GENETICS

Expression of Apoptosis-Related Genes in Human Oocytes and Embryos

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Purpose: The objective was to study whether apoptosis occurs in human embryogenesis.

Methods: Human viable, arrested, and nonviable embryos and immature, and nonfertilized oocytes donated by our patients were used to detect apoptosis by Tunel labeling, annexin staining, and single-cell reverse transcriptase-polymerase chain reaction (RT-PCR).

Results: DNA fragmentation and phosphatidylserine translocation, the two markers for apoptosis, were detected frequently in fragmented human embryos derived from in vitro fertilization-embryo transfer (IVF-ET). Using RT-PCR, apoptotic genes also were detected in these embryos. The frequencies of gene expression in viable embryos, arrested embryos, nonviable embryos, immature oocytes, and nonfertilized oocytes were: 7/8, 5/5, 5/6, 0/6, 0/3, for Bax; 8/8, 5/5, 7/7, 0/4, 0/5 for Fas; 2/8, 0/2, 0/3, 0/5, 0/3 for BCL-2; 0/8, 1/3, 0/2, 0/3, 0/2 for Fas-ligand; and 8/8, 17/17, 21/21, 24/24, 15/15 for actin, respectively.

Conclusions: Our preliminary data did not show a significant difference in the expression frequency of all studied genes between viable embryos and nonviable or arrested embryos. However, the expression of Bax and Fas was noticeably higher in nonviable embryos than in viable embryos as judged by the intensities of amplicons visualized after ethidium bromide staining. In addition, BCL-2 was only detected in viable embryos. Whether embryos quality is re-

lated to the regulation of BCL-2, Bax, and Fas expressions requires further study.

KEY WORDS: Single-cell reverse transcriptase-polymerase chain reaction; human embryos; apoptosis related genes; Tunel labeling; annexin V staining.

INTRODUCTION

Development of multicellular organisms is controlled not only by cell proliferation and differentiation, but also by the elimination of unwanted cells with minimal disturbance to the organism (1). This process of "programmed cell death" or "cell suicide" is called apoptosis. Apoptosis is characterized by chromatin aggregation, nuclear and cytoplasmic condensation, and partition of cytoplasm and nucleus into membrane-bound vesicles (2). This is a marked contrast to the inflammatory response during necrosis, where loss of plasma membrane integrity and release of cytoplasmic context into the surroundings accompanies cell death and provokes an inflammatory response (3). Recent evidence suggests that deregulation of apoptosis contributes to the pathogenesis of several human diseases including cancer (4), viral infections (5), autoimmune diseases (6), neurodegenerative disorders, and AIDS (7). This triggered our interest to study apoptosis in human embryogenesis and to study whether deregulation of apoptosis will improve embryo quality.

The hallmark of apoptosis is the translocation of phosphatidylserine (PS) on cell membrane (8) and

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nuclear breakdown by nuclear endonuclease (9). Assays were developed to detect apoptosis based on these morphological and biochemical changes. For instance, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (Tunel) to detect 3'-OH DNA breaks and annexin V staining to detect the translocation of phosphatidylserine. Both Tunel labeling and annexin V staining are commercially available and have been used widely for the detection of apoptosis in cell populations.

Fujino *et al.* (10), using Tunel labeling to detect DNA fragmentation in newly ovulated or cultured mouse oocytes, concluded that the rate of chromosomal fragmentation increased with the maternal age and duration of culture in vitro. He suggested that DNA fragmentation in these oocytes was due to the result of apoptosis, which might be one of the reasons for poor oocyte quality and low fertility in aged mice. The age-dependent acceleration of apoptosis in oocytes maintained in vitro requires the cumulus cells (11). However, Van Blerkom and Davis (12), using dual staining of Tunel and annexin V, reported that the occurrence of cytoplasmic chromosomal Tunel fluorescence is rare for both newly ovulated/aspirated and cultured MII stage mouse and human oocytes. Jurisicova *et al.* (13) also presented molecular evidence of apoptotic changes in human fragmented embryos using the 4,6-diamidino-2-phenylindole (DAPI)/Tunel method. They reported that approximately 75% of embryos with morphological fragmentation exhibited apoptotic changes.

Apoptosis is regulated by a group of apoptosis-related gene products such as BCL-2, Bax, Fas, and Fas-ligand. BCL-2 protein is a cell survival factor that prevents cell death or apoptosis (14,15). The cell survival-promoting ability of BCL-2 is hindered by Bax, which accelerates the cell death rate (16,17). Fas is a cell surface molecule homologous to the tumor necrosis factor- α (TNF- α) receptor (18–21). This receptor can be activated to trigger apoptosis by either a natural ligand (Fas ligand) (20–22) or anti-Fas antibody. Recent studies have demonstrated that these apoptosis-related gene products are involved in the massive loss of oogonia in the fetal ovary, follicular atresia, and luteolysis (23,24). Mice with targeted disruption in the Bax gene show abnormalities in granulosa cell apoptosis (25) and increased resistance to follicular atresia resulting in a dramatic extension of ovarian life span (26). In addition, mice carrying a null of Bax are more resistant to apoptosis induced by chemotherapy agent (27) and their primordial follicle pool also is markedly greater (28).

On the other hand, mice carrying a null allele of BCL-2 have a reduced number of primordial follicles and many of these lack oocytes (29). Spontaneous oocyte death occurring in vitro could be delayed by targeted expression of BCL-2, and overexpression of BCL-2 blocks the apoptotic response of oocytes (30) induced by chemotherapy agents. After immunolocalization of Fas antigen in humans and murine ovary, many investigators believed that Fas and Fas ligand may serve as a mediator of communication between cumulus cells and oocytes to induce oocyte apoptosis during atresia (31–33).

Furthermore, using advanced technologies such as reverse transcription–polymerase chain reaction (RT-PCR) and Northern and Western blot analysis, transcripts and proteins of these apoptosis-related genes have been detected in both animal and human oocytes and embryos (34–37). All these studies strongly suggest that apoptosis may occur not only during oogenesis but also during embryogenesis.

In fact, a majority of human preimplantation embryos derived by in vitro fertilization (IVF) exhibit some degree of cellular fragmentation most frequently between the two-cell to eight-cell stages (38). Both apoptotic and necrotic processes have been suggested as the cause of blastomere fragmentation in human embryos (39).

In this study, we used Tunel labeling and annexin staining and RT-PCR to detect the expression of apoptosis related genes in human embryos at various stages to demonstrate that apoptosis may occur in human embryos derived from IVF-ET.

MATERIALS AND METHODS

Human Embryos

This study was approved by the committee on Human Rights in Research of the New York Hospital–Cornell Medical Center Institutional Review Board (IRB) following the guidelines in accordance with the Helsinki Declaration of 1989 on human experimentation (IRB-approved protocol 0995-9970). Of the retrieved oocytes, 3% were either degenerated or immature, 16.5% were not fertilized, 10.7% were abnormally fertilized, and 69.7% were normally fertilized. After normal fertilization and culture in vitro for 3 days, the best-quality embryos were selected for transfer or for frozen embryo storage. After transfer, deselected day-3 embryos

with poor quality along with abnormally fertilized embryos and immature or nonfertilized oocytes were donated by patients for this research. Occasionally frozen embryos also may be donated by patients who had no intention of having more babies after their IVF attempts. Frozen embryos with intact blastomeres and normal cleavage rates 24 hr after thawing were classified as viable embryos. Embryos were defined as nonviable if they demonstrated >25% fragmentation at any given stage or <6 blastomeres after 72 hr of in vitro culture. Embryos were defined as arrested if they were completely arrested over a 24-hr period at any stage.

Tunel Labeling

Eighteen fragmented embryos at the one-cell to six-cell stages with fragmentation ranging from 10% to 70%, three blastocysts (two derived from 2 pronuclei and one from 3 pronuclei stage), and three viable embryos as well as three nonfertilized oocytes were used in this study. After fixation, permeabilization, blocking, and extensive washing, the embryos were subjected to Tunel labeling by using in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) according to the company's suggestion. Briefly, individual embryos were incubated with 50 μ l of Tunel reaction mixture containing 5 μ l of enzyme solution and 45 μ l of labeling solution (containing fluorescein conjugated nucleotide) for 60 min at 37°C. After incubation, embryos were washed extensively with phosphate buffered saline (PBS) buffer plus 0.3% bovine serum albumin (BSA). Photographic images were taken under a fluorescence microscope (Zeiss, Axioskop) with bright light and under UV light with FITC filter. These photographic images were further processed with digital deconvolution software for microscopy (Microtome, Vay Tek, Inc., Fairfield, IA) to remove the haze.

For each experimental setup, embryos incubated with label solution (without terminal transferase) instead of Tunel solution mixture were used as negative controls and embryos pretreated with DNAase (1 mg/ml, 10 min at room temperature) to induce DNA strand breaks were used as positive controls. Tunel labeling, as mentioned before, is a method to detect nuclear (3'-OH) DNA breaks. Therefore, DNA strand breaks were demonstrated by the presence of nuclear (green) signals.

Annexin Staining

Another 19 embryos at the one-cell to nine-cell stages with fragmentation ranging from 15% to 70% and two nonfertilized oocytes were used for study. ApoAlert annexin V apoptosis detection kit (Clontech, Palo Alto, CA) was used to stain these embryos and oocytes according to the manufacturer's recommendation. Embryos were washed well with PBS plus 0.3% BSA. Unfixed vital embryos were incubated with a mixture of 100 μ l binding buffer, 0.5 μ l FITC conjugated annexin V, and 0.5 μ l propidium iodide for 10 min. After incubation, the embryos were washed well before photographic images were taken under fluorescence microscope (Zeiss, Axioskop) with bright light and under UV light with DAPI/FITC/Texas Red triple filters. These photographic images were also processed with digital deconvolution software for microscopy.

This kit contains propidium iodide (PI) to determine the integrity of the plasma membrane and annexin V to detect the translocation of phosphatidylserine (PS) from the inner face to the outer surface of the plasma membrane. In apoptotic cells, the cells translocate PS from the inner face of the plasma membrane to the cell surface while maintaining membrane integrity (8). Once on the cell surface, PS can easily be detected by staining with a FITC conjugate annexin V, a protein that will specifically bind to PS. On the other hand, in necrotic cells, the broken plasma membrane allows PI, a DNA binding dye to enter the cell through broken membranes to bind cellular DNAs.

Costaining of PI and annexin V will allow apoptotic, necrotic, and viable cells to be distinguished. Apoptotic cells stain positive for annexin V (green) and necrotic cells stain positive for PI only (red), while viable cells with intact plasma membrane and no translocated PS stain negative for both annexin V and PI (colorless). Some necrotic cells can be stained positive for both annexin V (if the probe is internalized) and PI (due to the broken plasma membrane) and give off colors ranging from yellow to orange.

RT-PCR

Eight viable embryos (at the two-cell to nine-cell stage with 0% fragmentation), 17 arrested embryos (at the one-cell to four-cell stage with 10%–50% fragmentation), 21 nonviable embryos (at the two-cell to five-cell stage with 10%–90% fragmentation), 24 immature oocytes (at the GV stage) and 15 nonfertil-

ized oocytes were used to study their frequency of expression in apoptotic related genes by our recently published single cell RT-PCR method (40). Briefly, RNA of individual embryos or cells were extracted by phenol-chloroform-isoamylalcohol (25:24:1) and further purified by deoxyribonuclease treatment, phenol-chloroform-isoamylalcohol extraction, and ethanol precipitation. These purified RNAs were used for cDNA synthesis by (M-MLV) Moloney Murine leukemia virus reverse transcriptase random hexanucleotide or an oligo (dT) primer. Subsequently, the synthesized cDNA was separated into two to six equal portions to simultaneously detect multiple genes by PCR amplification with Taq DNA polymerase and specific primers (Table I). The amplified products were identified by sizing on 3% agarose gel and restriction enzyme digestion analysis.

Isolation of Blastomeres from Donated Human Embryos

Four donated frozen embryos at the four-cell, six-cell, seven-cell, and nine-cell stages were used to isolate their blastomeres in order to study the consistency of gene expression among the sibling blastomeres. These blastomeres were isolated from embryos by digestion with protease (250 U/ml) (Sigma, St. Louis, MO). After washing thoroughly with PBS, the isolated blastomeres were subjected to gene detection by RT-PCR.

Biopsy to Separate Fragments from Human Embryos

Eight embryos with fragmentation ranging from 30%–90% at the one-cell to five-cell stages were donated for this study. Embryos were biopsied with standard acid Tyrodes method (41). After piercing the zona pellucida with acid Tyrodes, embryonic frag-

ments were removed through the opening by micro-manipulated glass pipets. Both fragments and biopsied embryos were washed well with PBS before detection of gene expression by RT-PCR.

RESULTS

Apoptosis Occurs in Abnormal Human Embryos

As predicted, all our negative controls (without terminal transferase) were not stained and all the positive controls (pretreated with DNAase) were stained (data not shown). All arrested and fragmented embryos ($n = 18$) and blastocysts ($n = 3$) were stained and all viable embryos were not stained ($n = 3$). Figure 1 shows a few representative results of Tunel labeling on the studied human embryos. Nuclear green stains (shown by arrows) were detected in these deselected nonfertilized oocytes (Fig. 1a), arrested embryos (Fig. 1b), highly fragmented embryos (Fig. 1c and d), and abnormally fertilized embryos (Fig. 1e), but was not detected in a three-cell viable embryo (Fig. 1f), which may serve as a negative control of our Tunel labeling too.

Coexistence of Apoptotic, Necrotic, and Viable Blastomeres Within a Highly Fragmented Human Embryo

Embryos and oocytes were diagnosed, fixed, then stained with apoptosis detection kits containing annexin V and PI. In nonfertilized oocytes ($n = 2$), only polar bodies were stained red and all fragmented embryos ($n = 19$) showed the coexistence of apoptotic, necrotic, and viable blastomeres. Figure 2 shows one representative embryo after costaining with annexin V and PI. As mentioned previously, the

Table I. Amplification Conditions, Primers, and Products

Gene	Primers	Annealing temp. (°C)	Size of PCR products	No. of cycles
FAS	5' 5'-AGACTGCGTGCCCTGCCAAGA-3' 3' 5'-CAGGATTTAAGGTTGGAGATT-3'	56	413 bp	32
FAS Ligand	5' 5sp-TTCTTCCCTGTCCAACCTCTG-3' 3' 5'-TCTTCCCCTCCATCATCACCA-3'	60	750 bp	32
BCL-2	5' 5'-TTGTGGCCTTCTTTGAGTTTCG-3' 3' 5'-TACTGCTTTAGTGAACCTTTT-3'	60	332 bp	32
BAX	5' 5'-AGACAGGGGCCCTTTTGCTTC-3' 3' 5'-GAGCACTCCCGCCACAAAGAT-3'	58	482 bp	32

Fig. 1. Apoptosis occurs in abnormal human embryos: A nonfertilized oocyte (a), a three-cell stage arrested embryo (b), embryos with high fragmentation (c–e), and a blastocyst derived from 3 pronuclei embryo (3PN) (f) were subjected to TUNEL labeling. The presence of green stains on the studied oocyte and embryos illustrate that apoptosis does indeed occur during human embryogenesis. The upper panel shows pictures that were taken under fluorescence microscope with FITC filter and the lower panel shows the picture taken in bright field.

Fig. 2. Detection of apoptosis in a human embryo by costaining of annexin V and propidium iodide: A highly fragmented human embryo was dezonated, fixed, and then stained with ApoAlert Annexin V apoptosis detection kit. The result shows the coexistence of apoptotic, necrotic, and viable blastomeres in a dezonated embryo. After staining, pictures were taken under bright field (a) and under UV light with DAPI/FITC/Texas Red triple filters (b).

apoptotic blastomeres were stained positive for annexin V (green), necrotic blastomeres were stained positive for PI (red), and viable blastomeres (indicated by arrows) were stained negative, and some blastomeres with PS translocation and broken membrane were stained positive with both annexin V and PI and gave off colors ranging from yellow to orange when observed under a triple filter (FITC/Texas red/DAPI).

Detection and Identification of Amplified Products by Sizing on 3% Agarose Gels

Embryos or blastomeres were used to simultaneously detect Bax, Fas, BCL-2, and Fas-ligand expression by RT-PCR. The amplified products were identified by sizing on 3% agarose gels and visualized after

ethidium bromide staining (Fig. 3). In this study, 10 blastomeres isolated from two four-cell (two blastomeres from each embryo), one seven-cell (four blastomeres), and one nine-cell (two blastomeres) donated viable embryos were used to extract mRNAs and the extracted mRNAs were separated into 6 equal portions in order to detect the studied genes and actin simultaneously. Each lane represents the results of the same blastomere. In a majority of these blastomeres, Bax and Fas were detectable, whereas BCL-2, and Fas-L were not.

Frequency of Expression Apoptosis-Related Genes in Human Embryos

Viable embryos ($n = 8$), arrested embryos ($n = 17$), nonviable embryos ($n = 21$), immature oocytes ($n = 24$), and nonfertilized oocytes ($n =$

Fig. 3. Identification of the amplified products by sizing on 3% agarose gel: Ten blastomeres isolated from four donated viable embryos were used to simultaneously detect Bax, Fas, P53, BCL-2, Fas-ligand by RT-PCR. The amplified products were identified by sizing on 3% agarose gel and visualized by ethidium bromide staining. Each lane represents the results of one blastomere. Lane M: markers; lanes 1, 6: two sibling blastomeres from one four-cell embryo; lanes 2, 7: two sibling blastomeres from one four-cell embryo; lanes 3, 4, 8 and 9: four sibling blastomeres from one seven-cell embryo; and lanes 5, 10: two sibling blastomeres from one nine-cell embryo.

Table II. Frequency of Expression Apoptosis-Related Genes in Deselected Human Oocytes and Embryos

	Frequency of gene expression				
	Bax (+/n) ^a	Fas (+/n)	BCL-2 (±-n)	Fas-ligand (+/n)	Actin (+/n)
Viable embryo	7/8	8/8	2/8	0/8	8/8
Arrested embryos	5/5	5/5	0/2	1/3	17/17
Nonviable embryos	5/6	7/7	0/3	0/2	21/21
Immature oocytes	0/6	0/4	0/5	0/3	24/24
Nonfertilized oocytes	0/3	0/5	0/3	0/2	15/15

^a +/n, Number of samples with positive gene expression/of total samples.

15) were used to detect the expression of apoptosis related genes by RT-PCR. The extracted mRNAs were separated into two equal portions to detect one of the studied apoptosis genes and actin (used as control to insure the presence of mRNA). On the other hand, extracted mRNAs of viable embryos ($n = 8$) were separated into six equal portions to detect all four studied apoptosis related genes as well as actin simultaneously. Table II shows the frequency of expression (i.e., number of samples with positive detection/number of total samples) in human embryos.

Consistency of Gene Expression Among the Sibling Blastomeres

The sibling blastomeres isolated from donated viable embryos ($n = 4$) by protease treatment were used to study the expression of Fas, Fas-Ligand, Bax, and BCL-2 simultaneously. These results were shown in Table III. Fas and Bax were consistently expressed among sibling blastomeres. BCL-2 was not detectable in blastomeres of four-cell and six-cell embryos and was inconsistently expressed in blastomeres of the seven-cell and nine-cell embryos. Fas-ligand consistently was not expressed in any of the tested samples.

Expression of Fas and Bax in Fragments Biopsied from Human Embryos

The fragmented human embryos ($n = 8$) were micromanipulated to remove fragments. The biopsied embryos and the removed fragments were used to detect Fas and Bax gene expressions. Surprisingly, Fas and Bax were detected in embryos after biopsy, but only Bax was detected in the removed fragments (Fig. 4).

DISCUSSION

By using in situ Tunnel labeling techniques, we demonstrated that DNA fragmentation occurs frequently in deselected human oocytes and embryos. In general, brighter fluorescences were detected in the pronucleus of the oocytes or nuclei of intact blastomeres, with a few lighter fluorescences diffused to the ooplasm or cytoplasm. This may be due to the activation of endonucleases cutting nuclear genomic DNA into oligonucleosomal fragments with diffusion of small fragments into the ooplasm or cytoplasmic components (42). Brighter fluorescences also were found in the extruded first polar body and the attached sperms. Van Blerkom and Davis (12) and

Table III. Consistency of Gene Expression Among the Sibling Blastomeres

Type of gene	4-Cell embryo				6-Cell embryo				7-Cell embryo					9-Cell embryo						
	S1	S2	%	Consistency	S1	S2	%	Consistency	S1	S2	S3	S4	%	Consistency	S1	S2	S3	S4	%	Consistency
Fas	+	+		100%	+	+		100%	+	+	+	+		100%	+	+	+	+		100%
Fas-L	−	−		100%	−	−		100%	−	−	−	−		100%	−	−	−	−		100%
Bax	+	+		100%	+	+		100%	+	+	+	+		100%	+	+	+	+		100%
BCL-2	−	−		100%	−	−		100%	−	−	+	−		75%	+	−	+	−		50%

Fig. 4. Expression of Bax and Fas in the fragments biopsied from human embryo. Eight embryos were biopsied with standard acid Tyrode's method to remove embryonic fragments as shown in (a). Both fragments and biopsied embryos were used to detect the expression of Bax and Fas by RT-PCR as shown in (b). Each lane represents data of the biopsied embryo and its corresponding removed fragments.

Fujino *et al.* (10) also found that the first polar body was stained by TUNEL labeling in newly ovulated and cultured mouse oocytes. In highly fragmented embryos (i.e., Fig. 1c and d), bright fluorescences were found on the cell corpses as well as the intact blastomere hinting that these cell corpses may be apoptotic bodies containing DNA fragments. On the other hand, fluorescence stains were not found in intact viable embryos (Fig. 1e).

Though TUNEL labeling can detect 3'-OH DNA strand breaks, these breaks also may arise other than through apoptosis-associated endonuclease digestion. Thus, TUNEL labeling cannot be used as solid evidence for apoptosis. However, we cannot exclude the possibility that some of the detected DNA fragmentation may still be part of or as a consequence of the apoptotic process.

Phosphatidylserine translocation occurs in the early stage of apoptosis, preceding endonuclease digestion of DNA into oligonucleosome fragments and cellular fragmentation into apoptotic bodies. Unlike TUNEL label-

ing, PS translocation is an apoptosis specific marker. The translocated PS at the outer surface of the plasma membrane can be easily detected by FITC conjugated annexin V. However, the labeled annexin V also can be internalized to stain the inner PS, when the plasma membrane integrity is compromised due to necrosis.

To distinguish the binding of annexin V to the internalized or translocated PS, costaining with propidium iodide was used to check the integrity of the membrane. The apoptotic cells with the integrity of plasma membrane will be stained by annexin V only (green), whereas the necrotic cells, with broken membranes, will be stained by PI (red) or both PI and annexin V (ranging from yellow to orange).

Using costaining of annexin V and PI on embryos exhibiting morphologies similar to that of TUNEL labeling group, we confirmed that apoptosis occurs frequently in fragmented human embryos. This may hint that some of DNA fragmentation in human embryos detected by TUNEL labeling was apoptosis associated.

The costaining of annexin V and PI clearly demon-

strated the coexistence of apoptotic, necrotic, and viable sibling blastomeres within intact pellucida (Fig. 2). Thus, apoptosis may occur independently among the sibling blastomeres. While some may proceed further to secondary necrosis after apoptosis (43), others may or may not initiate apoptosis. Whether apoptotic or necrotic blastomeres could trigger the apoptosis of the adjacent blastomeres remains to be studied. Moreover, apoptosis seems to be embryo fragmentation related. It was detected mostly in the blastomeres with fragmentation but was not detected in intact viable blastomeres (Fig. 2).

Interestingly, costaining of annexin V and PI revealed that apoptosis did not occur in our studied nonfertilized oocytes ($n = 2$). Therefore, the detection of apoptosis by TUNEL labeling in oocytes remains questionable. Unfortunately, costaining and TUNEL labeling were not performed simultaneously on the same oocyte to verify the controversial reports that apoptosis in oocytes occurs frequently by Fujimoto (10) or rarely by Van Blerkom and Davis (12).

The combined data from our costaining and TUNEL labeling not only confirm that human embryo fragmentation is associated with apoptosis or programmed cell death (PCD) (13), but it also proves that apoptosis could occur in human embryos at stages prior to blastocyst formation (13). PCD was believed to occur at the blastocyst stage to eliminate redundant cells as part of normal development in mammalian embryos including human embryos (44–47), but does not occur at stages prior to the blastocyst stage (48). However, recent evidence indicates that poor-quality embryos from aged mice or controlled ovarian stimulation (34) underwent apoptosis regardless of the stage of the embryo. Normal mice embryos at stages prior to the blastocyst stage also underwent apoptosis if they were treated with a high concentration of apoptotic stimuli (49).

In blastocysts ($n = 3$), a substantial number of inner cell mass and trophectoderm cells were brightly stained. Figure 1e shows an example of a blastocyst derived from a 3 pronucleus embryo (3PN). It would not be surprising that the stained cells were apoptotic. However, whether apoptotic cells were more pronounced in this abnormal fertilized blastocyst than in the normal fertilized ones requires further study.

Apoptosis-related genes were detected in human embryos (Fig. 3 and Tables II and III). None of the apoptosis-related genes were detected in immature and nonfertilized oocytes (Table II). Bax and Fas genes were frequently expressed in both nonviable

and viable embryos (Table II). On the other hand, BCL-2, the survival gene, was only detectable in some viable embryos, but not in any of the nonviable embryos (Fig. 3, Tables II and III). Fas ligand was expressed in only one arrested embryo (Table II).

Failure of detection of transcripts of apoptosis-related genes in oocytes was in contrast to the reports that the maternally stored mRNAs and proteins of apoptosis-related genes were detected in both human and animal oocytes (35–37,39). This failure of detection may be due to the sensitivity of the detection method. Most of the other investigators performed either RT-PCR with pooled oocytes (ten to hundreds of oocytes) (34) or nested RT-PCR (35,36). Low amounts of mRNAs in oocytes and poor-quality embryos may not be detectable by our RT-PCR after separation of the extracted mRNAs from individual oocytes or embryos into two to six fractions for multiple gene detection.

Failure of detection also may be due to the time that nonfertilized oocytes were obtained for research. Unfortunately, our nonfertilized oocytes were cultured for 3 days after fertilization before being subjected for mRNA extraction. During this period of culture in vitro, the unstable mRNAs of apoptosis genes (half-life is approximately 2 hr) may have undergone degradation, thereby becoming virtually undetectable.

Lack of sensitivity may be the factor to explain why BCL-2 was not detected in our early viable embryos (< seven-cell stage), while other investigators were able to detect this gene in human embryos from the two-cell stage onward (35).

Though our condition was set for qualitative rather than quantitative analysis, samples with positive detection implied that more mRNAs were present than in samples with negative detection. Therefore, mRNAs of BCL-2 in viable embryos at stages later than the seven-cell stage (see Tables II and III) were higher than that of nonviable embryos and viable embryos of earlier stages (< seven-cell stages). Coincidentally, the seven-cell stage is the time when activation of human genomic DNA is initiated. Commencement of BCL-2 transcription at the time of genomic DNA activation also was reported in mouse (34).

Our preliminary data did not show a significant difference in the expression frequency of all studied genes between viable embryos and nonviable or arrested embryos. However, the expression of Bax and Fas was noticeably higher in nonviable embryos than

in viable embryos as judged by the intensities of amplicons visualized after ethidium bromide staining. In addition, BCL-2 was only detected in viable embryos (one seven-cell in Fig. 3; one seven-cell and one nine-cell embryo in Table II; one seven-cell and one nine-cell embryo in Table III). Whether embryos quality is related to the up-regulation of BCL-2 and the down-regulation of Bax and Fas expressions requires further study. In many somatic systems, cell death is based on the balance of cell death suppressors and activators. Recently, Jurisicova (34) demonstrated that embryos undergoing fragmentation alter the expression of apoptotic genes by elevating the expression of cell death genes (MA-3, P53, Bad, Bcl-xs) and reducing the expression of cell survival genes (BCL-2). Kawahara (50) demonstrated that Fas-induced apoptosis can be inhibited by BCL-2. However, our further study of the regulation of cell death suppressors and activators for improving embryo quality also should include the study of regulation of other genes in BCL-2 family such as BAD, BID, BCL-x, BCL-xs, BCL-xl, and so on and the regulation of posttranscriptional events such as translation, splicing, and protein synthesis.

The detection of apoptosis-related genes in human preimplantation embryos may hint that human embryos are capable of undergoing programmed cell death and constitutively express genes that are required to execute the death program. High expression of cell death genes (Fas and Bax) may confirm that oocytes and early embryos were inherently biased toward PCD (16,51,52).

Though Bax and Fas were highly expressed in preimplantation embryos, the fate (survival or death) of embryos is finally determined along with other factors such as external stimuli, internal defects, and activation of other apoptotic related genes. The balance in favor of survival may be restored by embryonic transcription or translational recruitment of maternal mRNAs encoding cell protectors. Embryos with inadequate maternal storages or failure to commence a relevant gene at the optimal time will die. This may explain the presence of transcripts of Bax and Fas in both viable and nonviable embryos. In viable embryos, maternally stored BCL-2 mRNA and proteins are sufficient to suppress the activity of Bax and Fas. Thus, embryos can survive and proceed to the six- to eight-cell stage to then initiate the transcription of BCL-2, which will further enhance embryo viability. This may explain why PCD is rarely found in the two-cell to the morula stage embryos obtained in vivo.

In contrast, embryos under external apoptotic stimuli or embryos with internal defects due to suboptimal controlled ovarian stimulation will greatly stimulate the expression of Bax and Fas. This overly expressed Bax and Fas will then trigger apoptosis to lead embryo fragmentation and change embryos to be nonviable.

Fragmentation occurs frequently in embryos produced by IVF (38,53). The viability of these embryos are inversely related to the percentage of fragmentation. Removal of these fragments has been suggested to enhance human embryo viability (54). Bax, the death gene, was highly expressed in these fragments (Fig. 4). Therefore, removing these fragments with high expression of Bax may contribute to the improvement of embryo quality.

In conclusion, DNA fragmentation and phosphotyrosine translocation, the two markers for apoptosis, were detected frequently in fragmented human embryos derived from IVF-ET. Apoptosis-related genes were detected in the studied human embryos. There was no significant difference in the expression frequency of the studied apoptosis-related genes between viable and nonviable embryos. However, the expression of Bax and Fas, the death genes, was noticeably higher in nonviable embryos than in viable embryos. On the other hand, BCL-2, the survival gene, was detected only in some viable embryos. Whether embryo quality is related to the regulation of BCL-2, Bax, Fas, and other related gene expressions requires further study.

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