Increased expression of the cyclin-dependent kinase inhibitor p27 in cleavage-stage human embryos exhibiting developmental arrest

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It is accepted that ~50% of embryos obtained after IVF arrest during the first week. Traditionally, chromosome abnormality and suboptimal culture conditions have been proposed as factors commonly associated with embryo arrest. However, even when considering ‘ideal’ conditions and embryos of only excellent morphology in vitro, there is still a significant incidence of embryonic arrest. There is considerable evidence that the nuclear protein p27, a member of the Cip/Kip family of CDK inhibitors, plays an important role in multiple fundamental cellular processes, including cell proliferation, cell differentiation, and apoptosis. The present investigation, using immunocytochemical techniques coupled with confocal microscopy, was undertaken to determine whether p27 could play a role in the arrest of 4–8-cell human embryos. A total of 28 preimplantation embryos at the 4–8-cell stage were investigated. Of these, 16 were diploid embryos showing cleavage arrest with no further progression, and 12 were normally developing embryos. There was a 2-fold increased expression of the cell-cycle inhibitor p27 in arrested embryos compared with control normally developing embryos. This study represents the first demonstration of an increased expression of p27 in cleavage-stage human arrested embryos.

Key words: arrested embryo/CDK inhibitors/embryo growth/IVF/p27

Introduction

Natural fecundity and implantation in the human are low compared with other species. Even with daily intercourse, most (63%) ovulatory menstrual cycles may be incapable of producing a conception (Wilcox et al., 1995) and 70% of conceptions fail to achieve viability, with an estimated 50% lost before the first missed menses (Hill, 1994). Similarly, in spite of improvements in assisted reproduction techniques, success rates remain low and implantation failure following embryo transfer is a major continuing problem in IVF. Data from recent large databases (Centers for Disease Control, 2000; European Society of Human Reproduction and Embryology, 2001) show that as many as 75–80% of embryos that are transferred to patients 2–3 days after IVF fail to implant in the uterus. Hardy et al. have reported varying degrees of cellular fragmentation and asymmetry in ~75% of embryos and developmental arrest during the first week in ~50% of embryos cultured in vitro (Hardy, 1993, 1999; Hardy et al., 2001). This high level of early embryonic death must contribute significantly to the observed low fecundity. Definite causes for this high early embryonic loss rate remain to be elucidated, but both chromosome abnormality (Munné et al., 1995; Wells and Delhanty, 2000) and suboptimal culture conditions (Hardy, 1993; Bavister, 1995) have been proposed.

Current concepts on the molecular mechanisms governing various stages of oocyte maturation, transition from maternal to embryonic control and the initial steps of pre-embryo development are unveiling new insights into reproductive medicine. Several lines of evidence suggest that the presence of a maturation promoting factor (MPF) is responsible for the resumption of meiosis in oocytes, and that meiosis arrests once more at metaphase-II owing to the action of an oocyte-specific protein kinase, c-mos, until fertilization, when meiosis is completed (Heikinheimo and Gibbons, 1998; Cohen et al., 1999). After fertilization, MPF continues to play a role in regulating the bi-phasic cell cycles of early cleavage divisions. While MPF is critical for the entry into mitosis, various cyclins and their respective kinases are responsible for the progression through the remainder of the cell cycle. Cell cycle transcripts and proteins, c-mos and cyclins have been identified in human oocytes and preimplantation embryos (Heikinheimo and Gibbons, 1998; Cohen et al., 1999).

The orderly progression of dividing mammalian cells through the various phases of the cell cycle is governed by a series of proteins called cyclins which function as positive regulatory subunits of a family of cyclin-dependent kinases (CDK), whose activity is inhibited by the CDK inhibitors (Tsilihas et al., 1999; Šgambato et al., 2000; Slingerland and Pagano, 2000). CDK inhibitors cause G1 arrest when overexpressed in transfected cells and are classified into two families: the Cip/Kip family members (p21, p27 and p57) which possess the ability to inhibit a variety of cyclin–CDK complexes and share partial structural similarity, and the Ink4 family members (p15, p16, p18 and p19) which are CDK4/CDK6–specific inhibitors (Sherr, 1994;
Sherr and Roberts, 1995). There is considerable evidence that p27 is a nuclear protein playing an important role in multiple fundamental cellular processes, including cell proliferation arrest, cell differentiation, and apoptosis. Moreover, p27 is a putative tumour-suppressor gene that appears to play a critical role in controlling the pathogenesis of several human malignancies and its reduced expression has been shown to correlate with poor prognosis in cancer patients (Tsibillas et al., 1999; Sgambato et al., 2000).

The present investigation, using immunocytochemical techniques coupled with confocal microscopy, was undertaken to determine whether p27 could play a role in the arrest of 4–8-cell human embryos, as this, to our knowledge, has not been previously studied.

Materials and methods

Embryo collection and culture

Human embryos deemed non-viable material for embryo transfer were obtained from 14 patients undergoing assisted fertilization at the Assisted Reproduction Unit of the Hospital Clinic of Barcelona, following written consent and Institutional Review Board approval. A total of 28 preimplantation embryos at the 4–8-cell stage was investigated. Of these, 16 were diploid embryos showing cleavage arrest on days 3 or 4 with no further progression, when compared with the previous 24 h (study group). As controls, we used 12 8-cell stage normally developing 4 day old embryos obtained from patients undergoing preimplantation genetic diagnosis; these embryos had been discarded from embryo transfer because they were affected.

Ovarian stimulation with gonadotrophins under pituitary suppression and oocyte collection were conducted as previously reported (Balasch et al., 2001). Preincubation and insemination of oocytes and embryo culture were carried out in IVF-20 medium (Scandinavian IVF Science, Gothenburg, Sweden) at 37°C in an atmosphere of 5% CO₂ and 95% air. Following assessment of normal fertilization, embryos were cultured in 50 µl microdrops under mineral oil (Ovopl-150; Scandinavian IVF Science) as previously suggested (Lane and Gardner, 1992). On day 2 or 3 following insemination, embryos were examined and their overall quality recorded. Embryo evaluation included morphological characteristics as reported elsewhere (Veeck, 1999) and nuclear status. Briefly, embryos were classified as: grade I: perfectly symmetrical with no fragmentation; grade II: perfectly symmetrical with slight fragmentation (<20% fragmentation of the total embryonic volume); grade III: uneven blastomeres with no fragmentation; grade IV: perfectly symmetrical or uneven blastomeres with gross fragmentation (>20% fragments); and grade V: totally fragmented. Multinucleation was considered for any embryo presenting >1 multinucleated blastomere. All evaluations were performed with use of an Olympus inverted light microscope with Hoffman Optics.

Immunocytochemistry

Prior to embryo fixation, the zona pellucida encapsulating the embryo was removed by 10 min pronase digestion (0.5% in Ham’s F-10 culture medium) (Fluka, Neu-Ulm, Switzerland) at 37°C. After three 5 min washes in phosphate buffered saline containing 5% bovine serum albumin (5% BSA-PBS) (Sigma, St Louis, MO, USA), zona-free embryos were fixed for 12 min in 4% paraformaldehyde (Fluka) diluted in 5% BSA-PBS at room temperature. After three more 5 min washes in 5% BSA-PBS, they were then permeabilized with 0.1% Triton X-100 detergent (Sigma) diluted in 5% BSA-PBS for 10 min at room temperature.

At the time of staining, embryos were washed again three times in 5% BSA-PBS and then incubated for 1 h at 37°C with an anti-p27 polyclonal antibody (10 µg/ml in 5% BSA-PBS containing 0.1% Triton X-100) (Ref.: Sc 528; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Under the same conditions, embryos were incubated with an anti-rabbit antibody conjugated with Cyanine 3 (30 µg/ml in 5% BSA-PBS containing 0.1% Triton X-100) (Ref.: 111–166047; Jackson, West Grove, PA, USA). Both steps were followed with 5 min washes in 5% BSA-PBS. Exposure to light was minimized during and following secondary antibody incubation. Afterwards, embryos were individually mounted on a coverslip in a microdrop mounting medium (Sigma) within an adhesive 1 mm paper ring. Before setting the embryos, coverslips had been treated with a slide adhesive solution, poly-L-lysine (0.1% in water, Sigma), for 10 min and kept at 60°C for 1 h. Finally, a slide was applied to the coverslip and sealed with nail varnish.

Control of antibody specificity was done by preincubation of the primary antibody with purified p27-GST (0.1 µg/ml, 30 min, room temperature). The background signal was determined by incubation of the fixed and permeabilized embryos in the absence of the primary antibody. Only cytoplasmic background was observed. Analysis and quantification of p27 was performed with the confocal microscope (see below). To standardize the different measures, the ratio between the nuclear staining and the cytoplasmic background was evaluated.

Confocal microscopy analysis

Whole mounted samples were examined on a TCS NT confocal laser scanning microscope (Leica, Heidelberg, Germany). The excitation source was an Argon/Krypton laser (excitation wavelength 568 nm). Fluorescence emission from Cyanine 3 was recorded by a detector behind a 590 nm long pass filter. Both excitation and emission light were focused through a ×63 Plan Apochromate oil immersion objective with a numerical aperture of 1.4. Optical sections were acquired, each one with an image resolution of 1024×1024 pixels. Image processing was carried out with the NIH Scion image analysis software. Images were converted to grey scale and levels of p27 were expressed as the ratio between the mean nuclear optical density value and the mean cytoplasmic optical density value. The laser power and the offset used were always the same. The PMT gain was changed in a few cases in order to acquire the image in the linear range of signal. In order to correct for these changes, p27 levels were always evaluated as the ratio between nuclear and cytoplasmic grey intensities. Image processing was carried out with Leica computer software. Levels of p27 were expressed as the ratio between the mean densitometric value of the red channel in the nucleus and the red channel in cytoplasm (background). These parameters were measured using the same Leica software.

Statistics

Results are presented as mean ± SEM. For statistical analysis the Mann–Whitney U-test and Fisher’s exact test were used as appropriate. Data were analysed by Statistics Package for Social Sciences (SPSS, Chicago, IL, USA).

Results

Embryo morphological characteristics in the two groups studied are summarized in Table I. Cell fragmentation was similar in both groups of embryos, but the proportion of embryos exhibiting multinucleated blastomeres was significantly higher in the arrested group (P < 0.05).

Figure 1. p27 expression in arrested (n = 16) and normally developing (n = 12) embryos (controls). Results are given as individual data points with each point representing the ratio between nuclear and cytoplasmic optical density values. The bars represent mean values. In the arrested embryos group, triangles represent those embryos having multinucleation, and open circles correspond to embryos with gross fragmentation.
Immunocytochemistry was used to investigate the expression of the p27 protein. As shown in Figure 1, the expression of p27 was significantly higher in the arrested embryos (mean ± SEM: 2.53 ± 0.22) than in the control group (1.15 ± 0.14) ($P < 0.0001$). High p27 immunostaining was observed among arrested embryos irrespective of embryo fragmentation and multinucleation (Figure 1). Only one arrested embryo (showing neither fragmentation nor multinucleation) had p27 levels within the range of the control group.

Representative immunocytochemistry results of p27 immunostaining of normally developing embryos, arrested embryos, and an arrested negative control (stained only with the secondary antibody) are presented in Figure 2. The staining pattern obtained with p27 antibody that had been preincubated with purified p27-GST and the embryo staining with the secondary antibody only were both characterized by a faint cytoplasmic signal (Figure 2). Interestingly, two control and one arrested embryo were obtained from the same couple, with the two normal embryos testing negative for p27 staining but the arrested embryo clearly expressing that protein (Figure 2c,f). There were no differences in the staining pattern between blastomeres within an embryo, with all (p27 positive embryos) or none (p27 negative embryos) showing expression of p27.

Discussion

At present, little is known about the molecular mechanisms regulating cell multiplication in human pre-embryos, partly because of the limited number of embryos. Only 30–40% of all zygotes produced in vitro develop successfully to the blastocyst stage (Jones, 2000), and the time of arrest for the majority of embryos appears to be between the 4- and 8-cell stages (Bolton and Braude, 1987). The present study is the first to demonstrate an increased expression of the cyclin-dependent kinase inhibitor p27 in cleavage-stage human embryos exhibiting developmental arrest. There was a 2-fold increased expression of the cell-cycle inhibitor p27 in arrested embryos as compared with control normally developing embryos.

Precise control of cell-cycle progression is believed to be critical for normal development. Development of the human conceptus is a tightly controlled event and one of the keys to its regulation might reside in the balance between the expression of cell-cycle promotors, such as cyclins, and cell-cycle inhibitors, such as CDK inhibitors. Thus, a recent study showed that strong expression of the cell-cycle inhibitor p27 and absence of expression of cyclin E in the syncytiotrophoblast might represent an important control mechanism in human placentation proliferation (Bamberger et al., 1999). The first embryonic cell cycles are characteristically rapid; in human embryos the duration of the first cell cycle is ~20–22 h. Accordingly, cyclins needed for mitotic cell division are among the first genes to be expressed following initiation of the zygotic gene expression (Heikinheimo and Gibbons, 1998). It has been thought that cell-cycle checkpoint mechanisms are not operating at this early stage of development and it has been hypothesized that they may also not be functioning during early cleavage divisions (Handyside and Delhanty, 1997; Hunt, 1998) as demonstrated by the high percentage of aneuploidy and mosaicism in human preimplantation embryos (Muné et al., 1995; Wells and Delhanty, 2000).

The high rate of cleavage arrest occurring at a stage coincident with the stage of activation of the embryonic genome (Braude et al., 1988; Tesarik et al., 1988) has led to the suggestion that failure of activation of the genome in the embryo as a whole or in a significant proportion of blastomeres within the embryo may

<table>
<thead>
<tr>
<th>Group</th>
<th>Embryos with no or slight fragmentation (grade I, II, III)</th>
<th>Embryos with gross fragmentation* (grade IV, V)</th>
<th>Multinucleated embryosb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrested embryos ($n = 16$)</td>
<td>14</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Control embryos ($n = 12$)</td>
<td>10</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
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*aNo difference with respect to embryo fragmentation was observed between groups.

*bMultinucleation was significantly higher in the arrested group ($P < 0.05$).

Figure 2. Confocal images of arrested and normally developing embryos immunostained for p27. Arrested (a–c) and normally developing (d–f) embryos were processed for p27 immunostaining and confocal analysis as indicated in the Materials and methods section. Optical sections showing the highest number of nuclei were selected. (a, c, e) non-fragmented and (b, d, f) fragmented embryos. (c and f) Two embryos from the same patient. (g) An arrested embryo incubated with only the secondary antibody. (h) An arrested embryo immunostained with p27-GST preincubated anti-p27 antibody.
be the cause of the cleavage arrest. However, cleavage-arrested human embryos have shown evidence of synthesis of some transcriptionally dependent proteins, suggesting that cleavage arrest in vitro is not always associated with failure of genome expression (Artley et al., 1992; Artley and Braude, 1993). Moreover, it has been found that, although there are differences in the protein synthetic pattern between blastomeres within an embryo, the blastomeres from an individual embryo appear to be synchronous in terms of their genome activity, with all or none appearing to have activated their genome. Thus, it has been stressed that abnormalities of cleavage and subsequent development may be caused by cytoplasmic anomalies induced by culture in vitro, and that failure of genome activation is unlikely to be a primary cause of failure, but may be induced as a secondary event (Artley and Braude, 1993). This notwithstanding, a recent mathematical model of human preimplantation embryo development where the balance between apoptosis and proliferation was applied has concluded that a better understanding of mechanisms causing embryo abnormalities during early cleavage stages would be much more important than trying to optimize culture media in order to improve success rates in IVF (Hardy et al., 2001).

This is the first study showing an increased expression of p27 in cleavage-stage human embryos exhibiting developmental arrest. It might be argued that the observed increase in multinucleation and the potential role of chromosomal abnormalities, both of which are more common in arrested embryos, are limitations of our study. However, neither multinucleation nor chromosomal abnormalities or DNA damage in mammalian cells have been associated with increased expression of p27 (Wang, 1998). In addition, 67% (10/15) of arrested embryos showing high p27 immunostaining were non-multinucleated in the present investigation. Further studies are warranted both to confirm our results, given the relatively limited sample size in the present investigation, and to elucidate the mechanisms underlying the increased expression of p27 in cleavage-stage human arrested embryos.

References
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