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Research

Involvement of theca cells and steroids in the regulation of granulosa cell apoptosis in rabbit preovulatory follicles

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Follicular atresia is characterized by a rapid loss of granulosa cells and, to a lesser extent, theca cells, via apoptosis. The aim of this study was to investigate the possible involvement of theca cell secretions in the regulation of apoptosis of rabbit granulosa cells. The annexin-V binding method based on externalization of phosphatidylserine to the outer layer of plasma membrane during apoptosis was used to detect apoptotic granulosa cells in flow cytometry. Regulation of apoptosis of granulosa cells was studied in three different culture systems: (i) isolated cultured granulosa cells, (ii) granulosa cells obtained from cultured preovulatory follicles and (iii) granulosa cells co-cultured with theca cells. The results of this study indicate that: (i) the rate of apoptosis of granulosa cells was significantly reduced when granulosa cells were co-cultured with theca cells or obtained from cultured preovulatory follicles in comparison with isolated cultured granulosa cells; (ii) FSH exerts its anti-apoptotic effect only on granulosa cells issued from cultured preovulatory follicles; (iii) ovarian steroids do not affect the percentage of isolated apoptotic granulosa cells; and (iv) the occurrence of an apoptotic process in rabbit theca cells could be upregulated *in vitro* by hCG and an analogue of the gonadotrophin second messenger cAMP. The results of this study indicate that in rabbits (i) steroids were ineffective *in vitro* in protecting isolated granulosa cells against apoptosis in comparison with observations *in vivo* in rats, and (ii) the presence of theca cells was efficient to reduce granulosa cell apoptosis but not sufficient to allow the anti-apoptotic effect of gonadotrophins observed in cultured follicles.

Introduction

During the course of ovarian development, only a small proportion of follicles ovulate; the majority are eliminated from the ovary by atresia (Hirshfield and Midgley, 1978). This process is essential and maintains a constant cell mass and homeostasis of the adult ovary. Degenerative changes in granulosa cells are the first morphologically recognizable sign of follicular atresia, and it is well known that apoptosis is the underlying event associated with the granulosa cell death (Hughes and Gorospe, 1991). Whether theca cells undergo apoptosis is controversial. It was previously understood that theca cells persisted in the atretic follicles and were incorporated into the interstitium; however, Hurwitz and Adashi (1992) demonstrated that theca cells are eliminated from the ovary in the same manner as granulosa cells. Indeed, studies *in vivo* show that apoptotic cell death occurs in theca interna of avian (Tilly *et al*., 1991), pig (Tilly *et al*., 1992a), rat (Palumbo and Yeh, 1994) and bovine atretic follicles (Isobe and Yoshimura, 2000; Yang and Rajamahendran, 2000). When apoptosis occurs in theca cells it always occurs in the later stages of follicular atresia and at a considerably lower rate than that of granulosa cells (Palumbo and Yeh, 1994; Logothetopoulos *et al*., 1995).

Whether a follicle ovulates or undergoes atresia is determined by the balance of signals that the theca and granulosa cells receive. Signals, including gonadotrophin hormones (LH, FSH), other paracrine signalling molecules, such as steroid hormones (androgens and oestrogens), and growth factors, regulate the synchronized patterns of granulosa cell proliferation and cell death (McGee *et al*., 1998). Apoptosis of granulosa cells is mainly regulated by gonadotrophins, as demonstrated *in vivo* in rats (Billig *et al*., 1994) or *in vitro* in intact follicle culture systems (Chun *et al*., 1994, 1996; Kaipia *et al*., 1996). When granulosa cells were isolated, FSH was ineffective at inducing apoptosis in rats (Aharoni *et al*., 1995), whereas it attenuated apoptosis in pigs (Guthrie *et al*., 1998) and cattle (Yang and Rajamahendran, 2000; Hu *et al*., 2001). In a comparative study, Maillet *et al*. (2002) found that the sensitivity of isolated rabbit granulosa cells to apoptosis was different from that of granulosa cells obtained from cultured intact follicles. This finding indicates that the regulation of apoptosis in rabbit granulosa cells may require paracrine interactions within the follicle.

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In the present study, a co-culture system was used to analyse the role of theca cells in the regulation of apoptosis of granulosa cells *in vitro*, and to determine the conditions that induce apoptosis in theca cells *in vitro*.

Materials and Methods

Reagents

Equine chorionic gonadotrophin (eCG) was purchased from Chrono-Gest (Intervet, Paris). Eagles' minimum essential medium (MEM), fetal calf serum (FCS), trypsine–EDTA and penicillin and streptomycin were purchased from Eurobio (Les Ulys). BSA, Hepes, EGTA, sucrose, collagenase (type II), hyaluronidase (type II), protease (type XIV), dibutyryl cAMP (dbcAMP) and specific antibodies for radioimmunoassay of oestradiol and progesterone were obtained from Sigma Chemical (St Quentin Fallavier). Purified ovine FSH (USDA-o-FSH-20, 4.453 iu mg^{-1}) was obtained through the National Hormone and Pituitary Program (NIDKK) and A. F. Parlow. hCG was obtained from Organon (Serifontaine). The narcotic Embutramide T61 was provided by Distrivet (Paris). The annexin V–fluorescein isothiocyanate (annexin V–FITC) kit was a Boerhinger–Ingelheim kit supplied by Coger (Paris).

Animals and treatments

HY female white rabbits, aged 10–12 weeks (Elevage Gastebled, Hottot Les Bagues), were housed individually for about 2 weeks on a 14 h light (06:00–20:00 h) : 10 h dark photoperiod with standard rabbit food and water available *ad libitum*. Animals were bred under standard conditions according to the instructions of Ministère de l'Agriculture et de la Pêche–Service Santé Animale (France).

The development of preovulatory follicles was induced in these rabbits by i.m. injection of 200 iu eCG on 2 consecutive days. The animals were killed by intracardiac injection of 2 ml of a narcotic (Embutramide T61) 4 days after the first injection of eCG.

The ovaries were excised and placed in MEM containing 20 mmol Hepes l^{-1}, 50 iu penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 0.1% BSA (MEM–0.1% BSA). The follicles were extirpated out (those containing clotted blood were discarded) and cleared of interstitial tissue under a stereomicroscope.

Collection of cells

Dispersed granulosa cells and theca cells were obtained as described by Féral *et al*. (1995). Briefly, follicles were cut in half in MEM–0.1% BSA containing 6.8 mmol EGTA l^{-1} and incubated for 10 min. Released granulosa cells and follicles were centrifuged together for 10 min at 300 ***g*** and resuspended in MEM–0.1% BSA containing 0.5 mmol sucrose l^{-1} and 1.8 mmol EGTA l^{-1} for 5 min before centrifugation again at 300 ***g*** for 10 min. Granulosa cells were gently scraped from the theca with a microspatula under a dissecting microscope. For co-culture experiments, follicular walls were cut into four and washed three times in MEM–0.1% BSA before being placed in a cell culture insert. For culture of isolated theca cells, follicular walls were minced with iris scissors and incubated in MEM–0.1% BSA containing 0.1% (w/v) collagenase and 0.1% (w/v) hyaluronidase for 10 min at 37°C. After the addition of 5 ml MEM–0.1% BSA, pieces of tissue were centrifuged at 300 ***g*** for 30 s. The supernatant consisting of the remaining granulosa cells was discarded. Subsequently, pieces of theca were dissociated in MEM–0.1% BSA containing 0.2% (w/v) hyaluronidase and 0.1% (w/v) protease for 10 min at 37°C. Histological and biochemical analyses indicated that the level of contamination of theca cells with granulosa cells was $< 1\%$ (Féral *et al*., 1990). The dispersed granulosa and theca cells were pelleted separately by centrifugation at 300 ***g*** for 10 min.

Culture of granulosa and theca cells

After two washes in MEM–0.1% BSA, granulosa or theca cells were plated in serum-free medium with or without gonadotrophin or steroids at the density of 3×10^5 cells per well in 1 ml in Falcon 24-well tissue culture plates for 72 h. Steroids were dissolved in ethanol. The final concentration of ethanol had no effect on the percentage of granulosa cell apoptosis. After culture, floating and attached cells were collected. Adherent cells were washed twice with warm PBS without Ca^{2+} or Mg^{2+} and treated for 5 min at 37°C with PBS containing 0.5 g trypsin l^{-1} and 0.2 g EDTA l^{-1}. When adherent cells became round as a result of trypsin action, they were gently agitated until all cells were detached from the plate. Trypsine activity was stopped by the addition of medium supplemented with 5% FCS, and cells were treated for apoptosis analysis.

Culture of follicles

Preovulatory follicles were dissected from ovaries as described above. Six follicles per treatment group were placed in Falcon 24-well tissue culture plates in serum-free conditions with or without gonadotrophin treatment and incubated for 72 h. After culture, the follicles of each treatment group were washed in MEM–0.1% BSA and dispersed granulosa and theca cells were obtained as described above. Dispersed granulosa and theca cells were washed in cold PBS before they were assessed for apoptosis.

Co-culture

A co-culture system was used to determine the effects of theca cells on apoptosis of granulosa cells. Briefly,

dispersed granulosa cells obtained as described above were plated at the density of 3×10^5 cells per well in a Falcon 24-well tissue culture plate. A cell culture insert (0.1 μm pore size for 24-well format, Falcon) containing pieces of theca was placed in each well above plated granulosa cells without any direct contact. Theca pieces placed in the culture insert were equivalent to eight preovulatory follicles.

Co-culture was conducted in serum-free conditions with or without gonadotrophins for 72 h. Control wells containing granulosa cells only and wells containing granulosa cells treated with 10^{-7} mol androstenedione l^{-1} were used for each experiment. At the end of culture, granulosa cells were collected by trypsinization as described above.

Detection of apoptosis

On the basis of externalization of phosphatidylserine to the outer layer of plasma membrane during apoptosis, apoptotic cells were quantified according to the manufacturer's instructions for annexin V–FITC kit as described by Maillet *et al*. (2002). After two washes with binding buffer, annexin V–FITC (3 μg ml^{-1}) and propidium iodide (PI) (400 ng ml^{-1}) were added to the cell suspension (3×10^5 cells per 500 μl) and incubated for 10 min in the dark. Granulosa and theca cells were analysed during the hour after incubation in a fluorescence-activated cell sorter (FACScalibur, Becton-Dickinson, Sunnyvale, CA) using a 15 mW argon laser emitting light at 488nm. The following settings were used: 475 V on photomultiplier tubes both for fluorochrome one (FL1) (FITC) and fluorochrome two (FL2) (PI). Because fluoresence intensity of FL1 could be detected on FL2 photomultiplier tubes and vice-versa, a compensation setting (FL1–%FL2) was used. Compensation settings FL1–%FL2 and FL2–%FL1 were 1.0 and 32.5%, respectively. Data analysis was performed with the Cell Quest software (Becton-Dickinson). Ten thousand cells were analysed in each treatment group.

Production of progesterone and oestradiol

Concentrations of oestradiol and progesterone in culture media of granulosa cells were quantified by radioimmunoassay using specific antibodies as described by Benhaïm *et al*. (1987). Oestradiol was measured after extraction with five volumes of ether, whereas progesterone was measured directly in the culture media. The sensitivity of the assay for oestradiol was 3 pg per well and the intra- and interassay coefficients of variation were < 4 and 10%, respectively. The sensitivity of the assay for progesterone was 5 pg per well and the intra- and interassay coefficients of variation were 5 and 9%, respectively. The results are expressed as steroids secreted by 10^4 cells during 72 h of culture.

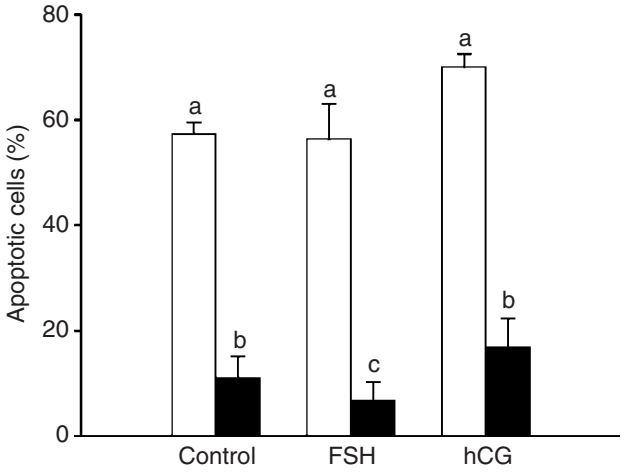

Fig. 1. Effects of FSH (5 ng ml^{-1}) and hCG (10 miu ml^{-1}) on apoptosis in (□) isolated rabbit granulosa cells and (■) granulosa cells scraped from rabbit preovulatory follicles both cultured for 72 h in serum-free conditions. Values are mean ± SEM of three experiments performed in triplicate for isolated cells or a single well for cultured follicles. Values with different superscripts are significantly different ($P < 0.05$).

Statistical analysis

Data regarding the production of steroids and the percentage of apoptotic cells were expressed as the means ± SEM of three experiments. In each experiment, granulosa cells were pooled from the ovaries of two rabbits, and each treatment was performed in triplicate for isolated cells or in a single well for cultured follicles. Data regarding the percentage of apoptotic cells obtained after annexin V binding analysis needed an arcsin transformation to obtain a normal distribution. Differences between treatment groups (raw data for the production of steroids or transformed data for the percentage of apoptotic cells) were assessed by a two-way ANOVA followed by Fisher's test and considered to be significant at $P < 0.05$.

Results

Involvement of theca in apoptosis of granulosa cells

When cultured in isolation in serum-free medium, a large number of granulosa cells were apoptotic (57 ± 2%). Addition of FSH and hCG had no significant effect on the percentage of apoptotic granulosa cells (Fig. 1). Granulosa cells were viable after this 72 h culture period because cell mortality determined by PI uptake was very low in the control cells (2.67 ± 0.57% dead cells) and did not change regardless of the gonadotrophin treatment (2.25 ± 0.59% dead cells with 5 ng FSH ml^{-1}, 2.97 ± 0.45% dead cells with 10 miu hCG ml^{-1}). When granulosa cells were isolated from follicles cultured in serum-free medium, the analysis

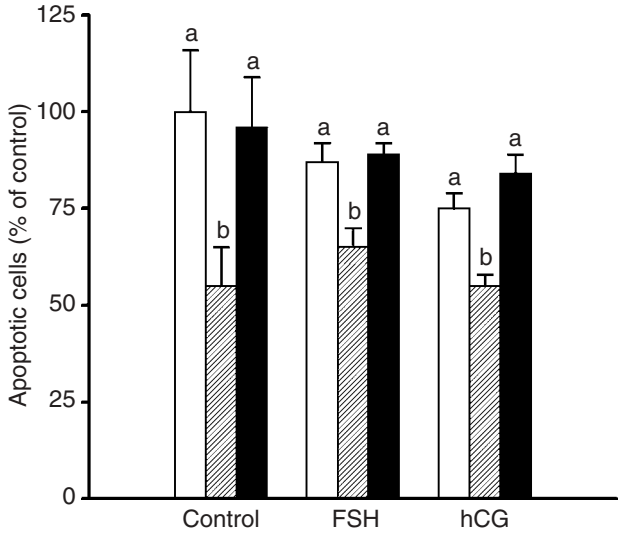

Fig. 2. Effects of FSH (5 ng ml^{-1}) and hCG (10 miu ml^{-1}) on apoptosis in isolated rabbit granulosa cells (□) cultured for 72 h in serum-free conditions in the presence of (▒) theca cells or (■) androstenedione (10^{-7} mol l^{-1}). Values are the mean ± SEM of three experiments performed in triplicate. Control = 37 ± 6% apoptotic cells. Values with different superscripts are significantly different ($P < 0.05$).

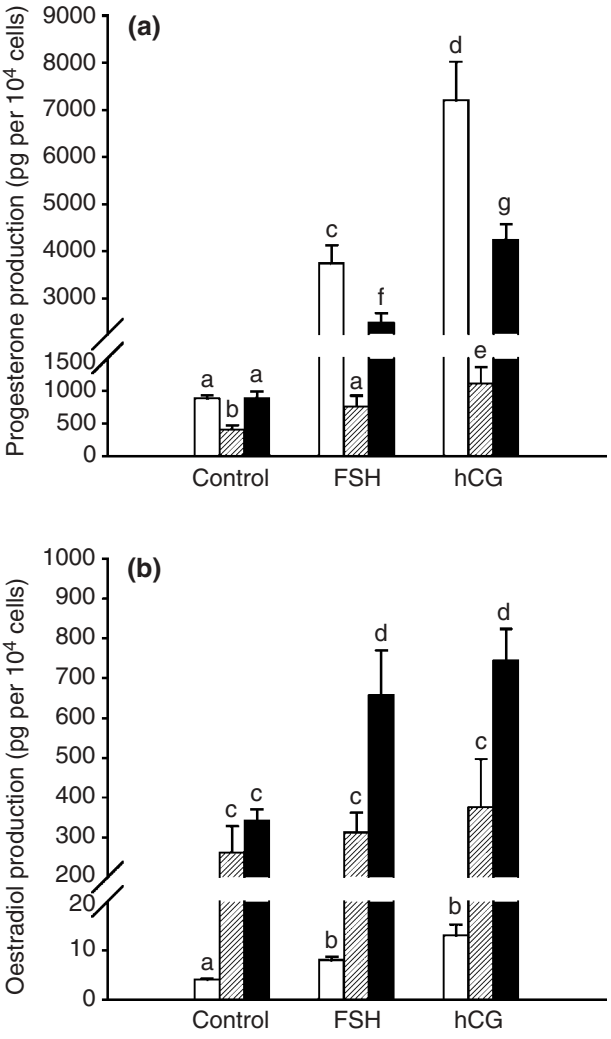

Fig. 3. Effects of FSH (5 ng ml^{-1}) and hCG (10 miu ml^{-1}) on (a) progesterone and (b) oestradiol production by isolated rabbit granulosa cells (□) cultured for 72 h in serum-free conditions in the presence of (▒) theca cells or (■) androstenedione (10^{-7} mol l^{-1}). Values are the mean ± SEM of three experiments performed in triplicate. Values with different superscripts are significantly different ($P < 0.05$).

of phosphatidylserine exposure showed 11 ± 4% of apoptotic granulosa cells and the addition of FSH in culture medium resulted in a significant decrease in the percentage of apoptotic granulosa cells. Addition of hCG did not affect the number of apoptotic cells (Fig. 1).

This first experiment illustrates the role of follicular integrity on apoptosis of granulosa cells. As oestradiol is the main steroid present in preovulatory follicles, its effect on apoptosis of cultured granulosa cells was examined. It was noted that increasing doses (10^{-12} to 10^{-7} mol l^{-1}) of oestradiol did not affect the percentage of apoptotic granulosa cells (data not shown). The effect of increasing doses (10^{-10} to 10^{-7} mol l^{-1}) of progesterone on apoptosis of granulosa cells was also examined, and this steroid did not affect apoptosis of rabbit granulosa cells (data not shown).

A co-culture system that allowed the modulation of granulosa cell apoptosis to be studied in the presence or absence of theca cells was used to investigate the involvement of theca cells in regulation of granulosa cell apoptosis. In the presence of theca, granulosa cell death was significantly decreased (1.6-fold versus control) (Fig. 2). When gonadotrophins FSH or hCG were added to the co-culture, the percentage of apoptotic granulosa cells did not change (Fig. 2).

Wells containing granulosa cells only were treated with 10^{-7} mol androstenedione l^{-1} to determine whether the effect of theca cells on granulosa cell death occurred via androgens produced by theca cells. It was noted that this dose of androstenedione did not affect granulosa cell apoptosis either alone or when granulosa cells were treated with gonadotrophins (Fig. 2). The addition of 10^{-8} and 10^{-6} mol androstenedione l^{-1} confirm that this steroid did not affect the percentage of apoptotic granulosa cells *in vitro* (data not shown).

Progesterone and oestradiol production by granulosa cells co-cultured with theca cells

In parallel to the study of apoptosis, the production of progesterone and oestradiol by granulosa cells during 72 h of culture was measured under different conditions (Fig. 3).

Granulosa cells produced 900 pg progesterone per 10^4 cells in serum-free medium (Fig. 3a). Co-culture with theca inhibited progesterone production, whereas the addition of androstenedione did not significantly affect progesterone accumulation. When granulosa cells were treated with 5 ng FSH ml^{-1} or 10 miu hCG ml^{-1}, progesterone production was increased 4.3- and eight-fold, respectively, compared with control cells. Co-culture of granulosa cells with theca in the presence of FSH significantly reduced the FSH-induced progesterone production, as did hCG. Treatment of granulosa cells with both androstenedione and FSH or hCG induced a 1.5- or 1.7-fold decrease in FSH-induced and hCG-induced progesterone production, respectively (Fig. 3a).

Granulosa cells produced very little oestradiol (4 pg per 10^4 cells) in serum-free medium (Fig. 3b). This production was positively regulated by FSH and hCG (2- and 3.3-fold increase, respectively). Co-culture of granulosa cells with theca induced a 65-fold increase in oestradiol accumulation. Theca-induced oestradiol production was mimicked by treatment of granulosa cells with 10^{-7} mol androstenedione l^{-1} (Fig. 3b). The addition of gonadotrophins to the co-culture did not affect theca-induced oestradiol production, but did induce a twofold increase in androstenedione-stimulated oestradiol production by isolated granulosa cells (Fig. 3b).

Regulation of apoptosis of theca cells

When preovulatory follicles were cultured in serum-free medium for 72 h, the percentage of apoptotic theca cells did not change significantly in the presence or absence of 5 ng FSH ml^{-1} or 10 miu hCG ml^{-1} (data not shown).

Isolated theca cells were cultured for 72 h in medium with increasing doses (0–50 miu ml^{-1}) of hCG to determine whether apoptosis of theca cells could be induced *in vitro* (Fig. 4). In the absence of serum, treatment with hCG induced a weak, but significant, increase in the percentage of apoptotic theca cells. The addition of serum in culture medium induced a twofold decrease in apoptotic theca cells and addition of hCG resulted in a slight increase in this rate (Fig. 4).

The effect of dbcAMP, an analogue of gonadotrophin second messenger cAMP on apoptosis of theca cells was determined (Fig. 5). A low (0.1 mmol l^{-1}) as well as a high dose of dbcAMP (5 mmol l^{-1}) induced an increase in the rate of apoptosis without change in the percentage of dead cells (PI + cells) (data not shown) excluding a possible cytotoxic effect of dbcAMP on theca cells. A low dose of dbcAMP induced a twofold increase in progesterone production by theca cells ($P < 0.05$ compared with control), whereas treatment with 5 mmol dbcAMP l^{-1} resulted in a 20-fold stimulation of progesterone secretion ($P < 0.001$ compared with control) (data not shown).

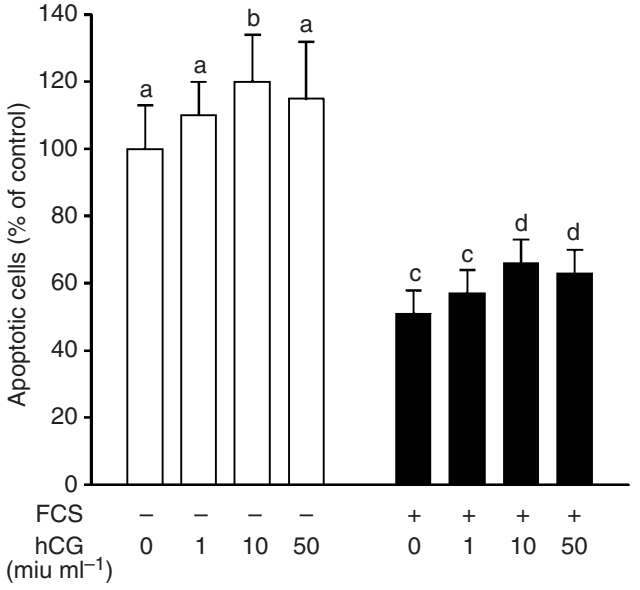

Fig. 4. Effect of increasing doses of hCG on apoptosis in isolated rabbit theca cells cultured for 72 h in the (□) absence or (■) presence of fetal calf serum (FCS). Values are the mean ± SEM of three experiments performed in triplicate. Values with different superscripts are significantly different ($P < 0.05$).

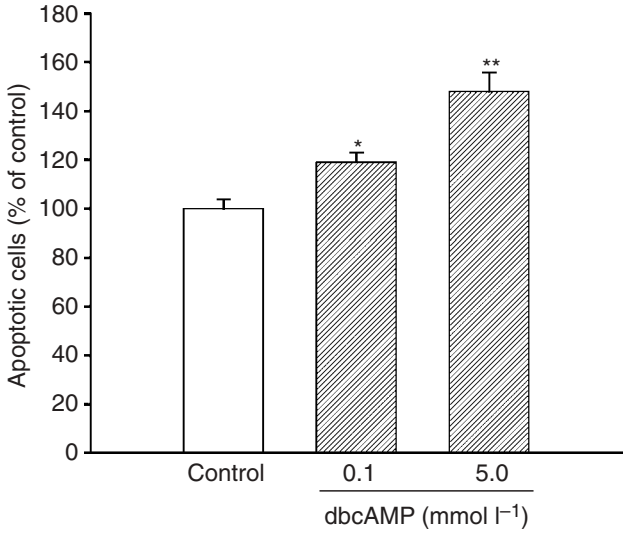

Fig. 5. Effect of dbcAMP on apoptosis in isolated rabbit theca cells cultured for 72 h in serum-containing medium. Values are the mean ± SEM of three experiments performed in triplicate. Control = 25 ± 3% apoptotic cells. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the control value.

Discussion

In the present study, the use of different culture models provided an opportunity to investigate the involvement of theca cells in granulosa cell apoptosis in the rabbit ovary and demonstrated that apoptosis could be induced and regulated in theca cells *in vitro*.

Maillet *et al*. (2002) reported that the regulation of apoptosis in granulosa cells appears to require paracrine interactions within the follicle. The present study used a co-culture system to show that theca cells could reduce the rate of granulosa cell death. This anti-apoptotic effect of theca cells was not sufficient to mimic the protective effect exerted in the follicle in so far as the rate of apoptosis of granulosa cells obtained from cultured follicle was lower than that of granulosa cells co-cultured with theca. The disruption of granulosa cell-to-cell contacts in cultured isolated cells could provide an explanation for these findings. Indeed, Peluso (1997) showed that single granulosa cells are more likely to undergo apoptosis *in vitro* when compared with clumps of granulosa cells.

In the present study, treatment of granulosa cells with androstenedione or oestradiol had no effect on apoptosis induced by serum deprivation regardless of the dose. Therefore, the observed protective effect of theca on apoptosis of granulosa cells could not be due to a direct or indirect effect of the ovarian steroids. *In vivo*, an apoptotic effect of androgens has been reported, whereas oestradiol prevented ovarian apoptotic DNA degradation in hypophysectomized rats (Billig *et al*., 1993). *In vitro*, Murdoch (1998) demonstrated an inhibition of oxidative stress-induced apoptosis by oestradiol in pig ovarian tissues. Thus, the results of the present study *in vitro* indicate that, oestradiol and androstenedione have an indirect effect on apoptosis of granulosa cells *in vivo*.

Progesterone is an important ovarian steroid which is required as a survival factor by many types of cell, in particular human granulosa cells (Makrigiannakis *et al*., 2000). In the present study, the percentage of apoptotic rabbit granulosa cells did not change with increasing doses of progesterone. This finding could be explained by the fact that expression of progesterone receptors in rabbit granulosa cells is weak before the LH surge (Iwai *et al*., 1991). In contrast, the anti-apoptotic effect of progesterone on human granulosa cells was exerted on cells from patients undergoing IVF and thus was obtained after LH surge.

However, co-culture of rabbit granulosa cells with theca or androstenedione inhibited progesterone production. This result is in agreement with inhibition of progesterone production by androgen observed in human and pig cultured granulosa cells (Batta *et al*., 1980; Lischinsky *et al*., 1983). Androgens inhibit progesterone synthesis via the inhibition of conversion of pregnenolone to progesterone (Evans *et al*., 1984).

Several factors produced by theca other than steroids could be involved in the protective effect of theca cells on granulosa cell death. For example, keratinocyte growth factor produced by theca cells (Parrott *et al*., 1994) suppresses apoptosis in cultured rat preantral follicles (McGee *et al*., 1999). Transforming growth factor alpha (TGFα) and epidermal growth factor, which are produced by theca–interstitial layer follicles (Kudlow *et al*., 1987; Skinner *et al*., 1987), were shown to inhibit the spontaneous onset of apoptotic DNA cleavage of granulosa cells obtained from rat preovulatory follicles (Tilly *et al*., 1992b).

The present study demonstrated that the presence of theca was not sufficient to allow the anti-apoptotic effect of FSH, indicating that anti-apoptotic paracrine factors are produced in the follicle but not by theca cells. Growth differentiation factor 9 (GDF-9) is a protein of the TGFβ/activin superfamily produced by oocytes in several species (Bodensteiner *et al*., 1999). GDF-9 stimulates follicle growth, but its potential role in the regulation of granulosa cell death remains to be elucidated (Erickson and Shimasaki, 2001). However, the action of FSH may require the presence of basal lamina in cultured intact follicles. Indeed, laminin, a protein which constitutes about 35% of total basement membrane proteins, was shown to inhibit apoptosis of rat and sheep granulosa cells (Aharoni *et al*., 1996; Huet *et al*., 2001).

The present study showed that apoptosis could be induced *in vitro* in isolated rabbit theca cells that had been cultured in serum-free medium. Foghi *et al*. (1997) demonstrated that apoptosis of isolated theca cells *in vitro* could be induced in rat preantral follicles, only in combined treatment with TGFα and TGFβ and not under serum-free conditions. These discrepancies could be due to different follicular stage studied, in addition to the different culture conditions.

The anti-apoptotic effect of gonadotrophins was shown *in vitro* in whole rat preovulatory follicles (Chun *et al*., 1994; Eisenhauer *et al*., 1995), but studies on isolated theca cells have not been performed. The present study failed to demonstrate any effect of hCG on apoptosis of theca cells in intact cultured preovulatory follicles, whereas in isolated rabbit theca cells, 10 miu hCG ml^{-1} resulted in a slight increase in apoptosis. This paradoxical effect is in agreement with the study of Aharoni *et al*. (1995) in which an apoptotic effect of hCG on preovulatory rat granulosa cells cultured in serum-free conditions was reported.

However, in the present study, dbcAMP stimulated apoptosis in isolated theca cells of rabbit preovulatory follicles *in vitro* without modifying theca cell viability, excluding a cytotoxic effect of dbcAMP. This analogue of cAMP was shown to induce apoptosis particularly in rat granulosa cells from preovulatory follicles (Aharoni *et al*., 1995) and in human granulosa cells (Makrigiannakis *et al*., 1999). A similar effect of dbcAMP was also observed in our laboratory in cultured granulosa cells obtained from rabbit preovulatory follicles (Maillet *et al*., 2002). It was shown that cAMP-induced apoptosis in rat granulosa cells involved the activation of tumour suppressor gene p53 (Keren-Tal *et al*., 1995).

Treatment of theca cells with dbcAMP caused a simultaneous increase in apoptosis and in progesterone production as observed in rabbit granulosa cells by Maillet *et al*. (2002), indicating the steroidogenic and

apoptotic processes are independent of one another, as suggested by Amsterdam *et al*. (1998).

In summary, the present study has shown that apoptosis could be induced and regulated *in vitro* in isolated rabbit theca cells. The use of a co-culture system made it possible to demonstrate that theca cells were effective in reducing rabbit granulosa cell apoptosis, and this protective effect did not appear to act via secretion of ovarian steroids. Other paracrine factors may be required for an additional inhibition of programmed cell death in the preovulatory follicles and their identification remains to be elucidated.

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