Induced apoptosis and expression of related proteins in granulosa cells from women undergoing IVF: a preliminary study


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BACKGROUND: Because apoptosis may be involved in the outcome of IVF, the expression of pro- and anti-apoptosis proteins in a model of induced granulosa cell (GC) apoptosis was evaluated in 25 women with normal FSH levels undergoing IVF. METHODS: After 1 day of culture, apoptosis was induced with interferon (IFN)-γ (200 IU/ml), followed 24 h later by an agonistic anti-Fas antibody (0.5 µg/ml). On day 3, apoptotic GC, identified by chromatin condensation and/or nuclear fragmentation after DAPI staining, were counted among 1000 cells in randomly chosen fields under UV microscopy, and enabled allocation of women into two groups with either low (group 1) or high (group 2) percentages of apoptosis (11.6/8064 4.8 and 59.5/8064 14.8% respectively; P < 0.001). Immunoblotting was used to evaluate the following in proteins: poly (ADP-ribose) polymerase (PARP), caspases 8 and 3, Bcl-2, heat shock protein (HSP) 70, Bax, Bak and Stat-1 (a protein known to be inducible by IFN-γ). RESULTS: Based on densitometric analysis of immunoblots, the PARP 116 kDa bands were respectively 4.3- and 33.3-fold lower for treated groups 1 and 2. Caspase 8, caspase 3 and HSP70 were expressed slightly less in treated group 2 than treated group 1. Densitometric analysis of bands corresponding to Bcl-2 showed respectively for treated groups 1 and 2, 3.2- and 2.5-fold decreases. Bak expression was similar in both control groups, and comparably lower in the two treated groups. With regard to Stat-1, densitometry showed 3.3- and 1.3-fold increases respectively in treated groups 1 and 2. CONCLUSIONS: These results suggested that Fas-mediated apoptosis of GC is accompanied by significant changes in proteins acting in apoptosis, and that this type of programmed cell death might play a potential prognostic role for women undergoing IVF.

Key words: apoptosis-related proteins/human granulosa cell/IVF/Stat-1 protein

Introduction

Reports that the demise of granulosa cells (GC) during follicular atresia and luteolysis might occur by programmed cell death (Tilly et al., 1991; Hsueh et al., 1994; Palumbo and Yeh, 1994; Piquette et al., 1994; Yuan and Judice, 1997) have suggested a potential role of apoptosis in human reproduction. Spontaneous apoptosis was observed in GC isolated from the follicular fluid (FF) of women undergoing IVF (Seifer et al., 1996; Sugino et al., 1996; Nakahara et al., 1997a). With regard to the relationship between apoptosis and IVF outcomes, a high incidence of spontaneous apoptosis of human GC has been associated with small-sized follicles (Sugino et al., 1996), lower ovarian reserves (Seifer et al., 1996), poor oocyte outcomes (Nakahara et al., 1997a) and a low pregnancy rate (Nakahara et al., 1997b; Oosterhuis et al., 1998).

In all these circumstances, however, the apoptosis pathway followed by human GC was not well defined. Biochemical analyses of animal and human GC have previously shown apoptosis to be regulated by caspases and Bcl-2 gene family members (Boone and Tsang, 1998; Kugu et al., 1998). The presence of the Fas receptor (Quirk et al., 1995) clearly indicated that this apoptosis pathway was also involved in GC isolated from women undergoing IVF.

The capacity of cultured GC to enter apoptosis after Fas induction has been investigated previously (Sifer et al., 2000). GC from 25 women undergoing IVF were incubated with interferon (IFN)-γ, a well-known Fas protein up-regulator (Choi et al., 1999; Moers et al., 1999), before being subjected to the action of an agonistic anti-Fas antibody. The percentage of each patient’s treated GC entering apoptosis allowed distinction to be made between two groups of women. In the present study, the expression of apoptosis-related proteins implicated...
in the Fas pathway in relation to the rate of apoptosis was analysed.

Materials and methods

FF samples were collected from 25 women, aged ≤40 years, who were undergoing oocyte retrieval for IVF procedures.

IVF stimulation procedure

All patients received the standard stimulation protocol, as described briefly below. First, hormonal down-regulation was started on day 3 of the menstrual cycle preceding IVF with a GnRH agonist, triptorelin (Decapeptyl®; Ipsen/Biotech, Paris, France), given i.m. at a daily dose of 0.1 mg for a minimum of 2 weeks. When the plasma estradiol level was ≤50 pg/ml, two ampoules of rFSH (follicitrophin α; Gonal-F®, 75 IU, Serono, Boulonne, France, or follicitrophin β; Puregon®; 100 IU, Organon, Saint-Denis, France) were administered i.m. every evening along with 0.05 mg of the GnRH agonist. Follicular development was monitored ultrasonographically with a 5 MHz transvaginal probe. The dose of gonadotrophin stimulation was then adjusted according to plasma estradiol levels and the size of ovarian follicles measured. When the plasma estradiol level surpassed 1800 pg/ml, and when at least two follicles with a diameter of 20 mm were observed on ultrasound examination, 10 000 IU of HCG (Gonadotrophine chorionique endoβ; Organon) were given i.m. to induce follicle rupture. Transvaginal follicular aspiration was performed 34–36 h later, under propofol (Diprivan®, Zeneca, Cergy, France) general anaesthesia.

Reagents

Monoclonal apoptotic agonistic anti-Fas/CD95 antibody (clone 7C11) was purchased from Immunotech (Marseille, France), and recombinant human IFN-γ from R&D Systems (Abingdon, Oxford, UK). For immunological studies, the following murine monoclonal antibodies were used: anti-caspase 8/FLICE immunoglobulin (Ig)G2a (clone B9-2); anti-poly (ADP-ribose) polymerase (PARP) IgG1 (clone B9-2); anti-poly (ADP-ribose) polymerase (PARP) IgG1 (clone C2-10) and anti-heat shock protein (HSP) 70 IgG2b (clone 5G10) from Pharmingen (San Diego, CA, USA); and anti-Stat-1 IgG1 from Transduction Laboratories (Lexington, KY, USA). Polyclonal rabbit antibodies to caspase 3/CPP32 and Bax (Pharmingen) and to Bcl-2 and Bak (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were also purchased. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and conjugated goat anti-rabbit IgG was from Immunotech, and the ECL Immunoblot detection system was from Amersham (Amersham, UK).

GC isolation and culture

After oocyte identification and isolation, all FF samples were collected, pooled for each woman and centrifuged at 500 g for 30 min. The supernatant was discarded and the pellet resuspended in 10 ml of phosphate-buffered saline (PBS) (Eurobio, Les Ulis, France). An aliquot of the suspension was layered onto 10 ml of a 50% Percoll® solution (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 100 g for 30 min to sediment the red blood cells. The isolated GC were aspirated from the interface, washed and resuspended in minimal essential medium (MEM) with Earle’s salts and glutaMAX (Gibco, Glasgow, UK) supplemented with 10% heat-inactivated fetal calf serum (Valbiotech, Paris, France) and antibiotics. Under our experimental conditions, lymphomyeloid cells were present only in the supernatant medium, this being verified as follows. GC, leukocytes and anti-CD45 antibody were mixed just before plating and on day 1 it was observed that, while GC were adherent, no lymphomyeloid cells had become attached. Purified GC were cultured for 1 day in 25 cm² vented culture flasks (Nunc, Roskilde, Denmark) at 37°C in humidified air with 5% CO₂, then washed with MEM to discard residual red and white blood cells. Fresh culture medium was added and incubation was continued according to the apoptosis-induction protocol.

Apoptosis induction

Cultured GC were divided into two aliquots. The first was treated to induce apoptosis, on day 1, with IFN-γ (200 IU/ml) and, on day 2, with the agonistic anti-human Fas monoclonal antibody (0.5 μg/ml). The second aliquot served as an untreated control. On day 3, treated and control GC were harvested by scraping, and the rate of apoptotic GC was measured by 4,6-diamidino-2-phenylinindole (DAPI) staining.

DAPI staining

After apoptosis induction, GC were fixed with 70% ethanol for 10 min at room temperature, smeared on a glass slide and air-dried. Cell nuclei were stained with 0.1 μg/ml DAPI (Sigma, St Louis, MO, USA) in PBS containing 0.1% Tween 20 (Sigma). The slides were washed three times in PBS, air-dried and mounted in Mowiol® solution (Calbiochem, San Diego, CA, USA). For each woman, GC with morphological characteristics of apoptosis, such as chromatin condensation and/or nuclear fragmentation, were identified and counted among 1000 cells in randomly selected fields using ultraviolet microscopy. DAPI staining of treated GC allowed identification of two groups of women based on the percentage of apoptotic cells (setting threshold at the median): group 1 (n = 12) had low percentages (mean 11.6 ± 4.8%) while group 2 (n = 13) had high percentages (mean 59.5 ± 14.8%; P < 0.001) (Sifer et al., 2000) (Figure 1). The percentages of apoptotic GC in the respective untreated control samples were comparable: 5.6 ± 2.7 versus 4.7 ± 2.7% (not
significant). The remaining treated and untreated cells from each group were pooled and subjected to immunoblotting. Results were analysed according to the group assignment.

**Immunoblot analysis**

After apoptosis induction, harvested GC were centrifuged at 500 g for 5 min. The pellets were resuspended in 1 ml of PBS and recentrifuged. Supernatants were carefully discarded and pellets weighed and frozen at –80°C until used in immunoblot analysis. To obtain membrane/cytoplasmic and nuclear extracts, cells were prepared as previously described (Andrews and Faller, 1991). Briefly, cell pellets were resuspended in 10 volumes of hypotonic buffer A [10 mmol/l HEPES–KOH (pH 7.9), 1.5 mmol/l MgCl2, 10 mmol/l KCl, 1 mmol/l dithiothreitol, 0.5 mmol/l spermidine, 1 mmol/l phenylmethylsulphonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml aprotinin], kept on ice for 10 min, vortexed and centrifuged at 500 g for 1 min at 4°C. The supernatant was considered to be the cytoplasmic extract. The nuclear pellet was treated with 5 volumes of hypertonic buffer B [20 mmol/l HEPES–KOH (pH 7.9), 25% glycerol, 0.4 mol/l NaCl, 1.5 mmol/l MgCl2, 20 µg EDTA, 4 mmol/l dithiothreitol, 1 mmol/l PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin] for 20 min on ice. Nuclear extracts were obtained after centrifugation at 18 000 g for 2 min at 4°C to separate non-extractable chromatin residue. The nuclear fraction was used to study the PARP cleavage, and the membrane/cytoplasmic fraction to evaluate the presence of caspases 3 and 8, Bcl-2, HSP70, Bax, Bak and Stat-1 proteins. Membrane/cytoplasmic or nuclear extract samples corresponding to 2 mg of cell pellet were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (7% polyacrylamide for PARP, HSP70 and Stat-1; 15% polyacrylamide for caspases 3 and 8, Bcl-2, Bax and Bak) and proteins were then transferred electrophoretically onto nitrocellulose membranes. To block non-specific sites, the membrane was incubated in 5% non-fat dry milk in PBS containing 0.1% Tween 20. Blots were incubated for 1 h at room temperature with the appropriate antibody, followed by an incubation with the corresponding HRP-conjugated antibody for 1 h, and then washed. Chemiluminescence detection was performed according to the manufacturer’s instructions and exposed to X-ray film for 5 s to 3 min for visualization. Immunoreactive bands for all proteins were evaluated densitometrically using Candela® software (Microvision, Evry, France) with an image analyser. For each protein of each group, the optical density was measured per surface unit (OD/mm²) and compared with its respective control, which was considered to be 100%.

**Results**

PARP was cleaved in both groups of treated GC compared with their controls (Figure 2). Densitometric analysis showed that the PARP-116 kDa bands were decreased 4.3-fold in group 1, and 33.3-fold in group 2. In contrast, the PARP-85 kDa bands were increased by only 2.8- and 2.2-fold respectively in groups 1 and 2. Caspase 8 (Figure 3a) and caspase 3 (Figure 3b) were weakly expressed in both treated groups compared with their controls: caspase 8 was 2.0- and 3.4-fold lower, and caspase 3 was 2.6- and 2.9-fold lower in groups 1 and 2 respectively. Densitometric analysis of bands corresponding to anti-apoptotic proteins Bcl-2 (Figure 3c) and HSP70 (Figure 3d) showed respectively for each protein and groups 1 and 2, 3.2- and 2.5-fold, and 1.5- and 1.7-fold decreases. Pro-apoptosis protein Bak (Figure 3e) was comparably expressed in both control groups, but a 1.8-fold decline was observed in group 2 compared with a 1.2-fold decline in group 1. Stat-1 (Figure 3f) in treated GC increased 3.3- and 1.3-fold respectively in groups 1 and 2. However, a 2.9-fold higher expression of Stat-1 was observed in the group 2 control compared with the group 1 control. Finally, the methodology used did not permit the detection of Bax in any group (data not shown).

**Discussion**

Several reports have documented a clear relationship between spontaneous apoptosis of human GC and IVF outcome (Nakahara et al., 1997b; Oosterhuis et al., 1998). Preliminary results obtained by the present authors (Sifer et al., 2000) did not confirm the existence of a relationship with spontaneous apoptosis. However, when GC apoptosis was induced, as was achieved previously, it was noted that women with lower
percentages of GC apoptosis had higher pregnancy rates. That observation led to an investigation of the expression of pro- and anti-apoptosis proteins in a model of induced apoptosis of GC. The patients in the present study were included in the previous report.

Immunoblot analysis demonstrated that differences also existed in the apoptosis proteins when comparing the two groups of women after separation on the basis of their percentages of apoptotic cells. PARP cleavage, which is an established marker of apoptosis (Oliver et al., 1998) and was (to the best of our knowledge) used here for the first time to investigate apoptosis of human GC, was markedly pronounced in group 2 treated GC—a finding that confirmed the DAPI results. However, for unknown reasons, the PARP 85-kDa band (which results from cleavage of the 116 kDa protein) appeared to be only slightly enhanced in groups 1 and 2.

Likewise, the expression of caspase 8 and 3 proforms was more strongly decreased for group 2 treated GC than group 1, thus indicating an enhanced activation of these enzymes in group 2 patients. These findings are not surprising because it has been well demonstrated that caspases 8 and 3 are directly associated with the Fas-mediated apoptosis pathway, as already reported in other cells (Nagata, 1997). Caspase 3 was previously detected in GC from women undergoing IVF (Izawa et al., 1998). However, in contrast to studies in rats where caspase 3 was present in GC atretic follicles but absent from healthy follicles (Boone and Tsang, 1998), the expression of this protein in human GC observed in the present study might suggest that caspase 3 is induced by gonadotrophins during ovarian stimulation, as outlined previously (Boone and Tsang, 1998).

With regard to the anti-apoptosis proteins, it is well established that Bcl-2 (Adams and Cory, 1998) and HSP70 (Samali and Cotter, 1996) can inhibit many apoptosis signals in very different cell types and in various organisms. Bcl-2 has previously been detected immunohistochemically in human ovaries (Lu et al., 1993; Rodger et al., 1995). It was found that this protein was weakly expressed in both control GC groups and decreased in both treated groups. Similarly, HSP70—which was clearly expressed in control group GC and particularly in group 2—was slightly decreased in treated groups 1 and 2. Therefore, it was concluded that Bcl-2 was probably not directly responsible for the lower percentages of apoptosis induced by IFN-γ and anti-Fas antibody in group 1.

In contrast, HSP70 may have played a positive role in the protection of GC in this experiment. Indeed, it has been reported that when HSP70 expression declined, cells became more sensitive to apoptosis (Robertson et al., 1999; Sreedhar et al., 1999).

With regard to the pro-apoptosis proteins, it was not possible to detect Bax by immunoblotting of cultured GC—a result which is in agreement with the reported absence of this protein in human GC from healthy follicles, as assessed by immunolocalization (Kugu et al., 1998). In contrast, Bak was well represented in both controls and, after treatment, its expression was slightly weaker in group 1 and more markedly diminished in group 2.

In the present study, IFN-γ was used to stimulate Fas expression, though it is well known that IFN-γ can induce apoptosis by itself (Stark et al., 1998) by activating the janus kinases (JAK), and the signal transducers and activators of transcription (STAT). This was the reason why, in the present study, Stat-1 was examined as it is particularly enhanced by IFN-γ. The present results showed that GC from both treated groups exhibited comparably enhanced Stat-1 expression, though group 2 control GC expressed this protein more strongly than group 1 control GC—a finding which might explain why Stat-1 enhancement was less marked in treated group 2 than in treated group 1. As reported previously, activation of the STAT pathway might cause apoptosis by overexpression of some caspases, particularly caspase 1 (Chin et al., 1997). However, this caspase was not investigated in the present study, and the difference observed between the two treated GC groups, according to the Stat-1 status in control samples, may reflect the synergistic expression of Stat-1 and Fas pathways. It is hypothesized that the stronger Stat-1 expression in group 2 control GC might facilitate apoptosis induction (Schindler, 1998).

The different expression of the anti- and pro-apoptosis proteins in cultured GC from women undergoing IVF might suggest a potential role for hormones in Fas-mediated apoptosis. Indeed, FSH is known to attenuate in-vitro apoptosis in cultured human GC (Matsubara et al., 2000; Sifer et al., 2001), whereas GnRH agonists, at different concentrations, increased the incidence of human GC apoptosis (Zhao et al., 2000; Sifer et al., 2001). However, those in-vitro results must be confirmed in vivo according to the women stimulation protocol. Finally, further studies are required to demonstrate a prognostic role for, and to understand the mechanism of, apoptosis of GC from women undergoing IVF—especially to explore the potential impact of hormonal regulation of interactions among the Fas-mediated pathway and cytokines.

Acknowledgements
The authors wish to thank Drs A.Neuraz, M.Naouri, S.Alvarez and A.Devaux for their help in data collection, Mrs P.Loiseau for scanning the blots and Mrs J.Jacobson for reviewing the English translation.

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919


Submitted on December 14, 2000; resubmitted on July 11, 2001; accepted on November 13, 2001