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Anti-Apoptotic Action of Insulin-Like Growth Factor-I During Human Preimplantation Embryo Development

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ABSTRACT

Insulin-like growth factor I (IGF-I) has been shown to increase the proportion of embryos forming blastocysts and the number of inner cell mass cells in human and other mammalian preimplantation embryos. Here we examined whether the increased cell number resulted from increased cell division or decreased cell death.

Normally fertilized, Day 2 human embryos of good morphology were cultured to Day 6 in glucose-free Earle's balanced salt solution supplemented with 1 mM glutamine, with (n = 42) and without (n = 45) 1.7 nM IGF-I. Apoptotic cells in Day 6 blastocysts were identified using terminal deoxynucleotidyl dUTP terminal transferase (TUNEL) labeling to detect DNA fragmentation and 4′-6-diamidino-2-phenylindole (DAPI) counterstain to evaluate nuclear morphology. The number of nuclei and extent of DNA and nuclear fragmentation was assessed using laser scanning confocal microscopy.

IGF-I significantly increased the proportion of embryos developing to the blastocyst stage from 49% (control) to 74% (+IGF-I) ($P < 0.05$). IGF-I also significantly decreased the mean proportion of apoptotic nuclei from 16.3 ± 2.9% (–IGF-I) to 8.7 ± 1.4% (+IGF-I) ($P < 0.05$). The total number of cells remained similar between both groups (61.7 ± 4.6 with IGF-I; 54.5 ± 5.1 without IGF-I). The increased number of blastocysts combined with reduced cell death suggests that IGF-I is rescuing embryos in vitro which would otherwise arrest and acting as a survival factor during preimplantation human development.

apoptosis, growth factors

INTRODUCTION

Human preimplantation embryos cultured in vitro are characterized by variable morphology and developmental potential. Following in vitro fertilization (IVF), only about 25% of transferred embryos implant [1] and <20% of women become pregnant [2]. In addition, in vitro studies show that during the first six days of preimplantation development approximately 50% of human embryos arrest [3]. Varying degrees of cytoplasmic fragmentation are observed in approximately 75% of human embryos [4], and this is associated with reduced blastocyst formation [5] and implantation [6]. The causes, mechanism and role of extracellular fragmentation and precise reasons for early embryonic loss remain unknown.

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Cell death has been observed during preimplantation embryogenesis both in vivo and in vitro in a range of mammalian species (reviewed in [5, 7]). Cell death is prevalent in human blastocysts with approximately 75% of embryos having one or more dead cells on Day 6 [8]. Cells and nuclei with the morphological features of apoptosis [9, 10], including cytoplasmic and nuclear fragmentation, chromatin condensation, DNA fragmentation and phagocytosis have been identified in human preimplantation embryos [5, 7, 8, 11–14]. It has been suggested that apoptosis may be involved in early embryonic arrest and that cytoplasmic fragments are equivalent to apoptotic bodies, i.e., the end-product of apoptosis [5, 14].

Suboptimal culture conditions may be implicated in preimplantation arrest and cell death. In particular, there is increasing evidence that growth factors play an important role in preimplantation development (reviewed in [15, 16]). For example, the preimplantation development of mouse embryos cultured in vitro is retarded when compared to that in vivo [17, 18]. Moreover, reduced incubation volume or culture of embryos in groups improve preimplantation development [19–22]. Furthermore, a range of polypeptide growth factor ligands and their receptors have been found to be expressed and produced in the reproductive tract or preimplantation embryo, including epidermal growth factor (EGF) [19, 23], transforming growth factor alpha (TGF-α) [23–25], insulin receptor [25, 26] insulin-like growth factor I (IGF-I) and its receptor (IGF-IR) [26–31] (reviewed in [15]). Generally, growth factors have been shown to promote blastocyst formation and development and increase cell number [19, 31–34]. Taken together, these findings suggest the presence of regulatory autocrine and paracrine pathways acting in vivo that may be 'diluted' or not present in the in vitro environment.

Recently, a number of studies have demonstrated that growth factors such as insulin, IGF-I, and TGF-α may play a key role in regulating levels of cell death in preimplantation embryos. De Hertogh et al. [35] found that blastocysts recovered from diabetic rats had a higher incidence of cell death compared to embryos from diabetic rats treated with insulin. Brison and Schultz [22] showed that mouse embryos cultured singly had a threefold higher incidence of cell death than that observed in embryos cultured in groups of 30. Supplementation of the culture medium with TGF-α decreased cell death but had little effect on cell proliferation or rate of development to the blastocyst stage suggesting that TGF-α acts as a survival factor during mouse preimplantation development. Further evidence for this was provided by the observation that levels of cell death were higher in the inner cell mass (ICM) of blastocysts from TGF-α-deficient mice compared to wild-type mice [36]. Herrler et al. [37] induced apoptosis in rabbit embryos by subjecting them to ultraviolet (UV) irradiation, which could be reduced by addition of IGF-I or insulin.

Moley et al. [38] demonstrated that both DNA fragmentation and expression of Bax (a death-promoting member of the Bcl-2 family) was increased in blastocysts from hyperglycaemic diabetic mice, which could be prevented by maternal insulin treatment.

IGF-I has been shown to be an important inhibitor of cell death in many cell types (reviewed in [39, 40]). IGF-I also promotes preimplantation embryo development, cell number and metabolism in many species, including mouse [21, 33], rabbit [37], cow [41, 42], pig [43], and human [31]. Lighten et al. [26, 31] showed that IGF-I is synthesised by the maternal tract and that human embryos express IGF-I receptors (IGF-IR) throughout preimplantation development but do not express the IGF-I ligand. Furthermore, they showed that addition of IGF-I to culture medium significantly increased the proportion of embryos developing to the blastocyst stage by 25% and that these blastocysts had a significant (59%) increase in the number of inner cell mass cells [31]. In order to determine whether IGF-I was acting as a mitogenic or an anti-apoptotic factor during human preimplantation development we examined apoptosis in embryos cultured in the presence and absence of IGF-I. Two techniques were used to quantify cell death: TUNEL to identify DNA fragmentation (a biochemical marker for cell death), in conjunction with DAPI staining to examine nuclear morphology.

MATERIALS AND METHODS

Human Embryo Collection

Spare embryos used for this study were obtained from the IVF program at the Wolfson Family Clinic, Hammersmith Hospital. Infertile patients undergoing IVF, who did not wish to cryopreserve their untransferred surplus embryos for future transfer, were asked to donate them for research; informed consent was obtained from both partners. The work was conducted under licence from the Human Fertilisation and Embryology Authority (HFEA) and local ethical permission was granted by the ethics committee of Imperial College School of Medicine, Hammersmith Hospital, London.

Women undergoing IVF treatment were superovulated with recombinant follicle-stimulating hormone (FSH; Gonal-F, Serono, Feltham, Middlesex, UK) after pituitary-gonadal suppression by luteinizing hormone-releasing hormone agonist (LHRH; Buserelin, Hoescht, Hounslow UK). Ten thousand IU of human chorionic gonadotropin (hCG; Profasi, Serono) was administered 34 h before oocyte retrieval. Oocyte-cumulus complexes were preincubated, inseminated, and observed the following day for the presence of pronuclei [44]. Embryos were cultured singly in 1 ml of Earle's balanced salt solution (EBSS; Gibco BRL, Paisley UK) containing 5.56 mM of glucose, 25 mM sodium bicarbonate (BDH, Lutterworth, Leics, UK), 0.47 mM pyruvate (Sigma, Poole, Dorset, UK), 37.5 U/ml of streptomycin (Sigma) and 97.5 U/ml penicillin (Sigma) and supplemented with 10% heat-inactivated maternal serum, under a gas phase of 5% CO_2, 5% O_2, and 90% N_2 at 37°C.

Surplus Day 2 embryos of good morphology, with intact blastomeres and minor or no fragmentation (grade II.5 and above according to Dawson et al. [1]), were selected for this study. Briefly, grading of morphology ranged from embryos with symmetrical and even blastomeres (grade I), through embryos with all the blastomeres intact, but some cytoplasmic fragmentation and/or unevenly sized cells (grade II), to embryos with one blastomere (grade III) or more (grade IV) completely fragmented. Totally fragmented or degenerate embryos were grade V.

Culture of Preimplantation Embryos with IGF-I

Day 2 spare human embryos of good morphology from each patient were allocated randomly and evenly to the control or experimental groups using block randomization [45]. Human recombinant IGF-I (ARM 4010, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) was solubilized using 0.005 M HCl (Analar BDH) in EBSS (see below) to make a 2.57-μM stock solution and stored at −70°C for up to 3 mo. Embryos were incubated individually in 5-μl droplets of custom-made glucose-free EBSS supplemented with 1 mM L-glutamine (Sigma) [46] and 4 mg/ml IGF-free bovine serum albumin (BSA; A-9647 Sigma), with or without 1.7 nM IGF-I [31] and overlaid with silicone fluid (Dow Corning 200/50cS; BDH). Due to limited availability of human embryos, the IGF-I concentration could not be titrated. The dose of 1.7 nM used was shown to enhance survival to the blastocyst stage and increase the proportion of ICM cells in human [31] and mouse embryos [33].

Culture media for both treatment groups were prepared weekly from stock solutions and were stored at 4°C prior to use. Embryos were cultured from Day 2 and transferred to fresh 5-μl droplets of culture medium every 24 h until Day 6. Assessment of embryo quality (grade I-V) and developmental stage was recorded and light photomicrographs taken twice daily.

Blastocyst Morphology

A grading system was devised in order to compare blastocyst morphology in the presence and absence of IGF-I (Fig. 1, A–C). Grade I blastocysts were of excellent morphology displaying a fully expanded blastocoele cavity, discrete inner cell mass (ICM), smooth trophectoderm (TE) and appeared to have numerous cells in the mural TE (Fig. 1A). Grade II blastocysts were of good morphology, but had a few excluded cells in the blastocoele cavity or a slightly diffuse ICM (Fig. 1B). grade III blastocysts had poor morphology, with many excluded fragments or cells in the cavity and/or between the TE and zona pellucida and no discrete ICM (Fig. 1C). The TE and ICM of grade III embryos could be very granular and irregular and intracellular vacuoles could be present in both cell lineages.

TUNEL and DAPI Labeling

All preimplantation embryos used in this study were followed and treated individually throughout the entire experimental procedure. Culture was terminated on Day 6 of preimplantation development when 'normally' developing embryos were expected to have reached the blastocyst stage. TUNEL labeling was based on that described by Brison and Schultz [22]. Embryos were transferred to a 96-well, U-bottomed tissue culture plate (Falcon, Becton-Dickinson, Cowley, Oxfordshire, UK) and washed twice in ~150 μl phosphate buffered saline (PBS; Gibco BRL) with 3 mg/ml polyvinylpyrrolidone (PVP; Sigma) (PBS/PVP, pH 7.4). The zona pellucida was removed with a brief incubation in acid Tyrode's (pH 2.3–2.4) (ZD-10 Scandinavian IVF Science AB; Hunter Scientific Ltd, Saffron Waldon, Essex) at room temperature [47] and embryos were washed in PBS/PVP. Zona-free embryos were fixed for 1 h at room temperature in PBS (Gibco BRL) containing 4% parafor-

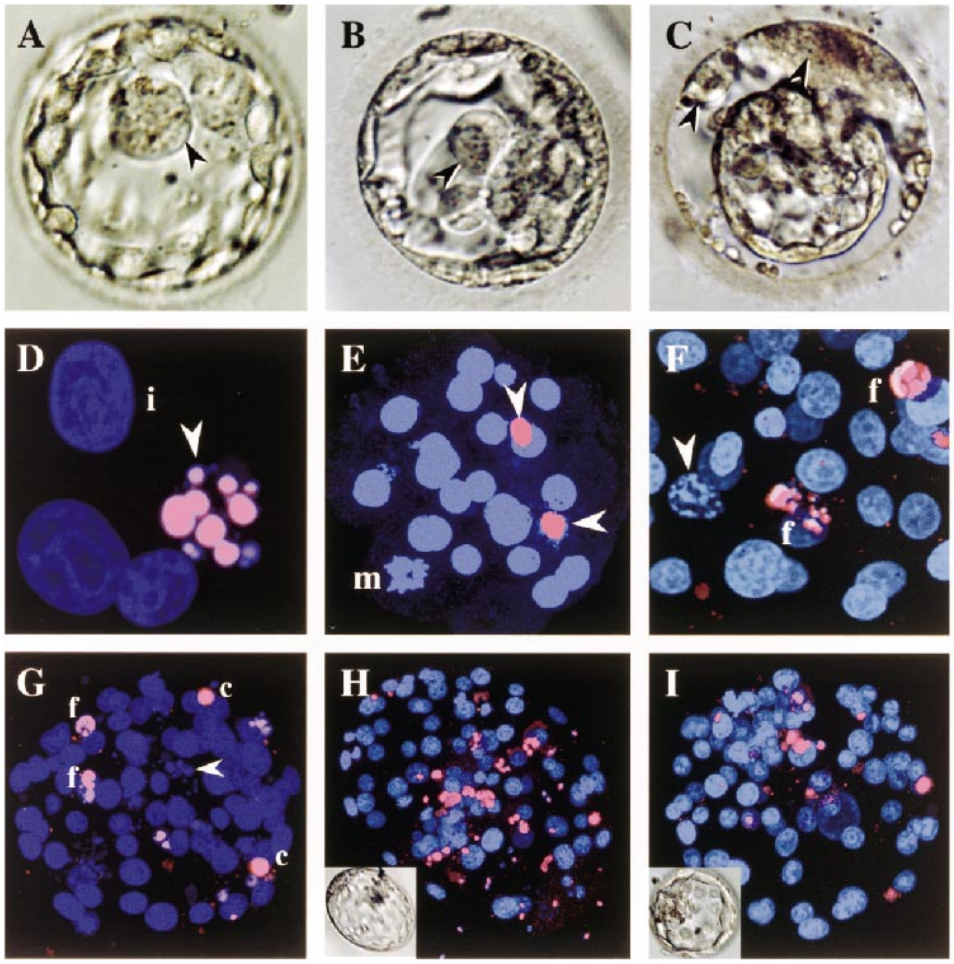

FIG. 1. Light micrographs and confocal sections showing blastocyst morphology and detection of apoptotic nuclei in Day 6 human embryos. Nuclear material is stained with DAPI (blue), TUNEL-labeled nuclei with DNA fragmentation are shown in pink. **A**) Grade I Day 6 expanded blastocyst (+IGF-I) with 62 nuclei, note discrete ICM (►). **B**) Grade II Day 6 blastocyst (-IGF-I) with 44 nuclei with excluded cell in blastocoele cavity (►). **C**) Grade III Day 6 blastocyst (-IGF-I) with 63 nuclei showing excluded cells between the zona pellucida and blastocyst (►). **D**) Confocal section from a Day 6 blastocyst showing TUNEL-labeled fragmented nucleus (►) and healthy interphase nucleus (i). **E**) Day 6 embryo with 29 nuclei, including 1 mitotic figure (m) and two TUNEL-labeled condensed nuclei (►). **F**) Day 6 hatching blastocyst (+IGF-I) with 95 nuclei showing two TUNEL-labeled fragmented nuclei (f) and a fragmented nucleus without TUNEL labeling (►). **G**) Day 6 blastocyst (+IGF-I) with 75 nuclei with two fragmented and TUNEL-labeled nuclei (f), two condensed TUNEL-labeled nuclei (c) and a fragmented nucleus without TUNEL-labeling (►). **H**) Confocal and light micrograph of representative Day 6 blastocyst (−IGF-I) with 82 nuclei, and a TUNEL index of 14.6% (close to average of 15%; Fig. 4). **I**) Confocal and light micrograph of representative Day 6 blastocyst (+IGF-I) with 79 nuclei, and a TUNEL index of 6.3% (close to average of 7%; Fig. 4).

maldehyde (Sigma), followed by two washes in PBS/PVP before permeabilization with 0.5% Triton X-100 (Sigma) in PBS for 1 h. Embryos were then briefly washed twice in PBS/PVP. The embryos were then incubated in 5 μl of fluorescein-conjugated dUTP and TdT (TUNEL reagents; Boehringer Mannheim, Roche Diagnostics, Lewes, East Sussex) for 1 h at 37ºC in a dark moist chamber. Positive controls were incubated in RQ1 DNase (50 U/ml; Promega, Southampton, UK) for 20 min at 37ºC and washed in PBS/PVP before TUNEL. Negative controls were incubated in fluorescein-dUTP in the absence of TdT. Following TUNEL, embryos were washed twice in 0.5% Triton (5 min each wash) and then one wash in PBS/PVP, mounted in Vectashield with DAPI (1.5 μg/ml) (Vector Laboratories, Peterborough, UK) and stored in the dark at 4ºC prior to laser scanning (LS) confocal microscopy.

Laser Scanning Confocal Microscopy

Fluorescence was detected using a Leica TCS NT-SP-UV LS confocal microscope (Milton Keynes). Each blastocyst was examined using serial scanning two channel confocal microscopy. To detect TUNEL labeling with fluorescein isothiocyanate-FITC label (excitation 488 nm) and DAPI staining (UV; excitation 350 nm), a red and blue colored Look Up Table was allocated, respectively. A Z series of optical sections was taken through each specimen, consisting of 25–36 optical sections at 2- to 3-μm intervals. This allowed the analysis of all nuclei within the same embryo. A projection image was created using Leica Scanware software resulting in blue (DAPI) nuclei and pink (TUNEL) nuclei. All confocal images were stored digitally as the original Z series.

Five nuclear morphologies were observed: 1) 'healthy' interphase nuclei that were either rounded or oval with a distinct nuclear outline and uniform DAPI staining but with no TUNEL labeling (Fig. 1D); 2) mitoses, which included cells at the prophase, metaphase or anaphase stages with visible chromosomes and which were counted as single nuclei (Fig. 1E); 3) condensed nuclei with intense DAPI staining, which were smaller than 'healthy' interphase nuclei and were uniformly TUNEL labeled (Fig. 1, E and G); 4)

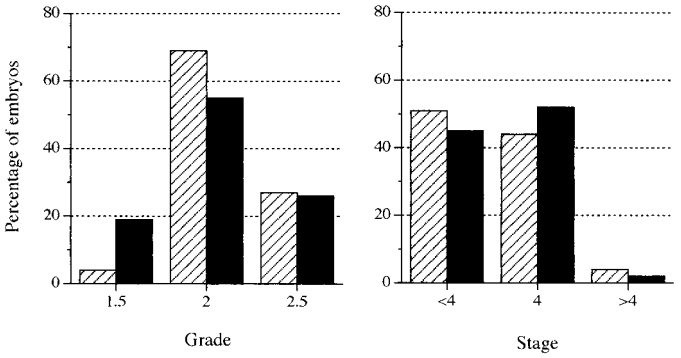

FIG. 2. Distribution of grade (left panel) and stage (right panel) of normally fertilized Day 2 human embryos randomly allocated to groups cultured in the presence (■, n = 42) and absence (▨, n = 45) of IGF-I.

fragmented nuclei which were both TUNEL labeled and fragmented into discrete clusters of membrane-bound vesicles (Fig. 1, D, F, and G); and 5) fragmented nuclei which were not TUNEL labeled (Fig. 1, F and G). Fragmented nuclei with or without TUNEL labeling (indicative of DNA fragmentation) were considered to be 'apoptotic' nuclei. Nuclei at interphase or undergoing mitosis were defined as 'healthy' nuclei. For each embryo, the number of nuclei, including these five apoptotic and healthy morphologies, were counted. The total number of nuclei included interphase, mitotic, TUNEL-labeled condensed, and fragmented nuclei with or without TUNEL (Fig. 1).

Mitotic, TUNEL, fragmented, and apoptotic indices were calculated for each individual embryo as follows:

$$\text{mitotic index} = (\text{no. of metaphases})/(\text{total no. nuclei}) \times 100;$$

$$\text{TUNEL index} = (\text{no. TUNEL-labeled nuclei})/(\text{total no. nuclei}) \times 100;$$

$$\text{fragmented index} = (\text{no. fragmented nuclei—TUNEL or not})/(\text{total no. nuclei}) \times 100.$$

The number of apoptotic nuclei included all nuclei that exhibited morphological features of apoptosis, i.e.,

$$\text{total number of apoptotic nuclei} = (\text{fragmented TUNEL-labeled nuclei}) + (\text{fragmented not TUNEL-labeled nuclei}) + (\text{condensed TUNEL-labeled nuclei});$$

$$\text{apoptotic index} = (\text{no. apoptotic nuclei})/(\text{total no. nuclei}) \times 100.$$

Statistics

Differences between the percentages of embryos reaching the morula and blastocyst stages were compared by χ^2-squared analysis. Differences in the distribution of total cell numbers per blastocyst, proportion of TUNEL-labeled nuclei, fragmented nuclei and mitoses were compared using the Mann-Whitney U test. Statview II (Abacus Concepts, Berkeley, CA) was used for statistical analyses.

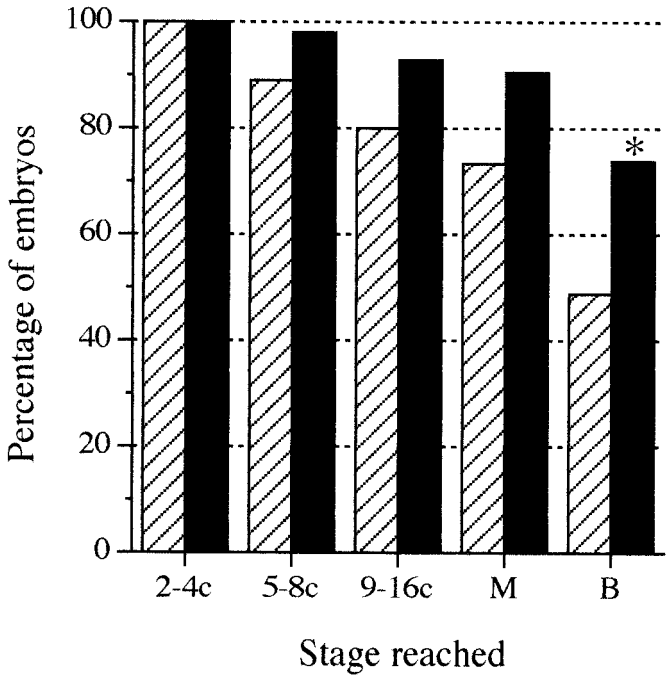

FIG. 3. Morula and blastocyst formation in the presence (■, n = 42) and absence (▨, n = 45) of IGF-I. IGF-I significantly enhanced blastocyst formation (*$P < 0.01$, χ^2).

RESULTS

Development of Human Embryos In Vitro

Eighty-seven normally fertilized embryos of good morphology from 32 patients were cultured in the presence (n = 42) or absence (n = 45) of 1.7 nM IGF-I from Day 2 until Day 6. Embryos were distributed randomly and evenly from each patient between the two media and were graded morphologically at the beginning of each culture experiment. There was no significant difference in the distribution of the grade or stage of embryos allocated to each group (Fig. 2).

Overall, out of a total of 87 embryos, 53 (61%) reached the blastocyst stage by Day 6. The remainder arrested mainly between the 8-cell and morula stages (Fig. 3). Supplementation of culture medium with 1.7 nM IGF-I significantly increased the proportion of embryos reaching the blastocyst stage (74% compared to 49% in the control group $P < 0.05$; Mann-Whitney) while decreasing the number arresting at the morula and other early cleavage stages (Fig. 3). Of the embryos that reached the blastocyst stage, the majority did so by Day 5 (70% in the presence of IGF-I and 59% in the absence of IGF-I, n.s.). There was no significant difference in the rate of development between Days 2 and 6 in the presence and absence of IGF-I.

Total Number of Nuclei in Day 6 Blastocysts

The total number of nuclei was counted for each blastocyst that remained intact on Day 6, 26/31 (84%) in the +IGF-I group and 17/22 (77%) in the –IGF-I group. Two blastocysts degenerated between Days 5 and 6 in the +IGF-I group and four in the –IGF-I group; thus counts were unobtainable. Furthermore, three blastocysts from the +IGF-I group and one from the –IGF-I group were lost during processing.

Blastocysts cultured in the presence of IGF-I had a similar total number of nuclei (61.7 ± 4.6; n = 26) to embryos

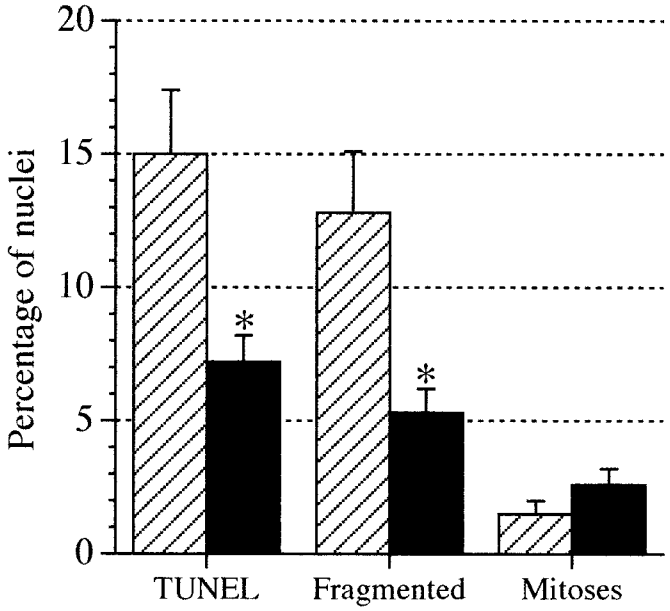

FIG. 4. DNA fragmentation (TUNEL), nuclear fragmentation (fragmented) and cell division (mitoses) in Day 6 human blastocysts following culture in the presence (■, n = 26) and absence of IGF-I (▨, n = 17). IGF-I significantly reduced DNA and nuclear fragmentation (*$P < 0.01$, Mann-Whitney). Values are mean ± SEM.

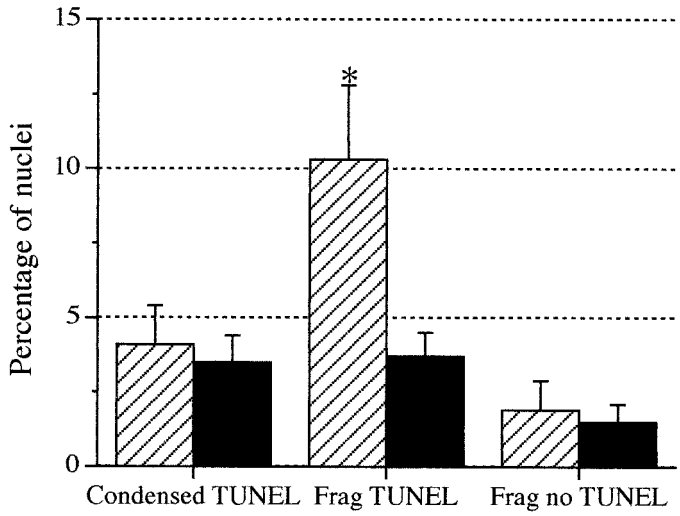

FIG. 5. Effect of IGF-I on apoptotic nuclear morphology in blastocysts. IGF-I specifically inhibits the formation of nuclei with both DNA and nuclear fragmentation (*$P < 0.005$, Mann-Whitney). Mean percentage of nuclei that were condensed and TUNEL labeled (condensed TUNEL), fragmented and TUNEL-labeled (Frag TUNEL), and fragmented nuclei with no TUNEL-labeling (Frag no TUNEL) in Day 6 blastocysts cultured in the presence (■, n = 26) or absence of IGF-I (▨, n = 17). Values are mean ± SEM.

cultured in its absence (54.5 ± 5.1; n = 17). The total number of nuclei ranged from 22 to 110 and 17 to 85 in the +IGF-I and –IGF-I groups, respectively. There was no significant difference in the mitotic index between the two groups (2.6 ± 0.6 with IGF-I; 1.5 ± 0.5 without IGF-I) (Fig. 4).

Apoptosis and Nuclear Morphology

The proportion of nuclei showing features of apoptosis (nuclear and/or DNA fragmentation) decreased from 16.3 ± 2.9% (–IGF-I) to 8.7 ± 1.4% (+IGF-I). The majority of fragmented nuclei (75% or more) were also TUNEL labeled. Comparing each marker of apoptosis separately, IGF-I significantly reduced ($P < 0.005$; Mann-Whitney) the percentage of fragmented nuclei from 12.8 ± 2.3 (control) to 5.3 ± 0.9 (Fig. 4). IGF-I also significantly decreased the proportion of TUNEL-labeled nuclei in blastocysts from 15.0 ± 2.4 to 7.2 ± 1.0 (Fig. 4). Figure 1, H and I show two representative Day 6 blastocysts with similar total cell numbers cultured in the absence and presence of IGF-I, respectively. These display the average TUNEL index for their respective groups.

The different apoptotic nuclear morphologies were categorized into three groups; fragmented and TUNEL labeled, fragmented with no TUNEL labeling, and TUNEL-labeled condensed nuclei. IGF-I had no effect on the proportion of condensed nuclei, or of fragmented nuclei with no TUNEL labeling (Fig. 5). However, blastocysts cultured in the presence of IGF-I had significantly lower proportions of nuclei that were both fragmented and TUNEL labeled (Fig. 5; $P < 0.005$; Mann-Whitney).

The nuclei in DNase-treated positive controls were all TUNEL positive. There was no staining present in the negative controls.

Blastocyst Morphology and Apoptosis

The number of grade II blastocysts were low in both the presence (n = 4) and absence (n = 4) of IGF-I, therefore the data for grade I and II blastocysts were amalgamated and considered to represent 'good morphology' blastocysts. 71% of blastocysts cultured in the presence of IGF-I were grade I and II, while 59% of control blastocysts were grade I and II (n.s.). The average total cell number in grade I blastocysts in the presence of IGF-I (71.7 ± 5.8) was similar to that in the absence of IGF-I (64.3 ± 7.8).

Blastocysts of good morphology (grades I and II) had fewer apoptotic nuclei than embryos of poor morphology (grade III), significantly so in the presence of IGF-I ($P < 0.01$; Mann-Whitney) (Fig. 6). IGF-I reduced the proportion of fragmented nuclei in blastocysts of good morphology ($P < 0.05$; Mann-Whitney) (Fig. 6); i.e., the reduction in apoptosis seen in the presence of IGF-I was seen even when comparing embryos of similar morphology and was not due to an increase in the proportion of blastocysts of good morphology.

Cytoplasmic Fragmentation

Cytoplasmic fragmentation was present at low levels in the majority of embryos used for this study (Fig. 2). Taking light micrographs twice daily allowed us to monitor fluctuations in the degree of cytoplasmic fragmentation. There was no increase in cytoplasmic fragmentation in embryos cultured in the absence of IGF-I.

DISCUSSION

We present evidence that IGF-I acts as a 'survival' factor during human preimplantation development. We found that 1.7-nM IGF-I significantly reduced the proportion of apoptotic nuclei in Day 6 human blastocysts (Fig. 4). Indeed, levels of nuclear and DNA fragmentation, as quantified by DAPI staining and TUNEL labeling, respectively, were reduced in the presence of IGF-I by 48% and 41%, respectively (Fig. 4). In addition, IGF-I significantly increased the proportion of embryos reaching the blastocyst stage by 25%, confirming previous work by Lighten et al. [31]. The total cell numbers in blastocysts cultured with (62 ± 5) and

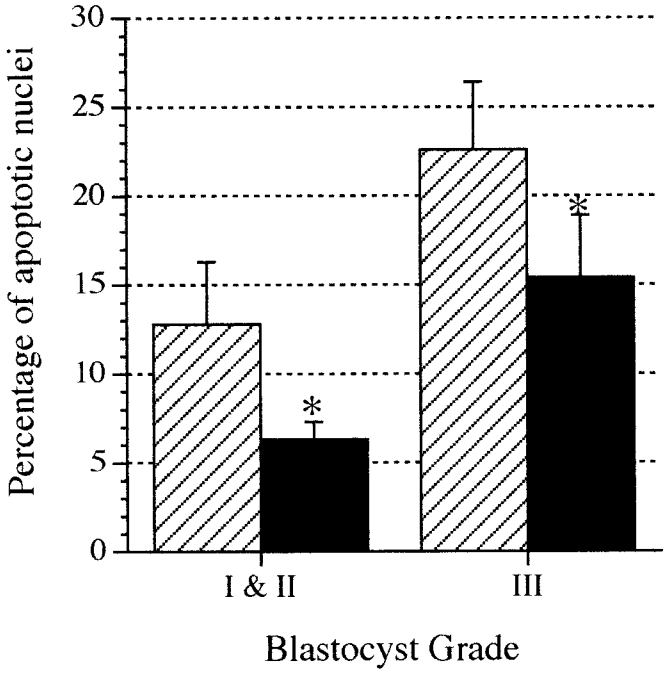

FIG. 6. Relationship between blastocyst morphology and the percentage of apoptotic nuclei in Day 6 blastocysts cultured in the presence (■, n = 26) or absence of IGF-I (▨, n = 17). The mean percentage of apoptotic nuclei in blastocysts of good morphology (I and II) (n = 19 and 12 in the presence and absence of IGF-I respectively) was compared to that in blastocysts of poor morphology (III) (n = 7 and 5, respectively). The percentage of apoptotic nuclei was significantly lower in blastocysts of good morphology cultured in the presence of IGF-I compared to similar blastocysts cultured in the absence of IGF-I (*$P < 0.05$), and compared to blastocysts of poor morphology (+IGF-I) (*$P < 0.01$). Values are mean ± SEM.

without (54 ± 5) IGF-I are in close agreement with those reported by Lighten et al. [31] (64 ± 5 and 54 ± 2, respectively). Labeling of embryo nuclei with DAPI did not allow us to confirm whether IGF-I specifically increased the cell number in the ICM (as was shown in [31]), or reduced cell death in this lineage. Studies in other species have shown that TGF-α significantly reduces cell death in the ICM of mouse blastocysts [22]. The anti-apoptotic properties of IGF-I have also been demonstrated in rabbit preimplantation embryos [37]. Herrler et al. [37] found that IGF-I reduced levels of cell death and increased cell number in rabbit preimplantation embryos with UV-induced DNA damage.

IGF-I is a powerful inhibitor of apoptosis via an IGF-I receptor mediated cell survival pathway (reviewed in [39, 40]). IGF-I, through activation of its receptor, has been shown to confer protection from apoptosis to a wide range of cells, including blastomeres [37], hemopoietic cells [48], fibroblasts [49, 50], neuroblastoma cells [51] and oligodendrocytes [24] (reviewed by [40]). Rodriguez-Tarduchy et al. [48] were the first to demonstrate the role of IGF-I as a survival factor for cells in culture that were IL-3-dependent. Further confirmation of the anti-apoptotic properties of IGF-I were provided by the study of c-myc induced apoptosis in Rat-1 fibroblasts [49], where it was found that IGF-I prevented death at any stage of the cell cycle even in the presence of a protein synthesis inhibitor. It has also been shown that reduction in the number of IGF-I receptors (IGF-Rs) causes extensive apoptosis while over-expression of IGF-Rs protects cells from apoptosis in vivo [52]. Interestingly, a direct correlation between expression of IGF-I ligand and receptor and developmental potential to the blastocyst stage in mouse preimplantation embryos has recently been observed [53].

We identified a range of different nuclear morphological features that could be either indicative of different stages of the degradation phase of the apoptotic pathway or the result of different apoptotic pathways being activated (Fig. 5). For example, DNA fragmentation may occur first in condensed nuclei, before the nucleus itself fragments. Fragmented nuclei without DNA fragmentation may represent the activation of another apoptotic pathway. Although there was no significant difference in the proportion of condensed nuclei with fragmented DNA and TUNEL-negative fragmented nuclei, there was a highly significant decrease (52%) of nuclei that had both DNA and nuclear fragmentation in blastocysts cultured in the presence of IGF-I. Our findings corroborate studies from other cell systems that have found that IGF-IR activation inhibits DNA fragmentation. There are a number of hypotheses for the mechanisms by which the IGF-I survival pathway achieves the inhibition of apoptosis (e.g., transcriptional activation of Bcl-2, inhibition of caspases, upregulation of DNA repair enzymes), and this again may reflect the existence of multiple pathways. IGF-I is known to bind to its receptor IGF-IR with highest affinity, activating tyrosine kinase function which in turn may activate a number of major signalling pathways (e.g., PI-3 kinase, p70 S6 kinase, MAP kinase) depending on the cell type. PI 3-kinase is emerging as the major effector pathway and activation of this pathway may result in phosphorylation and sequestration of pro-apoptotic factors such as BAD, preventing them from dimerizing with anti-apoptotic BCL-2 members in the mitochondrial membrane. Growth factor withdrawal would inactivate the kinase pathway, leaving BAD unphosphorylated and free to heterodimerize with BCL-2 and BCL-xL, leading to release of cytochrome c from the mitochondria, caspase activation, and apoptosis [54]. It is most likely that the nature of the trigger that activates the apoptotic pathway (growth factor deprivation, UV irradiation, abnormal chromosomal complement) will define the apoptotic signalling pathways acted on by a given survival factor.

The presence of nuclear fragmentation in mouse embryos in vivo [55] suggests that apoptosis occurs as a normal physiological process during normal preimplantation embryogenesis. In vitro culture has been shown to increase levels of cell death in mouse embryos [22] and levels of cell death can be reduced by altering the composition of the culture media [56]. It may be argued that by adding IGF-I to culture media we are 'rescuing' cells that are destined to die. IGF-I may be suppressing apoptosis triggered by genetic damage or suboptimal culture conditions. Alternatively, it may be inhibiting the elimination of cells with an inappropriate developmental potential. It has been proposed that ICM cells which retain the potential to form TE are eliminated by apoptosis during normal preimplantation development [55, 57]. The increased number of ICM cells in the presence of IGF-I [31] is consistent with inhibition of this proposed elimination.

We found that supplementation of culture medium with IGF-I also significantly increased the proportion of embryos reaching the blastocyst stage by 25%, yet we did not observe an increase in cell number despite seeing a significant decrease in cell death. It is possible that IGF-I is 'rescuing' embryos that would otherwise have arrested, and that the rescued embryos have a low cell number, reducing

the mean cell number in the +IGF-I group and masking any IGF-I induced increase in cell number. While optimizing culture conditions to ensure that healthy embryos reach the blastocyst stage is an important goal, it is of critical importance that mechanisms by which abnormal cells are eliminated are not overridden. Thus it is important to confirm that embryos cultured in the presence of IGF-I do not have an increased incidence of chromosomal abnormalities.

Blastocysts were classified according to their morphology (Fig. 1, A–C). Firstly, we found that embryos of good morphology (grades I and II) had a lower proportion of apoptotic nuclei in the presence of IGF-I than in its absence (Fig. 6). Secondly, the proportion of embryos of good morphology was not significantly different in the presence and absence of IGF-I. These two points demonstrate that IGF-I is not decreasing cell death rates by increasing the proportion of grade I blastocysts, but rather by reducing apoptosis per se. The incidence of cell death was correlated with embryo morphology, with embryos of poor morphology having a higher proportion of dead cells than embryos of good morphology (Fig. 6), confirming previous studies [8].

We have demonstrated that IGF-I reduces apoptosis during early human preimplantation development. Before adding IGF-I to culture medium it should be kept in mind that during in vivo conditions embryos are subjected to a ''soup'' of growth factors and their binding proteins. Nevertheless, while IGF-I supplementation of culture medium appears to be beneficial as it increases the number of embryos surviving and reaching the blastocyst stage, it is now important to firstly confirm that IGF-I is not overriding natural selection mechanisms and secondly to examine whether improvement of in vitro culture conditions will lead to a higher implantation and live birth rate.

The precise causes of apoptosis in preimplantation development remain unknown although the evidence presented here suggests that some cell death can be modulated by the environment. We do not believe that there is a single cause or role for cell death in preimplantation embryos but that apoptosis can be triggered by a variety of stimuli at different stages of development resulting in the elimination of cells by a number of apoptotic pathways.

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