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Role of the *Ped* Gene and Apoptosis Genes in Control of Preimplantation Development

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Purpose: *The properties of the mouse* Ped *gene and the genes that mediate apoptosis in mediating preimplantation embryonic survival were reviewed.*
Methods: *Preimplantation mouse oocytes and embryos were evaluated microscopically and biochemically for rate of development, degree of fragmentation, and gene expression to correlate these characteristics with embryo mortality. Biochemical assays included PCR for DNA analysis, RT-PCR for mRNA analysis, immuno-PCR for protein analysis, and TUNEL assay for assessment of apoptosis.*
Results: *Using the mouse as a model system we have identified a gene that controls the rate of development, the* Ped *gene. The* Ped *gene product is a class Ib major histocompatibility complex protein called the Qa-2 antigen. Research to understand the molecular mechanisms of Ped gene action and to identify the human homologue of the* Ped *gene is under way. We have also shown using the mouse model, that fragmented embryos show the morphological and biochemical characteristics of apoptosis. Genes in the two major gene families that regulate apoptosis, the caspase and Bcl-2 families, are expressed in mouse oocytes and preimplantation embryos.*
Conclusions: *Preimplantation embryonic survival depends on two major morphological parameters: rate of development and degree of fragmentation. A fast rate of development and a low degree of fragmentation lead to a better chance of producing live offspring. Both rate of development and degree of fragmentation are genetically controlled, the former by the* Ped *gene and the latter most likely by genes that mediate apoptosis. It seems probable that regulation of apoptosis will prove to be a major mechanism that mediates oocyte and preimplantation embryonic survival.*

KEY WORDS: *Ped* gene; apoptosis genes; mouse; preimplantation development.

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INTRODUCTION

The identification of genes that mediate important phenotypes in preimplantation embryos is critical for understanding mechanisms that confer a survival advantage to some embryos but not to others. Using the mouse as a model system we have identified one such gene, the *Ped* gene, that controls the rate of preimplantation cleavage division and subsequent embryo survival. In this article we review the properties of the mouse *Ped* gene, discuss future research directions, including the search for a human homologue of the mouse *Ped* gene, and present preliminary evidence that a new set of genes, those involved in apoptosis, is involved in mediating preimplantation embryonic survival.

THE MOUSE *PED* GENE

Discovery of the *PED* Gene

During the 1970's my laboratory had the opportunity to collect and evaluate more than 50,000 preimplantation mouse embryos from a variety of inbred mouse strains for our studies on preimplantation development. We observed that even though each of the strains had been superovulated with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) at the same time, at a given time post-hCG injection, some strains had embryos at a more advanced stage of development than others. For instance, at 89 h post-hCG a fast-developing embryo is at the blastocyst stage (approximately 32 cells), whereas a slow-developing embryo is at the morula stage (approximately 16 cells) of development (Fig. 1). We realized that the rate of development was correlated with the major histocompatibility complex (MHC) type of the mice and that

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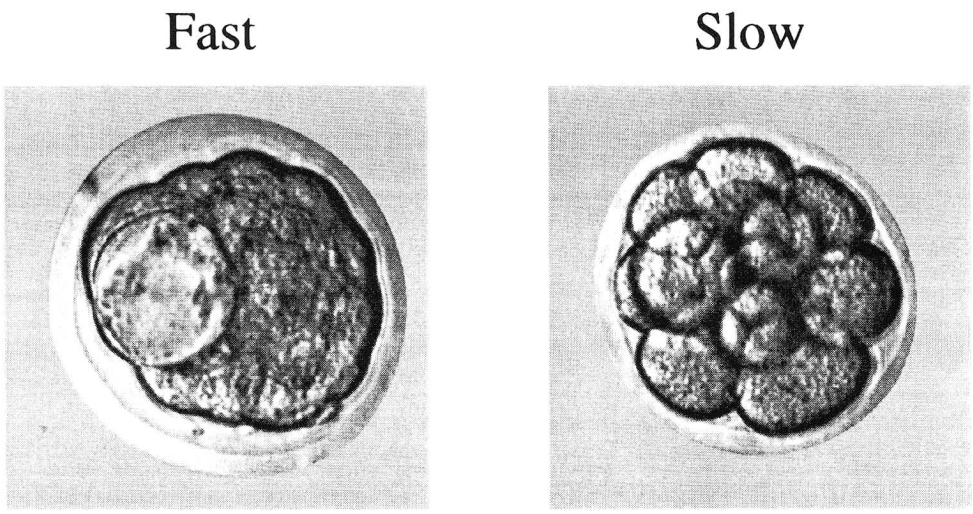

Fig. 1. Fast- and slow-developing mouse embryos. Embryos were collected at 89 hr post-hCG and photographed with a Nikon Diaphot microscope with Nomarski DIC optics at 300× magnification.

mice that were of the k haplotype always developed more slowly than mice with other MHC haplotyes. We hypothesized that there was a gene in the MHC, which we named *Ped* (preimplantation embryo development), that controlled the rate of cleavage of preimplantation mouse embryos (1).

The Mouse *Ped* Gene: DNA, RNA, and Protein

It was not easy to clone the *Ped* gene because only limited DNA sequence information is available for the mouse MHC. A combination of positional mapping, intuition, and luck allowed us to clone the gene in 1993 (reviewed in Ref. 2). We now know that the protein product of the *Ped* gene is a class Ib MHC protein called the Qa-2 antigen. The Qa-2 antigen is encoded by four similar genes, Q6, Q7, Q8, and Q9, but only the Q7 and Q9 genes are responsible for the *Ped* gene phenotype (3,4). Figure 2 shows the results of polymerase/chain reaction (PCR) analysis of genomic DNA from a prototype *Ped fast* mouse strain and a prototype *Ped slow* mouse strain. It can be seen that the strain with the *Ped fast* phenotype has the presence of the Q7 and Q9 genes, whereas the strain with the *Ped slow* phenotype has a deletion of both of these genes. In fact, the deletion includes all four Qa-2 encoding genes, Q6, Q7, Q8, and, Q9, but only Q7 and Q9 are transcribed in mouse embryos, as shown by reverse transcription (RT)-PCR (Fig. 3).

At the protein level, those strains that are *Ped fast* express Qa-2 antigen on the embryonic cell surface, whereas those that are *Ped slow* have the absence of Qa-2 antigen, as expected based on the known deletion of the Qa-2 encoding genes in the *Ped slow* strains. The level of Qa-2 antigen on the surface of embryos from *Ped fast* mice is below the level of detectability by immunofluorescence. However, Qa-2 antigen can be shown to be present on the embryonic cell surface by using multiple embryos in an enzyme-linked immunosorbent assay (ELISA) procedure (6) or by using single embryos in a recently described technique called immuno-PCR (7) (Fig. 4). The immuno-PCR technique has exquisite sensitivity and can detect fewer than 1000 molecules of protein on an embryo. The technique should be generally useful for the detection of any gene product that is expressed on the embryonic cell surface.

Recently, we have used immuno-PCR to examine *Tap-1* knockout mice for cell surface expression of Qa-2 antigen. TAP, a heterodimeric protein of TAP1 and TAP2 subunits, facilitates transport of small peptides into the endoplasmic reticulum (ER). In the ER, class I MHC molecules bind the peptides, and the class I MHC–peptide complex is then transported to the cell surface. We found that in *Tap-1* knockout mice, the Qa-2 antigen is expressed internally but not on the cell surface (Ke and Warner, unpublished). Importantly, the *Ped* gene phenotype of *Tap-1* knockout mice is *slow*, showing that Qa-2 antigen must be expressed on the cell surface to be functional.

The Qa-2 protein is an unusual class I MHC protein because it is linked to the embryonic cell surface by a glycosylphosphatidylinositol (GPI) linkage (7). If one cleaves Qa-2 antigen off the cell surface with phospholipase C, the rate of cleavage of the embryos is decreased. It remains to be determined whether add-

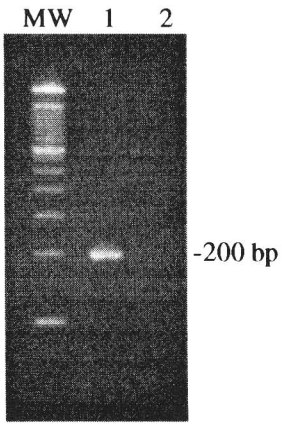

Fig. 2. PCR detection of the *Ped* gene at the DNA level. Individual embryos from a prototype *Ped* fast mouse strain, C57BL/6, and a prototype *Ped* slow mouse strain, CBA/Ca, were analyzed. Lane 1: 100-bp DNA ladder used as a size marker. Lane 2: PCR on a C57BL/6 blastocyst. Lane 3: PCR on a CBA/Ca blastocyst. To obtain embryo lysates for the PCR reaction, blastocyst embryos were first collected in Whitten–Biggers medium at 89 hr post-hCG and washed in PBS three times. Then individual embryos were transferred into a 0.5-ml thin-walled PCR tube in a small volume of phosphate-buffered saline (PBS) and 5 μl of lysis buffer [50 m*M* KCl 20 m*M* Tris–Cl (pH 8.3), 2.5 m*M* $MgCl_2$, 0.5% Tween 20, 100 μg/ml proteinase K] were added to the tube. The mixture was then incubated at 56°C overnight. To inactivate proteinase K, the tubes were heated at 99°C for 15 min. Five microliters of embryo lysate were used as the template for the PCR reaction. The primers used were designed to amplify a 200-bp sequence in exon 4 from the Q7 and Q9 genes. The upstream primer is 5′ CAGGTCT-TATGGTGCTGTCA 3′, and the downstream primer is 5′ GTGTAATTCTGCTCCTTC 3′. The PCR reaction was performed in a total reaction volume of 50 μl, including 5 μl of embryo lysate, 5 μl of 10× PCR buffer, 4 μl of dNTP (10 m*M* each), 2.5 μl of 25 m*M* $MgCl_2$, 1 μl of 10 μ*M* upstream primer, 1 μl of 10 μ*M* downstream primer, 31 μl of H_2O, and 0.5 μl of 5 U/μl Taq polymerase (GIBCO BRL, Gaithersburg, MD). The PCR was run in a Perkin–Elmer Cetus Thermo Cycler (Norwalk, CT) using the following conditions: 1 cycle at 96°C for 1 min for initial denaturation; 2 cycles at 96°C for 1 min for denaturation, 58°C for 30 sec for annealing, and 72°C for 30 sec for extension; and 40 cycles using a setting of 94°C for 1 min for denaturation, 58°C for 30 sec for annealing, and 72°C for 30 sec for extension, with a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 6% polyacrylamide gel followed by staining with ethidium bromide.

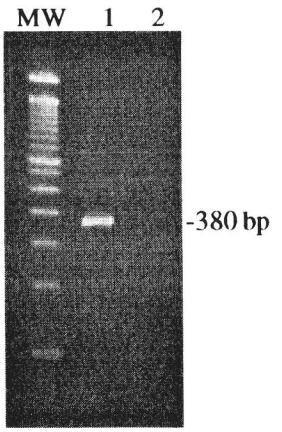

Fig. 3. RT-PCR detection of the *Ped* gene at the RNA level. Individual blastocysts from a prototype *Ped* fast mouse strain, C57BL/6, and a prototype *Ped* slow mouse strain, CBA/Ca. Lane 1: 100-bp DNA ladder was used as a size marker. Lane 2: RT-PCR on a C57BL/6 blastocyst. Lane 3: RT-PCR on a CBA/Ca blastocyst. To obtain embryo lysates for reverse transcription, blastocyst embryos were first collected in Whitten–Biggers medium at 89 hr post-hCG and washed in PBS three times. Then an individual embryo was transferred into a 0.5-ml PCR tube in a small volume of PBS and 2 μl of lysis buffer containing 10 m*M* dithiothreitol and 0.5% NP-40 and 0.5 U/μl RNase inhibitor were added. Then 1 μl of 50 μ*M* random hexamer primers and RNase-free water were added to bring the total volume to 8 μl. RNA was denatured at 70°C for 5 min and then annealed at 30°C for 5 min. To obtain cDNA, 4 μl of 25 m*M* $MgCl_2$, 4 μl of dNTP mixture (10 m*M* each), 2 μl of 10× PCR buffer II, 0.25 μl of 200 U/μl MuLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD) were added to bring the total reaction volume to 20 μl and the reaction mixture was incubated for 3 hr at 37°C. At the end of the incubation time the reaction was heated at 99°C for 5 min to inactivate the reverse transcriptase. Primers used for PCR were designed to amplify a 380-bp sequence from Q7/Q9 transcripts. This set of primers was designed in two exons (upstream primer, 5′ TGGTATTGCAGAGAAAGACCA 3′, located in exon 3 and downstream primer, 5′ ATCTCCCCCATCT-CAGGGTA 3′, in exon 4) so that the product amplified from the Q7/Q9 mRNA could be distinguished from the different size product (2400 bp) amplified from any contaminating genomic DNA or unprocessed RNA. The amplification of the cDNA was performed in a 100-μl reaction mixture containing 0.5 blastocyst-equivalent of cDNA, 6 μl of 25 m*M* $MgCl_2$, 8 μl of dNTP (10 m*M* each), 10 μl of 10× PCR buffer II, 2 μl of each primer (10 μ*M*), 0.5 μl of 5 U/μl Taq polymerase (GIBCO BRL), and sterile water. The cDNA was amplified in a Perkin–Elmer Cetus Thermo Cycler (Norwalk, CT) using the following conditions: 1 cycle at 96°C for 1 min; 2 cycles using a setting of 96°C for 1 min for denaturation, 58°C for 45 sec for annealing, and 72°C for 45 sec for extension; and 40 cycles using a setting of 94°C for 1 min for denaturation, 58°C for 45 sec for annealing, and 72°C for 45 sec for extension, with a final extension at 72°C for 5 min. The RT-PCR products were analyzed by electrophoresis on a 6% polyacrylamide gel, followed by staining with ethidium bromide.

ing Qa-2 antigen to the cell surface of embryos missing Qa-2 antigen by using a procedure called "protein painting" (8) will increase the cleavage rate of the embryos.

A Human Homologue of the Mouse *Ped* Gene

Mouse embryos that develop at a fast rate have a better chance of survival than slow-developing embryos. Data from human in vitro fertilization (IVF) clinics suggest that exactly the same situation exists for human embryos (2,9–11). It would, therefore, be beneficial for enhancing pregnancy outcome to find a

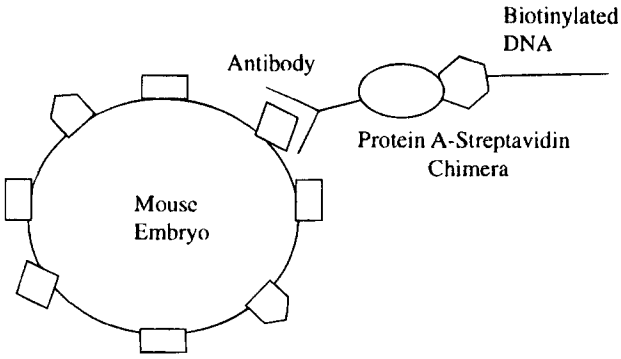

Fig. 4. Immuno-PCR assay for protein detection on single mouse embryos. In the procedure recently described by McElhinny and Warner (2), preimplantation embryos are collected and the zona pellucidae removed by a brief incubation in Acid Tyrode's (pH 2.5), followed by washing in PBS + 1% bovine serum albumin (BSA)(PBSA). The embryos are then incubated in the first antibody (0.05–0.5 mg/ml), or a control antibody at the same concentration, at 4°C for 1 hr. After extensive washing, the embryos are then incubated with a recombinant chimeric protein complex (protein A–streptavidin) at a concentration of 1×10^{-15} *M*, at 4°C for 1 hr, and then washed extensively. The protein A domain of the chimeric protein recognizes the Fc portion of the antibody, and the streptavidin domain binds to an added biotinylated fragment of DNA. The entire reaction mixture is subjected to PCR. If a band of the appropriate molecular weight appears on an electrophoresis gel after PCR amplification, this means that the first antibody bound to a specific molecule on the embryonic cell surface.

human homologue of the mouse *Ped* gene. We have initiated a search for the human homologue of the mouse *Ped* gene in collaboration with Dr. Carol Brenner and Dr. Jacques Cohen of the Saint Barnabas Hospital Institute for Reproductive Medicine, West Orange, NJ. We have tested, by RT-PCR, approximately 100 spare human embryos and found no correlation of human leukocyte antigen (HLA)-G expression with rate of development (Warner *et al.*, unpublished). Thus, it is very unlikely that HLA-G is the human homologue of the mouse *Ped* gene, despite the suggestion by other workers that this might be the case (12,13). Moreover, HLA-G is not attached to the cell surface by a GPI linkage, as would be predicted for a human *Ped* gene (Cao and Warner, unpublished). At this point, identification of the human homologue of the mouse *Ped* gene awaits further DNA sequence data from the Human Genome Project.

APOPTOSIS IN OOCYTES AND PREIMPLANTATION MOUSE EMBRYOS

There are two major criteria that determine preimplantation embryonic survival, rate of development, and degree of fragmentation (2,9–11). Rate of development is mediated by the *Ped* gene, as described above. However, the genes that control degree of fragmentation are just beginning to be elucidated. The genes that mediate embryonic fragmentation are likely the ones that mediate apoptosis because fragmented embryos have the typical pattern of fragmentation seen in apoptotic cells (Fig. 5). In addition, we and others have shown that it is possible to induce fragmentation in mouse blastocysts with staurosporine, a drug known

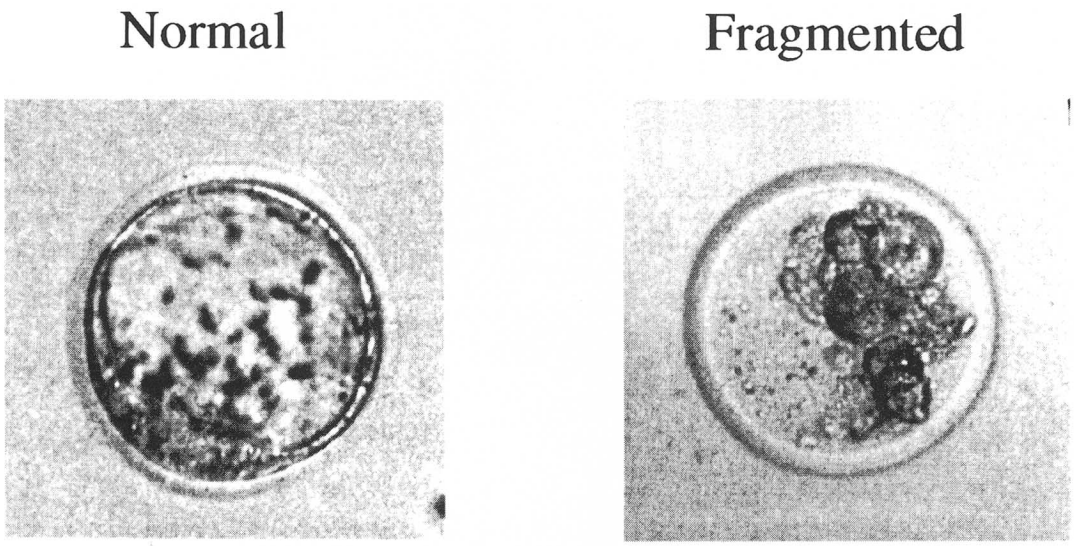

Fig. 5. Normal and fragmented mouse embryos. Embryos were collected at 89 hr post-hCG and photographed with a Nikon Diaphot microscope with Nomarski DIC optics at 300× magnification.

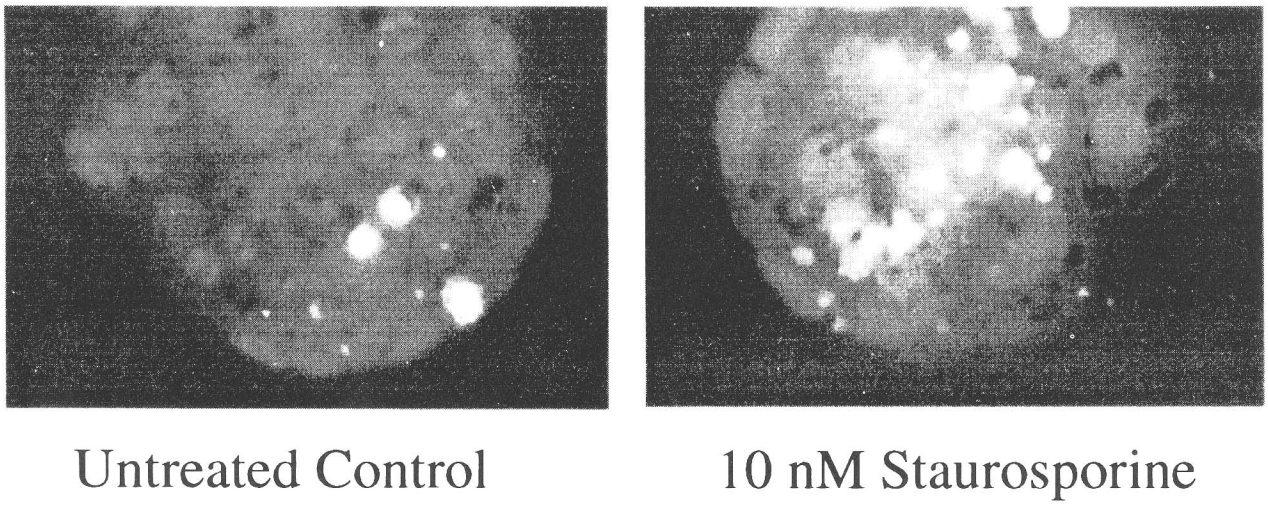

Fig. 6. Staurosporine (SP) induction of apoptosis in mouse blastocyts. Blastocysts were collected from C57BL/6 mice at 89 hr post-hCG into Whitten–Biggers (WB) medium and incubated for 6 hr at 37°C, 7% CO_2 either in WB only or in 10 *nM* SP in WB. After washing three times in WB, and then three times in PBS, the embryos were then fixed for 20 min at room temperature in 1% paraformaldehyde/PBS. The embryos were washed three times in PBS, permeabilized for 5 min in 0.1% Triton X-100/PBS, and washed again in PBS. At this point, the embryos were incubated in staining solution for 1 hr at 37°C, 7% CO_2, to begin the TUNEL assay using the Apo-Direct kit from Pharmingen. The staining solution contains TdT enzyme and fluorescein isothiocyanate (FITC)-dUTP, which will label fragmented DNA. The embryos were washed in rinse buffer and stained for 30 min in the dark in PI/RNase (Pharmingen), then washed an additional two times in PBS. They were then mounted onto microscope slides in a small volume of PBS and squashed gently with a glass coverslip. The embryos were then examined under UV light. Normal nuclei appear red from the propidium iodide staining, while apoptotic nuclei appear yellow from the FITC-dUTP.

to induce apoptosis (14, 15; Warner and McElhinny, unpublished). Mouse blastocysts treated with staurosporine show increased numbers of apoptotic cells compared to normal blastocysts, as shown by the TdT-mediated dUTP nick end labeling (TUNEL) assay (Fig. 6). Finally, oocytes that are allowed to age in vitro show an increase in fragmentation with time (Fig. 7) and these fragmented oocytes have been shown to be undergoing apoptosis (16). Taken together, the morphological and biochemical results suggest that both oocytes and preimplantation embryos can die by apoptosis.

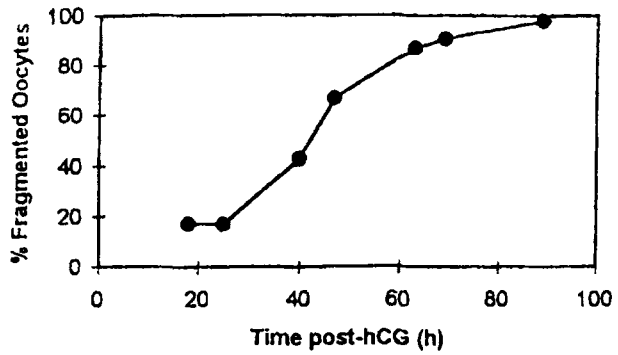

Fig. 7. Increase in fragmentation of oocytes with time in culture. Oocytes were collected at 18 hr post-hCG and cultured in vitro for 3 days. During the incubation time they were assessed at regular intervals for the percentage of fragmented oocytes.

As shown in Fig. 8, there are two major protein families involved in the regulation of apoptosis: those that mediate the proteolytic breakdown of the cells, the caspase family; and those that regulate the activity of the caspases, the Bcl-2 family (reviewed in Refs. 17 and 18). We have shown that many caspase (Tang and Warner, unpublished) and Bcl-2 (Exley and Warner, unpublished) family genes are expressed in oocytes and preimplantation mouse embryos. Work is under way in our laboratory to examine the mechanism of action of these genes in the control of apoptosis in oocytes and preimplantation embryos. The long-range goal is to be able to manipulate preimplantation embryonic survival by designing diagnostic and therapeutic reagents either to prevent or to enhance apoptosis in preimplantation embryos.

CONCLUSIONS AND CLINICAL RELEVANCE

The field of preimplantation genetics is a rapidly growing field making use of recent spectacular

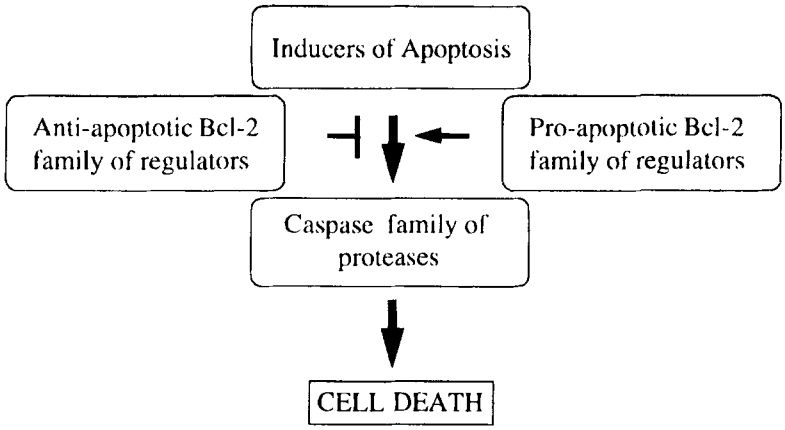

Fig. 8. Caspase and Bcl-2 family members mediate the apoptotic cell death pathway.

advances in techniques for gene identification, cloning, and sequencing. We are just beginning to identify the genes that regulate development and survival of preimplantation embryos. Preimplantation embryonic survival depends on two major criteria: rate of development and degree of fragmentation. Embryos with a fast rate of development and a low degree of fragmentation have a survival advantage over their slower developing and more fragmented counterparts. We have identified a gene in the mouse, *Ped*, that regulates the rate of preimplantation cleavage division and subsequent embryonic survival. We are currently searching for the human homologue of the *Ped* gene. Although we have not yet identified a human *Ped* gene, it seems unlikely that it is HLA-G. Because it is well documented from data from human IVF clinics that fast-developing embryos have a greater chance of leading to a successful pregnancy than slow-developing embryos, it is clinically important to identify the human homologue of the mouse *Ped* gene.

We have also begun to analyze genetic mechanisms of the second major criterion of embryo survival, degree of fragmentation. We have shown that fragmented oocytes and embryos show the morphological and biochemical characteristics of cells that have died by apoptosis. We are just beginning to define the genes that mediate apoptosis in oocytes and preimplantation embryos. There are several reasons why apoptosis is of clinical importance in early embryos. First, because apoptosis is a noninflammatory type of death, there is no deleterious effect on surrounding cells or tissues. Thus, in species with litters, the apoptotic demise of one embryo in the litter would not affect the others. Second, within a single embryo, a few defective cells could be eliminated without a deleterious effect on neighboring cells, thus assuring the overall survival of the embryo. Elimination of a few defective cells would be very important for embryos with mosaicism for chromosomal or genetic defects, which in the human population is estimated to affect up to one third of all embryos conceived (19). Defective cells would therefore be eliminated at a very early time point and not have the chance to undergo clonal proliferation leading to the possibility of mosaicism in the live-born baby. It is likely that regulation of apoptosis will prove to be a major mechanism that mediates oocyte and preimplantation embryo survival.

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REFERENCES

1. Verbanac KM, Warner CM: Role of the major histocompatibility complex in the timing of early mammalian development. *In* Cellular and Molecular Aspects of Implantation, SR Glasser, DW Bullock (eds). New York, Plenum Press, 1981 pp 467–470
2. Warner CM, Cao W, Exley GE, McElhinny AS, Alikani M, Cohen J, Scott RT, Brenner CA: Genetic regulation of egg and embryo survival. Hum Reprod 1998 (in press)
3. Xu Y, Jin P, Mellor AL, Warner CM: Identification of the *Ped* gene at the molecular level: The Q9 MHC class I transgene converts the *Ped slow* to the *Ped fast* phenotype. Biol Reprod 1994;51:695–699

4. Cai W, Cao W, Wu L, Exley GE, Waneck GL, Karger BL, Warner CM: Sequence and transcription of Qa-2 encoding genes in mouse lymphocytes and blastocysts. Immunogenetics 1996;45:97–107
5. Warner CM, Gollnick SO, Flaherty L, Goldbard SB: Analysis of Qa-2 antigen expression by preimplantation mouse embryos: Possible relationship to the *Ped* gene product. Biol Reprod 1987;36:611–616
6. McElhinny A, Warner CM: Detection of major histocompatibility complex class I antigens on the surface of a single murine blastocyst by immuno-PCR. BioTechniques 1997;23:660–662
7. Tian Z, Xu Y, Warner CM: Removal of Qa-2 antigen alters the *Ped* gene phenotype of preimplantation mouse embryos. Biol Reprod 1992;47:271–276
8. Warner CM: Methods for enhancing or reducing preimplantation embryo survival rates. U.S. Patent Application No. 60/053,499 (pending)
9. Bolton VN, Hawes SM, Taylor CT, Parsons JH: Development of spare human preimplantation embryos in vitro: An analysis of the correlations among gross morphology, cleavage rates, and development to the blastocyst. J Vitro Fert Embryo Transfer 1989;6:30–35
10. Levy T, Goldman JA, Dicker D, Ashkenazi J, Feldberg D: Very early pregnancy wastage in *in vitro* fertilization and embryo transfer (IVF-ET). J Vitro Fert Embryo Transfer 1991;8:250–253
11. Trounson A, Osborn J: *In vitro* fertilization and embryo development. *In* Handbook of In Vitro Fertilization, A Trounson, DK Gardner (eds). Boca Raton, FL, CRC Press, 1993, pp 57–84
12. Jurisicova A, Casper RF, MacLusky NJ, Mills GB, Librach, CL: HLA-G expression during preimplantation human embryo development. Proc Natl Acad Sci USA 1996;93:161–165
13. Jurisicova A, Casper RF, MacLusky, NJ, Librach CL: Embryonic human leukocyte antigen-G expression: Possible implication for human preimplantation development. Fertil Steril 1996;65;997–1002
14. Jacobson MD, Weil M, Raff MC: Role of Ced-3/ICE-family proteases in staurosporine-induced programmed cell death. J Cell Biol 1996;133:1041–1051
15. Weil M, Jacobson MD, Coles HSR, Davies TJ, Gardner RL, Raff KD, Raff MC: Constitutive expression of the machinery for programmed cell death. J Cell Biol 1996;133:1053–1059
16. Takase K, Ishikawa M, Hoshiai H: Apoptosis in the degeneration process of unfertilized mouse ova. Tohoku J Exp Med 1995;175:69–76
17. Nicholson DW, Thornberry NA: Caspases: killer proteases. TIBS 1997;22:299–306
18. Reed JC: Double identity for proteins of the Bcl-2 family. Nature 1997;387:773–776
19. Munne S, Alikani M, Tonkin G, Grifo J, Cohen J: Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. Fertil Steril 1995;64:382–391