3 (60)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>2 (n=42)</td>
<td>7</td>
<td>83±7</td>
<td>14±2</td>
<td>19±2</td>
<td>9 (21.4)</td>
<td>5 (12)</td>
</tr>
<tr>
<td>3 (n=4)</td>
<td>7</td>
<td>51±15</td>
<td>13±10</td>
<td>15±9</td>
<td>0</td>
<td>1 (25)</td>
</tr>
</tbody>
</table>

Numbers in third and fourth column represent a mean number of cells ± st.error. Cells were classified as apoptotic when chromatin condensation and change in nuclear shape was observed using DAPI. CDI was calculated as the proportion of cells showing apoptotic changes per embryo based on DAPI classification. The last two columns show the number of blastocysts with either depleted ICM cells or blastocysts with elevated CDI (above mean + st.err). None of the differences was statistically significant.

When cell number and cell death index (CDI) were compared between 2PN blastocysts with respect to culture medium, no significant differences were observed either at day 6 or at day 7 (Table III). Since there was no effect of medium type on PCD, further analysis with respect to cell death was done on pooled groups of embryos at day 6 or day 7 without taking into consideration the type of medium used. In most of the embryos, cell death appeared to be random. Dying cells were scattered in both the ICM and trophectoderm. However, we identified a subpopulation of embryos, 4/48 (8.3%) at day 6 and 9/42 (21.4%) at day 7, in which the majority of ICM cells had extensive chromatin condensation and/or fragmented DNA. The resulting complete depletion of ICM cells following implantation could lead to the condition
clinically recognized as “blighted ovum”, where the placenta but no embryo can be found.

We also observed that some blastocysts had an increased CDI. In the majority of blastocysts, this index did not exceed 14% (which represents the mean CDI of 13% + 1% SEM) on day 6 and 21% (which represents the mean CDI of 19% + 2% SEM) on day 7, respectively. However, in a subpopulation of embryos (10/48) at day 6 and (5/42) at day 7, the cell death index increased above this level (up to 56%), indicating widespread activation of PCD in these blastocysts. The different categories of cell death observed in these human blastocysts are illustrated in Figure 1.

Table III. Cell number and cell death in normally fertilized embryos that developed to the blastocyst stage.

<table>
<thead>
<tr>
<th>Day</th>
<th>Medium</th>
<th>Total Cell #</th>
<th>DAPI Apoptotic</th>
<th>TUNEL Apoptotic</th>
<th>CDI (%)</th>
<th>Depletion of ICM (%)</th>
<th>Elevated CDI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>KSOM</td>
<td>52 ± 4</td>
<td>7 ± 1</td>
<td>4 ± 0.3</td>
<td>1.5 ± 2</td>
<td>0/15 (0%)</td>
<td>2/15 (13%)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>HTF</td>
<td>54 ± 2</td>
<td>6 ± 1</td>
<td>3 ± 1</td>
<td>1.1 ± 2</td>
<td>3/33 (9%)</td>
<td>8/33 (24%)</td>
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<td></td>
<td>(n=33)</td>
<td>(n=33)</td>
<td>(n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>KSOM</td>
<td>84 ± 12</td>
<td>15 ± 3</td>
<td>7 ± 1</td>
<td>1.9 ± 3</td>
<td>5/19 (26%)</td>
<td>2/19 (11%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=19)</td>
<td>(n=19)</td>
<td>(n=16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>HTF</td>
<td>82 ± 9</td>
<td>14 ± 2</td>
<td>8 ± 2</td>
<td>1.9 ± 1</td>
<td>4/23 (17%)</td>
<td>3/23 (13%)</td>
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<td></td>
<td>(n=23)</td>
<td>(n=23)</td>
<td>(n=15)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers shown in the third, fourth and fifth column are mean number of cells ± st.error. Cells were classified as apoptotic by DAPI when chromatin condensation and change in nuclear shape was observed and as TUNEL apoptotic when DNA exhibited positive labelling indicative of DNA fragmentation. CDI was calculated as a proportion of cells showing apoptotic changes per embryo based on the DAPI classification. The last two columns show numbers of blastocysts with depleted ICM cells or with a higher rate of cell death (above mean + st.err). None of the differences was statistically significant.

6.3.2. Expression of genes associated with cell death in human blastocysts.

We analyzed the levels of expression of cell death suppressor genes (Mcl-1, Bcl-x, Bcl-w and Bag-1), cell death inducers (Bax and Hrk) and cell death executioners (Casp-2 and -3). After hybridization of cDNA obtained from 37 single blastocysts with probe for EF-1α, only those
giving strong signal (n=32) were used for further analysis. This population of 2PN embryos had a mean cell number 55±4 (with a range of 22-94 cells) and cell death index (based on DAPI staining alone) of 12±1% (range 0-27%). Eight of the 32 blastocysts (25%) showed extensive cell death in the inner cell mass.

Levels of cell death suppressor gene expression were variable between different embryos, often ranging from undetectable to very high expression (Fig 2.A.). All blastocysts analysed in this study had high levels of *Mcl-1* (1819±210 cpm with range 86-4806 cpm/embryo) and *Bag-1* (2623±707 with range of 38-6173 cpm/embryo) mRNAs. No blastocyst had undetectable transcripts of these two genes, consistent with the hypothesis that these two genes may be important for successful preimplantation embryo development. Both *Bcl-x* and *Bcl-w* had lower, but detectable levels of expression (193±42 and 207±58 cpm) in the majority of embryos with ranges of 0-1164 and 0-1699 cpm respectively and were undetectable in 2 and 4 blastocysts, suggesting that expression of these genes is not fundamental for development of the embryo to the blastocyst stage.

*Bax* was the most abundant "killer gene" found in human blastocysts with mean levels of 1129±180 cpm, and with only 1 out of 32 blastocysts having no detectable expression (Fig 2.B.) *Caspase-3* was detected in all blastocysts with a mean level of 437±100 cpm (range 32-2239 cpm). The remaining genes, *Hrk* and *Caspase-2*, showed lower but still detectable levels of expression (mean 63±10 and 343±74 with ranges of 0-230 and 0-1304 cpm respectively).

Subsequently, we analyzed the relationship between the level of expression of all genes and normal or dead cell number in the blastocysts, using multiple regression analysis. The levels of expression of all genes except one failed to show any relationship with either healthy or apoptotic cell numbers in the blastocysts. Interestingly, the only gene whose expression level
correlated significantly with number of apoptotic cells per blastocyst was Bcl-x (P=0.005). After graphing Bcl-x levels versus number of dead cells per blastocyst 5 samples showing higher level of Bcl-x expression with few dead cells were observed. The mean level of Bcl-x expression in this group of blastocysts was significantly different from the rest (Kolmogorov-Smirnov test P<0.0001, see Fig 3.). This suggests that there may be two distinct population of blastocysts; those with high (n=5) or low (n=27) Bcl-x expression.

Table IV. Correlation between pairs of gene expression

<table>
<thead>
<tr>
<th>gene</th>
<th>Bag-1</th>
<th>Bax</th>
<th>Bcl-w</th>
<th>Bcl-x</th>
<th>Casp2</th>
<th>Casp 3</th>
<th>Hrk</th>
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<td>0.52</td>
<td>-0.24</td>
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The data shown are partial correlation coefficients and their P values derived by Sperman’s Rank correlation analysis.

Since it has been shown previously that cell death genes work as heterodimers (for see review by Jacobson , 1997), we also analyzed ratios of cell death suppressor and inducer pairs including Mcl-I/Bax, Mcl-I/Hrk, Bcl-x/Bax, Bcl-x/Hrk, Bcl-w/Hrk, Bcl-w/Bax (see Table IV.). Interestingly, there was a significant correlation between the levels of expression for some pairs of genes suggesting that they work in the same pathway or influence each other. These included Bag-1/Bcl-x (P=0.003), Bcl-w/Bcl-x (P<0.001), Hrk/Bcl-x (P=0.003), Mcl-1/Casp-3 (P=0.003) and Bax/Casp-3 (P<0.001). In all the cases correlations were positive. Thus, when a higher level of Bax expression was observed, this was accompanied by an increased level of Casp-3.
expression. However, there appeared to be no relationship between these ratios and the CDI of the blastocysts (results are summarized in Table IV.). Because blastocysts contain two different cell lineages (ICM and TE), that express different levels of some genes associated with cell death, we also analyzed blastocysts with depleted ICM cells separately and compared them with the remaining blastocysts, but again no statistically significant correlation with gene expression was observed.

6.4. Discussion.

PCD observed during blastocyst formation has been suggested as the first apoptotic event during mammalian development (Parchment 1993). However, the extent of cell death at this stage has never been adequately quantified. Our results on rates of cell death in the human blastocyst are very similar to the previously published report of Hardy et al., (1989). However, in some normally fertilized human blastocysts (14.4%), one of the two embryonic lineages (ICM) is preferentially eliminated leaving only precursor cells for the placenta. Such blastocysts may initiate implantation, but will create only extraembryonic tissues with an empty embryonic sac (blighted ovum), as is occasionally observed after infertility treatment (Jovych and Matson, 1988). Lack of ICM cells was previously described in human embryos cultured in vitro and was attributed to abnormal allocation of cells into the ICM and TE lineages (Winston et al., 1991). We propose that in some embryos, ICM cells are established in early developing blastocysts, but that they are eliminated subsequently via apoptosis. Because TE cells have extremely well developed phagocytic capabilities (Drake and Rodger, 1987), apoptotic bodies produced by dying ICM cells may be actively engulfed and degraded (Hardy et al., 1996).
Therefore, the remaining cells of the blastocyst are not affected by cytoplasmic debris produced by dying cells. The higher rate of cell death in human ICM cells was also observed by Hardy et al., (1989), but Hardy subsequently reported that cell death occurs with equal frequency in trophectoderm and ICM cells (1997).

Another developmental scenario which we observed in 16.6% of 2PN blastocysts, appeared to be caused by excessive activation of the PCD cascade in both cell lineages. These blastocysts, with extensive cell death, would likely be unable to initiate implantation and would not be recognized as a pregnancy. Thus, approximately 29% of human blastocysts at day 6 and 33% of blastocyst at day 7 appear to be affected by extensive PCD which would compromise their developmental potential.

Several published reports have confirmed that the cell death rate in mouse blastocysts is elevated when embryos are cultured \textit{in vitro} compared to \textit{in vivo} development. Brison and Schultz (1997) also observed higher rates of cell death when embryos were fertilized \textit{in vivo}, but cultured \textit{in vitro} as single embryos or in small groups. This is consistent with previous observations showing increased rates of blastocyst formation when a number of embryos are cultured together or when smaller volumes of medium are used (Lane and Gardner, 1992). Also, the cell death rate is elevated in blastocysts fertilized and cultured exclusively \textit{in vitro} (Jurisicova et al., 1998). This observation suggests that, apart from early exposure to potential detrimental effects of the culture conditions themselves, exposure of oocytes to a high concentration of sperm may also be detrimental to further embryo development, as previously proposed for human embryos by Dumoulin et al., (1992).

We have reviewed possible triggers of cell death in preimplantation embryos (Jurisicova et al., 1995b, 1997). Based on work published in the past five years on the deleterious effects of
high concentrations of glucose and phosphate on early preimplantation embryo development (for review see Bavister 1995), we proposed that high glucose coupled with depletion of glutathione and generation of reactive oxygen species could be responsible for increased rate of PCD in mammalian blastocysts (Jurisicova et al., 1997). This hypothesis is consistent with recent reports of decreased ICM number in diabetic rats (Pampfer et al., 1990) and increased PCD in rat blastocysts exposed to a high concentration of glucose (Pampfer et al., 1998). However, in the present study, we did not observe any difference in the rate of cell death in human blastocysts cultured in the usual concentration of glucose (2.78 mM in HTF medium) and in a low glucose medium (0.2 mM in KSOM). There was a trend towards a higher rate of ICM depletion in HTF medium in day 6 blastocysts, but this was not statistically significant.

During normal mouse preimplantation development, there appears to be only two stages that are susceptible to PCD, the late one cell stage and the blastocyst stage (Jurisicova et al., 1998). Blastomeres of early cleaving embryos seem to be quite resistant to induction of PCD by the protein kinase inhibitor staurosporine, suggesting that if these cells contain cell death machinery, it is strongly suppressed (Weil et al., 1996). Interestingly, both TE and ICM cells of the blastocyst lose this resistance, and when treated with staurosporine, they behave identically to somatic cells, suggesting a shift from strong suppression of apoptosis in cleavage stage embryos to activation of the apoptotic pathway in blastocysts (Weil et al., 1996). Thus, during the first developmental commitment event to either TE or ICM at the blastocyst stage, the cells also acquire the ability to undergo PCD.

Because cell death is genetically programmed, it is of interest to analyze the expression of genes associated with cell death and possibly define which genes are important for survival of preimplantation embryos. The majority of the analyzed genes showed no significant correlation
between their levels of mRNA expression and cell death in human blastocysts. There are several possible explanations for this. First, nuclear condensation and changes in nuclear shape are late events in cell death. Thus, most of the cell death decisions have already happened by the time of assay, and therefore it may be too late to correlate the level of expression for different transcripts with the cell death rate. Since RNA is also a target of degradation during cellular destruction, our cDNA pool might not have reflected active regulation of cell death, because dead cells may not have contributed intact mRNA to the RNA pool. Alternatively, cell survival or death decisions involving the genes we analyzed in blastocysts may be regulated by post-translational modifications. Phosphorylation, conformational changes, protein-protein and co-factor interactions with protein products involved in cell survival have already been shown in somatic cells.

The majority of blastocysts appeared to have well-balanced cell death machinery, since we found a highly significant positive correlation between levels of expression of several interactive partners of cell death protectors e.g., Bag-1/Bcl-x and Bcl-x/Bcl-w. On the other hand, increased levels of transcript for the cell death inducer Hrk were accompanied by elevated Bcl-x expression, probably balancing the effective killing potential of Hrk. Furthermore, higher levels of Bax correlated with increased levels of the protease Casp-3 which acts late in the apoptotic cascade, but not with Casp-2 expression.

In order to determine whether the function of cell survival genes is important for survival and proper development of the mouse, several genes analyzed in this study, have been disrupted by targeted mutations. Disruption of the cell death inducer, Bax, did not affect embryo development, but caused male sterility due to increased cell death in testis, while it increased resistance to follicular atresia in female ovaries (Knudson et al., 1995). Furthermore, oocytes
obtained from Bax-/- females are more resistant to apoptosis induced by chemotherapeutic agents, suggesting that this protein is involved in execution of PCD in oocytes, and possibly in early embryos (Perez et al., 1997). However, cell death rates in Bax-/- blastocysts have not been examined yet.

Mcl-1, a member of the Bcl-2 family (Kozopas et al., 1993), is abundantly expressed during human preimplantation embryo development. Moreover, all blastocysts had detectable levels of expression for this gene. Interestingly, in the Mcl-1 knock-out mouse model, blastocysts without a functional copy of the Mcl-1 gene could be detected at day 4 in the reproductive tract, but they failed to implant and were unable to grow in vitro at any time point of their development (Rinkenberger et al., 1997). We are currently examining whether early human embryos that fail to grow in vitro express any Mcl-1 mRNA and protein.

Another gene with a high level of expression throughout preimplantation development is Bag-1. The protein product of this gene is an interactive partner with several Bcl-2 family members (Takayama et al., 1995, 1997). It has also been reported to associate with growth factor receptors (Bardelli et al., 1997) and steroid receptors (Zeiner and Gehring, 1995). Bag-1 enhances the ability of Bcl-2 proteins to suppress cell death (Takayama et al., 1995) and conveys cell survival signals from activated receptors (Bardelli et al., 1996, Clevenger et al., 1997). All human blastocysts analysed in our study expressed the Bag-1 gene, but the levels of expression were extremely variable. A dramatically decreased level of Bag-1 gene expression has also been detected in fragmented mouse embryos, suggesting that this gene may be one of the regulatory components of the cell death pathway in mammalian preimplantation embryos (A. Jurisicova, in preparation).

One of the Bag-1 interactive partners, Bcl-x, was discovered through its sequence
homology to Bcl-2 (Boise et al., 1993). Bcl-x was the only gene in this study that showed significant correlation with cell death rates in human blastocysts. The experiments described above detected total Bcl-x polyA mRNA expression based on hybridization to 3’ untranslated region probes, and therefore could not distinguish between the different splicing products of the Bcl-x gene. Alternative splicing within the coding region of the Bcl-x gene results in production of two distinct proteins with contradictory functions Bcl-xL and Bcl-xS (Boise et al., 1993). Bcl-xS lacks the BH1 and BH2 domains of the Bcl family, and therefore likely has different interactive partners than Bcl-xL. Bcl-xL was reported to be a major cell death protector during post-implantation embryonic development (Garcia-Gonzales et al., 1995). A targeted mutation of the murine Bcl-x gene that disrupts both Bcl-xL and Bcl-xS causes mid-gestation (day 13) embryonic lethality due to increased rates of cell death in neuronal and hematopoetic cell lineages (Motoyama et al., 1995).

Very little is known about the mode of action Bcl-xS. The presence of Bcl-xS renders cells more susceptible to apoptotic signals even with the simultaneous expression of Bcl-xL (Minn et al., 1996, Heermeier et al., 1996). Thus Bcl-xS is able to counteract the protective effect of Bcl-xL. However, in somatic cells, this molecule does not seem to mediate its action through competitive binding with other Bcl-2 family members (Minn et al., 1996), suggesting that it may have other interactive killer partners. We observed that Bcl-xL is abundantly expressed during normal murine preimplantation embryo development. Interestingly, we detected expression of the Bcl-xS transcripts in a subset of fragmented murine embryos, suggesting that this protein may be involved in altering the Bcl-gene family balance and thus promoting cell death (Chapter 4.). Based on statistical analysis of Bcl-x expression in human blastocysts, we identified two populations of blastocysts, those with high and those with low Bcl-x expression.
We speculate that in the group of high expressors that contain very few dead cells, factors other than Bcl-x may be involved in regulation of cell death. In the low-expressor group, we propose that the Bcl-xL to Bcl-xS transcript ratio has been altered in favor of Bcl-xS. Thus, increasing levels of Bcl-xS transcript via alternative splicing, would upset the otherwise balanced cell death machinery maintained by the presence of Mcl-1, Bcl-w, Bag and Bcl-xL on one side and Bax or Hrk on the other side. Thus, it will be of considerable interest to determine whether Bcl-xS mRNA is being translated and whether its protein product localizes to cells undergoing apoptosis.
6.5. Figure Legends.

Figure 1. Expanded human blastocysts analyzed for apoptotic cells by assessing chromatin status and fragmentation of DNA.

A. Embryo was stained with nuclear dye diamidino-phenolindol (DAPI). Normal nuclei have pale blue staining in contrast to nuclei with condensed chromatin showing bright white staining. Very few cells shown signs of apoptosis.

B. Human blastocyst with extensive activation of PCD in many cells, as shown by bright staining with DAPI.

C. Blastocyst with preferential elimination of ICM cells shown with DAPI.

D. The same embryo after TUNEL using biotinylated nucleotides and streptavidin - Texas Red conjugate. The same cells that showed intense DAPI signal are also labelled after TUNEL as indicated by the bright red fluorescence.
**Figure 2.** Expression of cell death suppressor genes (A.) and cell death inducer genes (B.) in population of expanded 2PN fertilized human blastocyst. The panels show the data expressed in units of bound cpm. Bars denote the mean (SEM of expression obtained from 32 blastocysts.)
Figure 3. Correlation between $Bcl-x$ expression and number of dead cells in blastocysts. $Bcl-x$ levels are expressed in units of bound cpm. Two populations of blastocysts with either high level (squares=5) or low level of $Bcl-x$ expression (dots=27) are shown.
Chapter 7. Discussion.

7.1. *Mammalian Embryo Fragmentation is a Result of Activated PCD.*

Using combined nuclear staining and analysis of integrity of chromatin, 75% of human fragmented arrested embryos displayed hallmarks of apoptosis. Other cellular features that were found in fragmented human embryos by transmission and scanning electron microscopy were also consistent with apoptosis. These included the presence of undegraded cell corpses with dense cytoplasm, multiple cellular fragments that contained normal appearing cytoplasmic organelles, and dense masses resembling condensed chromatin. Corpses were not phagocytosed and were always found in the intercellular space within the zona pellucida. Some of the cellular fragments showed secondary necrotic changes with disrupted cellular membranes and swollen cytoplasmic organelles. Necrotic changes were also evident in some non-apoptotic blastomeres. No chromatin abnormalities, except for occasional multinucleation, were observed in morphologically normal appearing human embryos.

The population of embryos studied consisted of early cleavage stage embryos which arrested and failed to develop to the blastocyst stage *in vitro.* The distinguishing feature of these embryos was excessive blastomere fragmentation, which was easily visible under a dissecting microscope. Embryos having reached variable stages of development were processed 24 hours after the last observed cleavage. Therefore, embryos with asynchronously dying blastomeres were sampled at different times with respect to the first apoptotic event that produced the original fragments. This may account for the different categories of embryos observed in our population. Embryos with a few fragments but no condensed DAPI/TUNEL signals may represent an earlier stage of apoptosis than those with both fragments and condensed DAPI/TUNEL signals. Embryos showing evidence of necrotic changes may be more advanced in the apoptotic process,
although we cannot rule out the possibility that some of the cells/embryos died through arrest-mediated necrosis rather than apoptosis.

Blastomeres probably have no phagocytic capability since we found cell corpses in the intercellular space, but none, engulfed by other cells. Alternatively, blastomere corpses may not promote phagocytosis, possibly through inability to express the cell surface molecules, apogens, responsible for recognition of apoptotic cells. This hypothesis was favoured by Hardy (1997), who observed that cells of human blastocysts are able to phagocytose apoptotic cells produced during the blastocyst stage (Hardy et al., 1996), but do not seem to recognize and phagocytose un-incorporated blastomeres arrested in development at earlier cleavage stages. Thus, within developing early preimplantation embryos, cell corpses and fragments which are not phagocytosed effectively may undergo secondary necrosis, which, in turn, may trigger arrest and subsequent necrosis of surrounding blastomeres. Since the embryos in the present study failed to reach the blastocyst stage, it is possible that fragmented embryos that reach the blastocyst stage may have the ability to deal with cell corpses more effectively than their arrested counterparts.

The nuclear and cytoplasmic changes observed in fragmented embryos support the hypothesis that PCD and resultant apoptosis is responsible for the excessive cellular fragmentation in human embryos. These conclusions have recently been confirmed by reports from various laboratories using different approaches for detection of apoptosis (Guerin et al., 1997, Rimarachin et al., 1997).

Previous attempts to trigger apoptosis in normal blastomeres of healthy murine embryos yielded interesting results. Both oocytes and blastomeres of 2- and 4-cell stage murine embryos seem to be unique in their resistance to staurosporine induced apoptosis (Weil et al., 1996). After prolonged treatment with high doses of staurosporine and cycloheximide, both blastomeres and
oocytes died, suggesting the presence of a heavily suppressed cell death pathway within these cells (Weil et al. 1996). However, these authors did not report the presence of cellular budding in 2-8 cell mouse embryos exposed to various apoptosis inducing agents. We have tried to trigger cell death in late 1-cell zygotes and in 2-cell- and 4-cell-stage blastomeres by H_2O_2, sodium butyrate, and by generating reactive oxygen species with xanthine/xanthine oxidase, all without success. All of these agents have been shown to initiate apoptosis in somatic cells as well as in blastocysts (Parchment et al., 1991; Forrest et al., 1994; Hague et al., 1997). However, treatment of preimplantation blastomeres resulted in cell arrest that subsequently led to death with hallmarks more consistent with primary necrosis than with apoptosis. We have observed DNA condensation, and fragmentation (assessed by DAPI/TUNEL) in some of these embryos, but no evidence of cytoplasmic blebbing (A. Jurisicova and A. Weissman, unpublished observations). Since staurosporine treatment results in activation of Caspase-3, staurosporine induced cell death can be suppressed by peptides such as zVAD-fmk, which inhibit caspases in many cell types. Treatment of mouse blastomeres with staurosporine, cyclohexamide and zVAD-fmk had no effect on death rates (Jacobson et al., 1996) and it was proposed, therefore, that blastomeres may not express caspase enzymes. However, we detected expression of both Caspase-2 and -3 during preimplantation development. This observation further supports our hypothesis that staurosporine activation of cell death in blastomeres of preimplantation embryos is not a bona fide apoptotic process.

7.2. PCD during blastocyst development: How Many Cells can Die without Compromising Normal Development?

Several articles previously described the morphological appearance of dead cells in
mammalian embryos at the blastocyst stage. Total cell number and cell death in human blastocysts (evaluated by nuclear staining only) were previously studied by Hardy et al. (1989), but no conclusion was reached regarding the physiological range of cell depletion. We have further explored this issue of cell death in human and murine blastocysts with the objective of defining how many cells die during development and how that may affect the future developmental potential of the embryo.

In the mouse, the majority of freshly flushed blastocysts contain at least one cell exhibiting signs of apoptosis (Hardy, 1997). We have observed that the usual proportion of dead cells eliminated by PCD during mouse blastocyst formation in vivo or in vitro ranges between 3-5%. These rates are consistent with the previously published work of Handyside and Hunter (1988) as well as of Brison and Schultz (1997). Devreker and Hardy (1997) reported lower rates of cell death in blastocysts cultured in KSOM medium without glutamine in comparison to other commonly used media. Interestingly, in vitro culture and maternal age both increased the cell death index in in vitro cultured embryos up to 12% indicating that both factors are responsible for increased rates of cell death during preimplantation embryo development. Recently, TGF-α was reported to be one of several survival factors, which may be able to decrease the frequency of cell death at the blastocyst stage (Brison and Schultz, 1997a). Moreover, embryos with a knockout of the TGF-α gene cultured in vitro or maintained in vivo have a several-fold increase in the rate of cell death at the blastocyst stage (Brison and Schultz 1997b).

In the human, our observed rates of blastocyst cell death are two-fold higher than those in mouse blastocysts, even when they are cultured in what appears to be the most suitable medium for human blastocyst formation, KSOM (Erbach et al., 1994, Sommers et al., 1995).

In the majority of human blastocysts, cell death appeared random. Dying cells were
scattered in both ICM and trophectoderm. However, we identified a subpopulation of human embryos (14%), in which the majority of ICM cells had extensive condensation of chromatin and fragmented DNA. The resulting complete depletion of ICM cells could lead clinically to a spontaneous abortion classified as a so called “blighted ovum” (Jovych and Matson, 1988). Interestingly, we have never observed ICM depletion via PCD in mouse blastocysts, suggesting either that this developmental mechanism does not affect murine embryos as frequently as it does humans, or that the KSOM medium may be more suitable for mouse than for human preimplantation embryos. Furthermore, we have also observed that about 16% of human blastocysts had an increased cell death index. In the majority of blastocysts, this index did not exceed 10% and 23% on days 6 and 7, respectively. However, in some blastocysts, the cell death index increased markedly up to 56% indicating widespread activation of PCD.

PCD observed during blastocyst formation has been suggested as the first apoptotic event during mammalian development (Parchment, 1993). In our opinion, the mammalian blastocyst is the first embryonic stage at which PCD is a normal component of development, being responsible for cellular remodelling. However, PCD and apoptosis are initiated earlier in some human embryos as a suicide program to destroy the embryo. In addition, at the blastocyst stage, one of the two embryonic lineages (ICM) may be preferentially eliminated, leaving only precursor cells of the placenta. Such a blastocyst may initiate implantation, but will result in a placenta with an empty embryonic sac. If, on the other hand, too many cells activate their PCD cascade, further blastocyst development in both cell lineages can be compromised leading to failure of implantation.

Certain murine embryonal carcinoma cell lines with different developmental potential will selectively die, or survive and participate in further development, when deposited into the
blastocoele cavity. Cells that solely exhibit embryonic potential are more resistant to apoptosis than cells with the capability of forming trophoderm. These results led to the speculation that PCD within blastocysts may be designed to eliminate redundant ICM cells with trophodermal potential (Pierce et al., 1989). Furthermore, in an attempt to isolate factors responsible for this selective killing by the blastocoele fluid, extracellular hydrogen peroxide generated via polyamine oxidation was suggested to be a mediator of PCD in the blastocyst (Pierce et al., 1991).

7.3. Possible triggers of PCD during mammalian preimplantation embryo development.

Fragmentation and death of human embryos through apoptosis suggests that they may have a pre-programmed response to external stimuli, or else this may be a natural response to internal defects. Are these stimuli/defects abnormally high in IVF, and can they be reduced? Or is PCD and embryo wastage an intrinsic property of many human embryos? If IVF is at least partially to blame, then by manipulating different culture parameters it may be possible to determine the triggers of apoptosis. One possible trigger for mammalian embryo fragmentation is the artificial environment for fertilization and cleavage utilized during IVF. For example, hormonal stimulation leading to asynchronous nuclear/cytoplasmic maturation, inappropriate culture conditions, and exposure to high concentration of sperm may all contribute to excessive activation of PCD.

7.3.1. Factors associated with IVF.

Our experiments suggest that only 12% of in vivo fertilized murine embryos harvested from young (12 week old) females of the outbred CD-1 strain contain fragments similar to those observed in human embryos. Interestingly, the frequency of early embryo fragmentation did not change when 24-26 week old females were used. However, when maternal age reached 40-44
weeks, the frequency of fragmentation doubled, suggesting that abnormalities in the embryos derived from old eggs were responsible for PCD. Maternal age has been previously associated with a higher rate of chromosomal aneuploidies in oocytes and embryos (Zenzes et al., 1992), precocious dissociation of chromatids in Metaphase II arrested human oocytes (Lim and Tsakok, 1997), increased rate of global DNA damage (Fujino et al., 1996; Lopes et al., 1998/a) and increased rate of spindle abnormalities due to abnormal follicular environment (Van Blerkom et al., 1996). We would also like to propose that with increased maternal age supporting ovarian somatic cells as well as growing oocytes are not as metabolically active and fail to endow the mature egg with sufficient amounts of maternally stored products. Subsequently, lack of or insufficient deposition of maternal products results in abnormal embryonic development, leading to elimination of the embryo by PCD.

Murine embryo fragmentation was increased almost 3-fold by IVF in all age groups compared to in vivo fertilization, implicating in vitro culture conditions and possibly exposure to a high concentration of spermatozoa as possible external triggers of cell death. In addition, both paternal and maternal genetic background was also shown to affect the frequency of early embryonic cell death. Reactive oxygen species (ROS), such as H$_2$O$_2$, have been implicated as mediators of apoptosis in somatic cells (Ratan et al., 1991) and in murine embryonic carcinoma (Pierce et al., 1991). Mouse oocytes and embryos on their own are able to generate small but measurable amounts of H$_2$O$_2$ which increase during the transition from the 2 to 4 cell stage. This rise in H$_2$O$_2$ may be attributed to the in vitro culture conditions where O$_2$ concentrations are relatively high (Nasr-Eshafani et al., 1990). Whether a similar situation exists in vivo, where the O$_2$ concentration is low, is currently unknown. Recently, it was reported that fragmented human embryos contain higher intracellular level of ROS than unfertilized oocytes or morphologically
normal human embryos (Hwang et al., 1997). However, at present, it is not clear whether a high concentration of ROS is a reason for, or a consequence of, an activated cell death program.

The healthy unfertilized oocyte contains glutathione, a tripeptide, which effectively protects against reactive oxygen radicals. Glutathione levels decrease during fertilization and cleavage to the blastocyst stage, when glutathione synthesis is renewed. Levels of glutathione are significantly decreased in embryos cultured in vitro compared to those developing in vivo (Gardiner and Reed, 1994). The exposure to greater concentrations of ROS from the culture conditions or from sperm may contribute to the rapid depletion of glutathione and thus may disturb the redox balance in early embryos. Glutathione levels seem to be affected by the apoptosis. An intracellular decrease of glutathione in somatic cells precedes the onset of apoptosis (Beaver and Waring, 1995) and compounds that increase intracellular glutathione, such as β-mercaptoethanol, or that stimulate activity of glutathione peroxidase (like N-acetyl-L-cysteine) can prevent the induction of apoptosis (Ferrari et al. 1995; Tilly et al. 1995).

Although the combination of phosphate and glucose seems to interfere with early preimplantation embryo development in some strains of mice and in other mammalian species (Bavister 1995; Scott and Whittingham, 1996), almost all media used for human embryo culture contain phosphate and relatively high concentrations of glucose. As already mentioned above, unfertilized mouse oocytes contain a high concentration of glutathione. Recently, a link between glucose toxicity and glutathione metabolism was established. Rat postimplantation embryos exposed to a high concentration of glucose were reported to have decreased levels of glutathione most likely due to decreased activity of glutathione synthetase (Trocino et al., 1995). Glucose inhibition of glutathione synthetase, with depletion of intracellular glutathione, may lead to activation of PCD by accumulation of ROS and subsequent cellular damage. Additionally, since
mitochondria are readily damaged by oxygen radicals (see review Barnet and Bavister, 1996), it is likely that increased exposure of the embryo to ROS may decrease the number of active mitochondria. Since mitochondrial replication does not occur until the blastocyst stage (Dvorak and Tesarik, 1985), each blastomere receives a finite number of oocyte-derived mitochondria which decreases by 50% with each cell division. Therefore, a critical reduction in mitochondrial content could occur after several cell divisions, leading to impaired energy production for the blastomeres and initiation of PCD. Additional evidence for the protective role of glutathione during development comes from supplementation of embryo culture media with agents such as β-mercaptoethanol or cysteamine. These compounds increase glutathione metabolism, during bovine oocyte maturation as well as throughout embryo development and they have been reported to result in an increased rate of blastocyst formation in vitro (Matos et al., 1995).

Co-culture techniques using a variety of epithelial cells improved embryo morphology with a decreased amount of fragmentation and an increased rate of blastocyst formation in vitro (Bongso et al., 1991, 1994). However, it is unclear exactly what underlying mechanisms may mediate the positive effect of co-culture. Cells in co-culture may secrete growth factors or other embryotrophic products which foster embryonic growth. For example, leukaemia inhibitory factor (LIF) was shown to be released by Vero cells. This cytokine plays a crucial role in mouse blastocyst implantation (Stewart et al., 1992) and is able to suppress PCD in primordial germ cells (Pesce et al., 1993). However, our results indicate that LIF does not have a significant protective effect on blastocyst formation in vitro in human embryos (Jurisicova et al., 1995a). Alternatively, co-cultured cells may remove and metabolize undesirable toxins and decrease oxygen concentration in the medium, thus protecting embryos from oxidative stress. This is in agreement with the observation that the rate of embryo fragmentation is decreased by co-culture
in comparison with regular culture conditions (Weimer et al., 1989).

Routine IVF practice is to incubate oocytes with 50,000-100,000 washed sperm overnight, and to assess fertilization the next morning. In contrast, normal in vivo fertilization probably involves exposure of the oocyte in the fallopian tube to a thousand-fold fewer sperm. It is possible that prolonged exposure to a large number of sperm may expose the oocyte to enzymes and other factors released during the acrosome reaction which may be detrimental to embryo development (Dumoulin et al., 1992) and be responsible for triggering PCD. For example, hyaluronidase, a component of the acrosome, will activate oocytes (Kaufmann 1983) and is known to be toxic to embryos cultured in vitro with prolonged exposure (De Silva et al., 1990). A second potential mediator of PCD during IVF is oxidative stress generated by the sperm suspension as discussed above.

Furthermore, intrinsic sperm factors, such as integrity of the sperm DNA may also contribute to the high rate of embryo demise. Experiments in our laboratory (Sun et al., 1997) demonstrated that in normal fertile men a low percentage of sperm (<4%) contain fragmented DNA. In contrast, up to 75% of sperm from infertile men have fragmented DNA, and the percentage was negatively correlated with semen analysis parameters, fertilization and cleavage rates in IVF (Sun et al., 1997, Lopes et al., 1998b). It is possible that sperm with damaged DNA can penetrate oocytes in IVF procedures. Perhaps more importantly, it is likely that some sperm with fragmented DNA are selected for intracytoplasmic sperm injection (ICSI), especially since extremely poor quality semen is the indication for this technique (Lopes et al 1998b). Although it is not known whether sperm with fragmented DNA can fertilize an oocyte, a number of possible outcomes could ensue: First, the oocyte may fail to be fertilized normally with no or partial paternal chromatin decondensation. It has been shown (Balakier et al., 1993, Van Blerkom et al.,
1994) that between 20% and 50% of apparently unfertilized oocytes, or in zygotes with 1 PN presumably parthenogenetically activated in IVF, have had unrecognized sperm penetration with failure of chromosomal decondensation and pronucleus formation. Moreover, our experiments (Lopes et al., 1998a), as well as experiments performed in other laboratories (Rybouchkin et al., 1997) showed that partial decondensation of paternal chromatin was observed in oocytes that remain unfertilized after ICSI and these often exhibited fragmented DNA. Second, fertilization could occur but the zygote arrests at the pronuclear or late 1 cell stage. Third, fertilization could occur with cleavage and subsequent apoptosis and fragmentation. All three outcomes are seen in our present IVF and ICSI programs.

Other abnormalities associated with the paternal contribution to the developing embryo could possibly activate cell death (such as lack of a paternal centrosome contribution or other abnormalities involving chromatin remodelling (Van Blerkom et al., 1995, Hewitson et al., 1996), or abnormal patterns of early paternal gene expression due to improper methylation of DNA Doerkson and Trasler 1996)

PCD in human embryos may result from inherent abnormalities of the oocyte or sperm. For example, if chromosomal aneuploidy is present, PCD may be a protective mechanism to prevent implantation and further development of an abnormal embryo. Genetic imbalance of some gene critical product(s) resulting from chromosomal anomalies could be responsible for activation of PCD. Of interest in this regard, cytogenetic observations of spare human embryos confirmed the presence of a spectrum of nuclear anomalies, e.g. multinucleated or anucleated blastomeres, and flocculent and fragmented nuclei, (Winston et al 1991, Hardy et al 1993). A wide range of chromosomal abnormalities including premature chromosome condensation, aneuploidy and polyploidy has been reported in spare human embryos (Papadoupoulos et al
1989. Zenzes and Casper, 1992). Compared to embryos with good morphology, a higher incidence of these cytogenetic abnormalities was found in embryos with fragmentation (Michaeli et al. 1990, Pellestor et al. 1994). However, karyotyping blastomeres from highly fragmented embryos has not always been successful (Zenzes et al. 1992), possibly because of the rapid DNA degradation by endonucleases during PCD. Only 29% of such fragmented human embryos produced readable chromosomal spreads. In addition, chromosomes can be artifactually lost during the metaphase spread, resulting in false diagnoses of hypoploidy by karyotyping. An alternative technique is to use labeled centromeric DNA probes (fluorescent in situ hybridization; FISH) for chromosomes, which are commonly affected by non-disjunction. Using a multicolour FISH technique, several chromosomes can be detected simultaneously and counted with fluorescence microscopy in interphase cells. However, one has to be very careful about interpretation of these results. It is possible that chromosomes in which the centromeric DNA has already begun to be cleaved may contain multiple signals. We believe that the presence of fragmented DNA may explain the observation of FISH signals of up to 20N in human monospermic fertilized embryos with extensive cellular fragmentation (Munne et al., 1994). A correction factor could be applied, however, by using TUNEL in the same cells to determine which nuclei have already initiated DNA fragmentation and which have intact DNA.

7.3.2. Factors associated with oocyte development.

Could oxygen tension, follicular fluid pH and granulosa cell death from distinct follicles influence and reflect developmental competence of oocytes? Gaulden (1992) hypothesized that oocyte chromosomal aneuploidy may originate as the result of a deteriorating intrafollicular environment secondary to hypoxia. It is known that oxygen tension in follicular fluid decreases as the follicle expands to its normal mature size of approximately 2 cm in diameter (Fisher et al
1992). Some rapidly enlarging follicles may outpace their blood supply resulting in decreased follicular fluid oxygen concentration and lowered intrafollicular pH which could adversely affect the nuclear spindle in the developing oocyte. Morphological changes associated with apoptosis appear to be preceded by cellular acidification (Gotlieb et al. 1995) which is consistent with the decreased cellular pH detected in some human oocytes (Van Blerkom, 1995).

Evidence of apoptosis in granulosa cells from some but not all aspirated follicles during human IVF has been reported (Piquette et al., 1994). In this small study, no correlation between the extent of cell death in granulosa cells, follicle size, color of follicular fluid or fertilizability of the oocytes was observed. Unfortunately, no observation of embryo quality or developmental potential was recorded. Recently, a correlation between a low rate of apoptosis in granulosa cells and pregnancy rates in patients undergoing IVF treatment was reported (Nakahara et al., 1997). Unfortunately, cell death assessments were done on pools of cells from all follicles of one patient, and thus, it was not possible to compare quality of embryos originating from follicles with low versus higher rates of cell death in accompanying granulosa cells. Apoptosis of granulosa cells may reflect a premorbid follicular state, which will be expressed as poor developmental competence of the oocyte upon fertilization, and thus might be used as an indicator for selection of suitable embryos for transfer.

Controlled ovarian stimulation using exogenous gonadotropins to obtain multiple oocytes may lead to an accelerated follicular response, resulting in "biochemical immaturity" of the cytoplasm of the oocytes. Embryos arising from such oocytes may not be able to proceed through development if, for example, their PCD "machinery" is unbalanced due to abnormal deposition of maternally stored products.

Using mouse embryos as the experimental system, we observed that embryo
fragmentation occurs between activation of the oocyte and the 2-cell stage. This timing is slightly different from that of fragmentation in human embryos, which occurs between the 2 to 8 cell stage. However, rodent embryos activate their genomes earlier in development at the 2-cell stage (Flach et al., 1982) while human embryos seem to initiate transcription from the embryonic genome one to two cleavages later at 4-8 cell stage (Braude et al., 1988, Tesarik et al., 1988).

Thus, it appears that in both rodents and humans, PCD and the resulting fragmentation precedes or is coincident with the activation of the embryonic genome. This also may explain why some fragmented human embryos are able to establish a viable pregnancy, but fragmented mouse embryos never proceed further in development than the blastocyst stage (unpublished observation). In the human, when cell death occurs in some blastomeres, other blastomeres may successfully suppress cell death via proper activation of the embryonic genome. These intact totipotential blastomeres can then re-populate the embryo and give rise to all the cell types needed for normal development. This suggestion is consistent with our observation that fragmented mouse embryos never proceed further in development. The chances for successful development are lower if cell death occurs at the 2-cell stage, since the amount of necrotic cytoplasm resulting from secondary necrosis of apoptotic bodies may damage the remaining blastomeres. In the mouse, since PCD occurs at the one cell stage, it is always lethal. Thus, the fundamental difference in the timing of the transition from the maternal to embryonic genomes observed during preimplantation development in mouse and human embryos ultimately has an impact on PCD effects on reproductive outcome. In the mouse, 10-20 oocytes are ovulated during a natural cycle, often resulting in litter of 10-12 viable pups (Rugh 1990). In humans, a single egg is usually released in each cycle. It is possible that blastomere cell death at a later developmental stage is a mechanism of giving the single human embryo an additional chance for elimination of
abnormal cells and subsequent survival.

It is known that specific interactions established during fertilization by sperm penetration and later zygotic genome activation are required for further embryonic development. Interference with these processes may lead to initiation of PCD and elimination of abnormal embryos. Oocytes seem to contain a built in program for apoptosis which remains quiescent until the mature ovulated oocyte undergoes activation. Oocytes arrested at metaphase II, which fail to be fertilized and do not resume meiosis, become necrotic within 2-4 days. However, once the killer program is triggered by or after oocyte activation, it can be suppressed only by proper signal(s), possibly originating from the oocyte or generated by the embryo itself. This system may ensure that only normal embryos proceed to the implantation stage and it could be one of the mechanisms preventing development of parthenogenetic embryos as observed in females lacking a functional c-mos gene (Colledge et al., 1994).

Interestingly, mouse embryos that undergo apoptosis not only contain low levels of mRNA for cell death supressors, but also appear to have augmented their maternal stores of factors that may promote PCD. We observed the production of Bcl-xS transcripts in some embryos undergoing fragmentation, and also detected an increase in expression of MA-3 and p53 transcripts. In embryos that are destined to develop, such increases in the expression of cell death inducer genes would be counteracted by increased expression of cell death repressors. Consistent with this prediction, we observed an apparent increase in the hybridization signals obtained with the probe for Bcl-x, in mouse embryos and also obtained evidence for a low level of transcription of the embryonic Bcl-2 gene. Bcl-x expression was entirely attributable to the protective Bcl-xL form of the transcript in embryos successfully proceeding through the one-cell stage. In addition, we detected maternal expression of Bcl-x, Bcl-2, and Bcl-w. Thus, embryonic expression of
protector genes augments the maternally deposited products of these genes.

7.4. Can Aspiration of Cellular Fragments Improve the Developmental Potential of Remaining Blastomeres?

During the course of this study, we have also observed that fragmented human embryos exhibit a polarity, with a tendency for normal appearing blastomeres to be clustered on one side of the embryo and fragments on the other. This observation suggests the possibility that cells originating from one parental blastomere are preferentially being eliminated. One possible explanation could be asymmetric cleavage of the zygote into two cytoplasmically distinct blastomeres. Unequal division of cytoplasmic components could occur during the first cleavage of the zygote to a 2-cell embryo, resulting in 2 separate cell lineages with different developmental potential. Subsequent cell divisions could result in varying resistance to apoptosis depending on the number of mitochondria (containing products of the Bcl gene family) in each cell, or on the amount of ROS scavenger activity (e.g. reduced glutathione) each daughter cell receives. If unequal division of mitochondria occurs at the first cell division, a critical shortage of these cellular organelles could occur in the subsequent cell line derived from the deficient sibling blastomere. Cohen et al (1994) reported successful aspiration of fragments from human embryos and transfer of the remaining intact blastomeres in an attempt to rescue these embryos. However, the results of preliminary experiments performed in our IVF laboratory involving aspiration of cellular fragments from 24 human 4 to 8-cell stage embryos, in which at least 30% of the volume of the embryo was filled with fragments, suggested that most of the remaining normal appearing blastomeres will continue to fragment. This finding supports the idea of a pre-programmed set of
events within these embryos with commitment of the embryo to die. However, a small proportion of embryos did go on to blastocyst formation after aspiration of fragments.

8. Conclusions.

We have demonstrated that apoptosis occurs in human embryos prior to the blastocyst stage, and is clearly detrimental to the subsequent developmental potential of these embryos. Further work is needed to determine the nature of both intrinsic and extrinsic triggers of PCD and potential protective measures. Our experiments with fragmented embryos are still preliminary, but we believe that they do show a trend towards active regulation of cell death decisions on a transcriptional level. However, it is possible that fragmented embryos originated from oocytes with unbalanced protection machinery (e.g. contained elevated levels of maternally stored Bad, p53 or Bcl-xS mRNA) as a consequence of hormonal recruitment of follicles otherwise pre-destined to undergo atresia via apoptosis. Embryos arising from such fertilized oocytes may not be able to suppress the activated over-powering build-up of the cell death program by signals from the embryonic genome. Once we can identify "natural" triggers of embryo fragmentation, it will be easier to carry out these experiments, since we might be able to assess the pattern of gene and protein expression prior to active degradation of the cell and its compartments. Application of this research to clinical IVF may lead to methods of selecting IVF embryos with normal developmental potential and to improved implantation and pregnancy rates in the future.
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