Reproductive BioMedicine Online (2011) **23**, 440–448

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ARTICLE

FSH modulatory effect on human granulosa cells: a gene–protein candidate for gonadotrophin surge-attenuating factor

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Abstract Previous evidence indicates a homology of gonadotrophin surge-attenuating factor (GnSAF) to the carboxyl terminal of human serum albumin (HSA) and the ability of human granulosa cells to produce mRNA transcripts corresponding to this fragment, but the underlying mechanism is still unknown. This study investigated the role of FSH *in vitro* in the expression of the carboxyl terminal of HSA by human luteinized granulosa cells. Cells were cultured on poly-L-lysine-coated microscope slides in the absence or presence of 10 ng/ml FSH, followed by in-situ hybridization and immunocytochemistry. In the presence of FSH, mRNA transcripts corresponding to the carboxyl terminal of the HSA gene and corresponding protein could be detected in comparable intensity to that seen by hepatic HepG2 cells (positive control). Significantly lower expression was detected in granulosa cells cultured without FSH addition ($P < 0.01$), but no expression was detected in HeLa cells. These results demonstrate for the first time that FSH stimulates the expression of the carboxyl terminal fragment of the HSA gene and corresponding protein in human luteinized granulosa cells. Therefore, the carboxyl terminal of HSA has a functional role in the ovary and this further supports the notion that this HSA fragment is a GnSAF-bioactive entity.

KEYWORDS: FSH, GnSAF, human granulosa cells, human serum albumin

Introduction

Gonadotrophin surge-attenuating factor (GnSAF) is a non-steroidal ovarian substance that, in women undergoing ovulation induction, reduces the LH responsiveness to GnRH and hence attenuates the preovulatory LH surge without affecting basal gonadotrophin secretion (Messinis and Templeton, 1989, 1990). A line of evidence dating back to

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2011.06.005

the late 1970s supports the existence of such a factor in humans as well as in other species (Ferraretti et al., 1983; Geiger et al., 1980; Koppenaal et al., 1992; Messinis et al., 1985, 1986; Messinis and Templeton, 1989, 1991; Schenken and Hodgen, 1983; Sopelak and Hodgen, 1984). It has been suggested that FSH is the main hormone that stimulates the production of GnSAF (Fowler and Price, 1997; Messinis et al., 1991, 1993, 1994a,b). From a physiological point of view, it has been postulated that GnSAF, by antagonizing the stimulating effect of oestradiol on the pituitary, controls the amplitude of the LH surge and the coordination of the LH signal with follicular development and steroidogenesis in women and other mammals (Fowler et al., 2001, 2003; Messinis and Templeton, 1991; Messinis et al., 1985, 1998).

Five attempts have been made to date to purify and characterize bioactive GnSAF molecule(s), using as sources Sertoli cell-conditioned medium (Tio et al., 1994), porcine follicular fluid (Danforth and Cheng, 1995), human follicular fluid from women undergoing ovulation induction (Mroueh et al., 1996; Pappa et al., 1999) and human granulosa-luteal cell-conditioned medium (Fowler et al., 2002). The proposed sequences refer to protein molecules of varying sizes from 12.5 to 69 kDa. One of these studies found an amino acid sequence of purified GnSAF from human follicular fluid that showed homology to the carboxyl terminal of human serum albumin (HSA) (Pappa et al., 1999). Following that study, the recombinant polypeptide 490—585 of HSA, referring to the carboxyl terminal of the protein, displayed GnSAF bioactivity *in vitro* (Tavoulari et al., 2004). GnSAF seems to be produced by the granulosa cells (Fowler et al., 2002) and recent findings support the suggestion that GnSAF is the carboxyl terminal of HSA as human luteinized granulosa cells possess the ability to express mRNA of HSA (Karligiotou et al., 2006). The mechanism of this expression, however, is still not known. Because FSH is considered the main stimulus of GnSAF production, the present study was undertaken to investigate further the ability of human granulosa cells to express the carboxyl terminal of the HSA gene *in vitro* with particular reference to the possible role of this gonadotrophin.

Materials and methods

Primary cells

The luteinized granulosa cells used in the procedures described below, were pooled from follicular fluid aspirated from 130 infertile women, 20—40 years old, undergoing ovulation induction for IVF. The infertility was caused by various aetiologies (42 cases of tubal factor, 12 cases of endometriosis, 33 cases of male factor, 38 cases of unexplained infertility and five cases of combined male and tubal factor infertility). The patients gave written informed consent and the local ethics committee approved the project.

The cells were isolated from the follicular fluid with consecutive washes with Dulbecco's phosphate-buffered saline (PBS; Biochrom, UK), by initially separating the follicular fluid in 6 ml aliquots in the collection tubes. Then, they were continuously washed with 20 mmol/l PBS and left to settle for 2—3 min at room temperature in sterile conditions, in order for the blood cells to be removed. Care was taken to meticulously remove as much blood as possible, which is the only cell contaminant in follicular fluid. Subsequently, the supernatant follicular fluid was aspirated and discarded and the granulosa cells were pooled and used either for RNA and protein extraction or culture (Hillier et al., 1991).

Cell cultures

The granulosa cells obtained from the follicular fluid of 50 out of the 130 women, as described earlier, were cultured on poly-L-lysine-coated microscope slides in Roswell Park Memorial Institute (RPMI) medium without phenol red (Sigma, UK), supplemented with 2 mmol/l L-glutamine (Gibco/BRL, UK), 10% (v/v) fetal bovine serum (Gibco/BRL, UK) and 100 IU/ml penicillin/streptomycin (Gibco/BRL, UK) at 37°C in an atmosphere of 5% CO_2 in air with extra humidity for 72 h. The granulosa cells were cultured in the absence or the presence of 10 ng/ml FSH. Cell cultures with major blood cell presence were rejected (Hillier et al., 1991). Each culture reflected a single follicular fluid sample pooled from one woman. HepG2 cells, a well-characterized human hepatic cell line, were used as a positive control for HSA expression. The cells were cultured on poly-L-lysine-coated microscope slides in Dulbecco's modified Eagle's medium (Sigma, UK) medium with the same supplements and incubation conditions. Finally, the established human cell line HeLa, was used as negative control. This cell line was cultured on poly-L-lysine-coated microscope slides in RPMI medium without phenol red with the same supplements and incubation conditions.

Extraction of RNA and cDNA synthesis

All types of RNA were extracted from luteinized granulosa cells, peripheral blood leukocytes, HepG2 and HeLa cells. Cytoplasmic and nuclear RNA were extracted by the vanadyl ribonucleoside complex method (Davis et al., 1986). Total RNA was extracted using the quanidinium isothiocyanate method (RNAzol; Sigma, UK), subjected to DNase I (Promega, UK) in order to avoid DNA contamination and stored at −70°C until use (Chomczynski and Sacchi 1987).

In-vitro reverse transcription of 1 μg of RNA to cDNA was performed using Moloney murine leukaemia virus reverse transcriptase (Gibco—BRL, UK) and random hexamers as primers. As a control for the presence of amplifiable RNA, 5 μl of the reverse-transcription cDNA product was amplified by polymerase chain reaction (PCR), using primers specific for the β-actin gene, as previously described (Karligiotou et al., 2006). Both in the reverse-transcription reaction and the ensuing amplification reactions, recommended measures to prevent cross-contamination of samples were followed (Kwok and Higuchi, 1989). In addition for each experiment, a control with no template was used to check for the presence of any contamination.

PCR amplification of HSA cDNA sequences

Ten microliters of the reverse-transcription reaction mixture was amplified by PCR using primers specific for exons

12 and 13 of the HSA gene as previously described (Karligiotou et al., 2006). In each experiment, cDNA from human leukocytes and HeLa cells were used as negative controls, while HepG2 cells were used as a positive control. The leukocytes were considered the only possible cell contaminants in the human follicular fluid.

Analysis and purification of PCR products

Determination of the specific PCR products was achieved by direct sequencing employing the dideoxy-chain termination method (Sanger et al., 1977) using the modified version of T7 DNA polymerase (Sequenase 2.0; US Biochemicals, Cleveland, Ohio, USA). The PCR product of interest was excised and purified by the QIAquick Gel extraction kit (Qiagen, USA) according to the manufacturer's instructions.

Standard labelling of PCR products and in-situ hybridization

In-situ hybridization (ISH) was performed using the Dig High Prime DNA Labeling and Detection Starter kit (Roche, Germany), following the manufacturer's instructions with certain modifications. The labelling reactions of the purified PCR products were performed according to the standard labelling reaction for DNA in solution. Briefly, 1 μg PCR product was labelled with digoxigenin (DIG) using the DIG High Prime DNA labelling kit (DIG Application Manual for non-radioactive ISH, Chapter 4: standard labelling reaction for DNA in solution). Cells were prepared, fixed and permeabilized on poly-L-lysine-coated microscope slides. The slides then were fixed in 4% formaldehyde, 5% acetic acid and 0.9% NaCl, dehydrated through a graded series of alcohol, dried and used immediately for hybridization. Post-fixed with 1% formaldehyde and treated at 37°C with 0.1% (w/v) pepsin in 0.1 mol/l HCl for 10 min to increase permeability to macromolecules. The cells were cultured for 3 days and then hybridization was performed in 60% deionized formamide, 30 mmol/l sodium citrate, 5% dextran sulphate, 10 mmol/l EDTA, 25 mmol/l NaH_2PO_4 (pH 7.4) and 250 ng/μl sheared salmon sperm DNA.

The DIG-labelled probe DNA was denatured at 80°C shortly before use and added to the hybridization solution at a concentration of 5 ng/μl. Ten microliters of the hybridization mixture (hybridization solution/denatured probe) was added to the fixed, permeabilized cells and covered with an 18 × 18 mm coverslip. The hybridization was performed overnight at 37°C. After hybridization, the slides were washed at room temperature in a solution of 60% formamide, 300 mmol/l NaCl and 30 mmol/l sodium citrate by shaking to remove the coverslips. Ten different sets of simultaneous cultures were performed. Each set comprised of one culture of granulosa cells in the presence of FSH and one in the absence of FSH, a culture of HepG2 and one culture of HeLa cells.

Immunofluorescent detection

The detection procedure used the HNPP Fluorescent Detection Set (Roche, Germany), following the manufacturer's instructions. In detail, immunological detection was performed by adding 100 μl of alkaline phosphatase at a dilution of 1:500 (v/v) and using a coverslip. The slides were incubated in a moist chamber for 1 h at 37°C, followed by consecutive washes and incubation of the slides in the detection buffer for 10 min. Then 100 μl of HNPP/Fast Red mixture were added to the slides which were incubated at room temperature for 30 min and then washed with ddH_2O for 10 min. Counterstaining and mounting were performed in the dark room where the slides were incubated for 5 min at room temperature with (4,6-diamidino-2-phenylindole) (DAPI) diluted in PBS at a concentration of 5 mg/ml. The slides were washed with water and air dried in the dark. Then, 20 μl anti-fading solution were added to the slides which were covered with coverslips and sealed. Detection was performed with a fluorescent microscope (Axionplan; Zeiss, USA) at a wavelength of 540–590 nm with the use of the TRIC filter (encompassing the rhodamine filter) and the DAPI filter sets. The image analysis was performed with the *In situ* Imaging System computer program (MetaSystems, Altlussheim, Germany).

Western blot analysis

Western blot analysis was performed as described by Sambrook et al. (1989). In short, granulosa cells were pooled from the follicular fluid of the remaining 80 women who underwent ovulation induction for IVF and they were cultured in the presence or absence of FSH for 72 h, as described by Hillier et al. (1991). Cell cultures with major blood cell presence were rejected, as previously described, and protein extracts with low yield (<40 μg) were excluded. Therefore, only 40 samples were used for the blotting experiments. After protein extraction, equal amounts of protein (40 μg) were separated by 8–13% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The blots were blocked and exposed to custom-made rabbit anti-carboxyl-terminal HSA (rabbit cat. no. 45111; Biosynthesis Incorporation, USA) polyclonal primary antibody overnight at 4°C at a concentration of 10,000 μg/ml, followed by 1 h incubation at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase (Abcam, UK). The primary antibody was ordered and was custom made to have specificity for the carboxyl terminal of HSA, according to the protein sequence from amino acid residues 490–585 (GenBank M12523; Minghetti et al., 1986). Ten separate blots were performed, each employing four different protein samples. Each protein sample referred to granulosa cells recovered from a single follicular fluid sample. Detection was carried out using the electrochemiluminescence reaction (Pharmacia, UK). To avoid contamination with tissue proteins, human liver tissue as a positive control was excluded and only HepG2 cells were used.

Combined DNA ISH and immunocytochemistry for the simultaneous detection of DNA and protein

Immunocytochemistry (ICC) and ISH were performed on cells that were prepared, fixed and permeabilized on poly-L-lysine-coated microscope slides as described earlier using the DIG High Prime DNA Labeling and Detection Starter

kit with some modifications. The aim of the experiment was to simultaneously detect on the same cells the mRNA using the DNA probe as well as the protein corresponding to the carboxyl terminal of HSA. The slides were fixed in cold methanol (−20°C) for 5 s, then in cold acetone (4°C) three times for 5 s and then air dried. Ten different sets of simultaneous cultures were performed. Each set comprised of one culture of granulosa cells in the presence of FSH and one in the absence of FSH, a culture of HepG2 and one culture of HeLa cells.

Detection of antigen by immunocytochemistry

The slides were incubated for 10 min at room temperature with PBS/Tween/NGS (PBS containing 0.05% Tween 20 and 2−5% normal goat serum), followed by 45 min at room temperature with rabbit anti-carboxyl-terminal HSA polyclonal primary antibody in PBS/Tween/NGS at a 1:1000 dilution. Subsequently, the slides were washed twice with PBS containing 0.05% Tween 20 for 5 min. Then they were incubated for 45 min at room temperature with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC; Abcam). Finally, the slides were washed twice with PBS containing 0.05% Tween 20 for 5 min and once with PBS for 5 min.

Cell processing for in-situ hybridization

The slides were washed for 2 min at 37°C with 10 mmol/l HCl. The samples were then digested with pepsin and washed twice at 37°C with 10 mmol/l HCl. Post-fixation of the samples was performed for 20 min at 4°C with PBS containing 1% paraformaldehyde. The slides were then washed for 5 min with PBS and twice for 5 min with 2× saline sodium citrate. The DNA probe hybridization and detection steps were followed as described earlier. The detection was performed with a fluorescent microscope at a wavelength of 540−590 nm with the use of the TRIC filter (encompassing the rhodamine filter) and the FITC and DAPI filter sets. The image analysis was performed with the *In situ* Imaging System.

Statistical analysis

Labeling intensity and cellular staining was independently evaluated by two observers. Intensity and distribution of ISH and ICC staining was used to classify samples as positive or negative for expression of gene and protein fragments.

Hybridization signals were evaluated on five different culture slides for each different cell type/culture condition. On each slide, 100 intact, well-preserved and non-overlapping cells were evaluated by at least two investigators. Statistical comparisons were performed using Statistical Package for Social Sciences version 19.0 (SPSS Inc., Chicago, IL, USA). All tests were two-tailed and differences in mean areas were considered significant for a *P*-value of less than 0.01 by the paired Student t-test. The results are shown as mean ± standard deviation.

Results

ISH was performed using a non-radioactive DIG-labelled PCR product as probe, corresponding to the 12.5-kDa 95-peptide carboxyl terminal of HSA (490−585 amino acids). The PCR product represented the gene area encoding for the amino acid sequence that was found to have GnSAF bioactivity and be homologous to the carboxyl terminal of HSA (Karligiotou et al., 2006; Pappa et al., 1999). Immunocytochemistry was performed for the detection of the HSA carboxyl terminal fragment using a custom-made polyclonal antibody specific for the carboxyl terminal of HSA.

RNA transcripts corresponding to exons 12 and 13 of HSA gene were detected both in messenger as well as in cytoplasmic RNA from granulosa cells (**Figure 1**). The addition of FSH in cultured granulosa cells increased the number of HSA transcripts corresponding to the carboxyl terminal of HSA protein, homologous to GnSAF factor.

Simultaneous cell cultures of granulosa cells, in the absence or the presence of FSH at the concentration of 10 ng/ml, HepG2 cells (positive control) and HeLa cells (negative control) were developed. Under fluorescent microscopic visualization, the nucleus of the cells showed a blue colouring corresponding to the DAPI dye, while the red colouring, due to the Fast Red dye, was emitted only when the probe used hybridized to a corresponding mRNA produced by the cell. In particular, the red colour was indicative of the presence of the mRNA corresponding to the carboxyl terminal of the HSA gene produced by the cell. HSA mRNA transcripts were detected, as expected, in the HepG2 cells (**Figure 2A**), while there was no detection of mRNA transcripts in HeLa cells (**Figure 2B**). Granulosa cells cultured with FSH produced red-coloured dots indicating the presence of carboxyl terminal HSA mRNA transcripts (**Figure 2C**). The granulosa cells positive for HSA mRNA expression after treatment with FSH accounted for 80 ± 3.3% of the total measured cells, while those detected

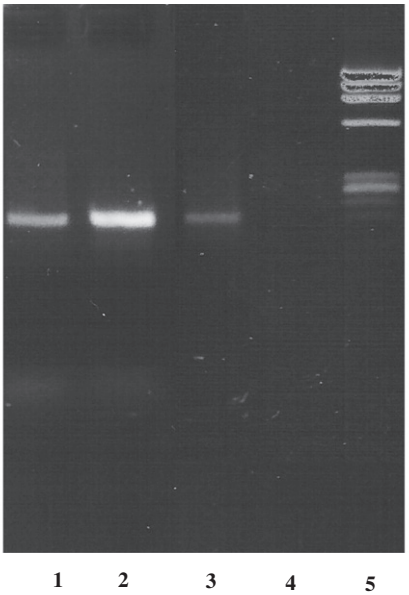

Figure 1 RNA transcripts corresponding to the region between exons 12 and 13 of the human serum albumin gene after PCR amplification (196 bp). Lane 1 = granulosa cells cultured with FSH; lane 2 = HepG2 cells (positive control); lane 3 = granulosa cells without FSH; lane 4 = negative control; lane 5 = marker φ174 DNA/*Hae*III.

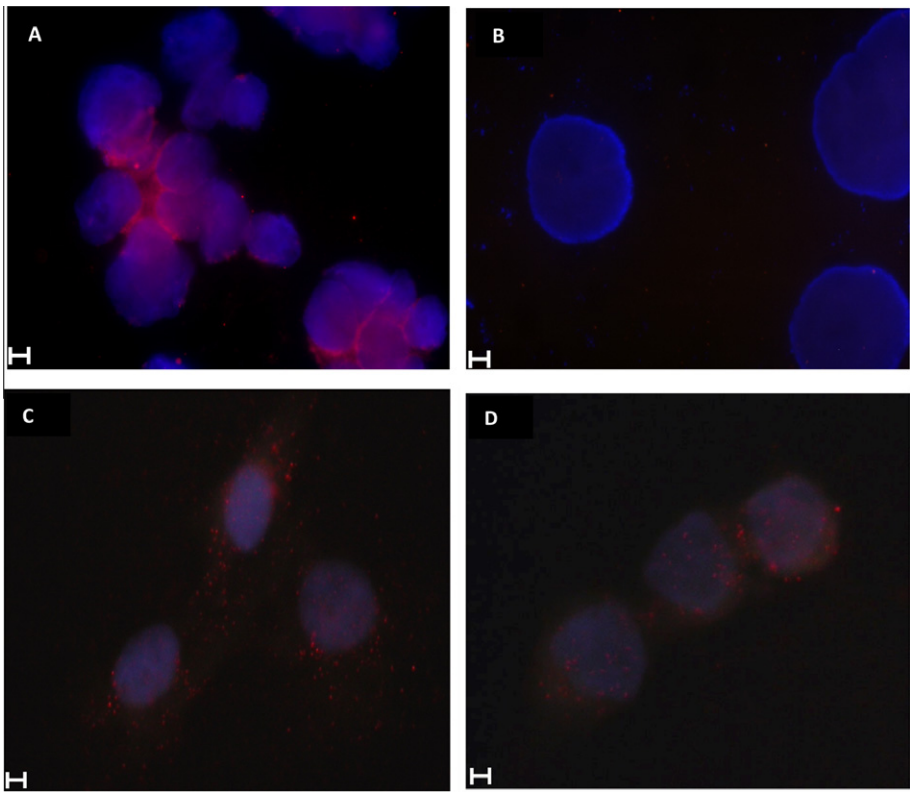

Figure 2 In-situ hybridization of HepG2 cells (positive control) (**A**); HeLa cells (negative control) (**B**); granulosa cells with and without FSH (**C**, **D**, respectively) using the carboxyl terminal of human serum albumin protein (exons 12—13) as the PCR probe labelled with FastRed (red dots). Cells were coloured with blue (4,6-diamidino-2-phenylindole) dye. Bars = 100 μm.

in granulosa cells cultured in the absence of FSH accounted for only 18 ± 4.8% of the total measured cells (**Figure 2D**). The expression observed in cells cultured in the presence of FSH was significantly higher ($P = 0.0002$) compared with that seen in granulosa cells cultured in the absence of FSH. The quantity of carboxyl terminal HSA mRNA transcripts in granulosa cells cultured with FSH was similar to that produced by HepG2 cells, which are the main production site of HSA mRNA transcripts (**Figure 2A**, **C**). The presence of FSH enhanced the production of HSA transcripts allowing for their detection in granulosa cells, while the addition of FSH in cultured HeLa cells did not induce any HSA mRNA production (results not shown for HeLa cells).

The protein corresponding to the carboxyl fragment of HSA was also detected in the same pattern. Western blotting was performed on protein extracts from luteinized granuolosa cells after 72 h culture with and without FSH and compared with protein extracts from HepG2 cells. The custom made antibody produced a band at the 64-kDa region corresponding to the whole of the HSA protein as well as a distinct band corresponding to the 12—14-kDa region of the carboxyl terminal (**Figure 3**). These results validated the anti-carboxyl-terminal HSA-specific antibody which was consecutively used for the ICC experiments.

As employed in the ISH, similar cell cultures were developed for the combined DNA ISH and ICC. The aim was to have a simultaneous detection of mRNA transcripts and protein under fluorescent microscope visualization. The cells again showed a blue colouring corresponding to the DAPI dye, while the red colouring, due to the Fast Red dye, was emitted only when the DNA probe used hybridized to a corresponding mRNA produced by the cell. Green colouring corresponded to the carboxyl terminal of the HSA protein produced by the cells indicative of the primary antibody's hybridization. The simultaneous presence of mRNA transcripts and protein, gave a yellow-green colouring as a result of the mixture of the red and green colours.

Results comparable to those observed with ISH were detected when both the DNA probe and the specific antibody for the carboxyl terminal of HSA were used. In detail, there was no signal for HeLa cells (**Figure 4B**) but a very strong signal of either combined yellow-green colouring or green and red colouring, representing the protein and the mRNA transcripts respectively, for the HepG2 cells (**Figure 4A**) and the granulosa cells cultured in the presence of FSH (**Figure 4C**). On these slides, 87 ± 3.5% of the granulosa cells showed the presence of both the protein and the mRNA (**Figure 4C**) at a similar extent to that seen in HepG2 cells (90 ± 3.5%). In addition, the expression observed was significantly higher in comparison to the granulosa cells cultured in the absence of FSH, where both protein and mRNA transcripts were detected (**Figure 4D**), but at a significantly lower percentage (22 ± 5.3%) of the total number of cells counted ($P = 0.0001$). These findings are similar to those found when only ISH was performed. Once more it was seen that the presence of FSH enhanced the production of such

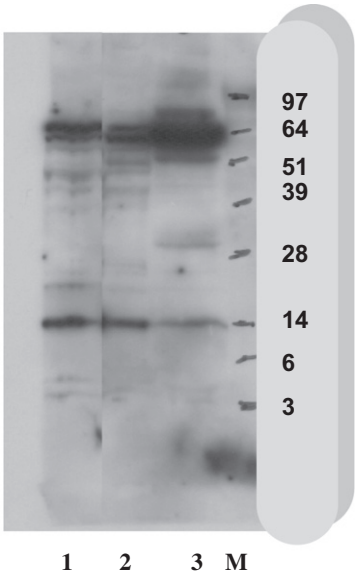

Figure 3 Western blot for the detection of the carboxyl terminal of the human serum albumin (HSA) protein in protein extracts. Lane 1 = HepG2 hepatic cell line; lane 2 = granulosa cells cultured without FSH; and lane 3 = granulosa cells after 48 h culture with FSH. The antibody used was anti-COOH human serum albumin specific for the carboxyl terminal of the HSA protein. The 12–14-kDa and 64-kDa signals correspond to the HSA carboxyl terminal and the whole of HSA, respectively. M = molecular weight markers.

transcripts and that of the corresponding protein, while the addition of FSH in cultured HeLa cells did not produce such mRNA transcripts and/or protein (results not shown for HeLa cells).

Discussion

Following previous findings regarding the nature of GnSAF and the gene encoding this protein, this research has provided additional evidence of the ability of human granulosa cells to produce the carboxyl terminal of the HSA gene *in vitro* as well as its corresponding protein (Karligiotou et al., 2006; Pappa et al., 1999; Tavoulari et al., 2004). The current study investigated the expression of HSA mRNA transcripts and protein in human luteinized granulosa cells cultured *in vitro* and the mechanism that may control the production of these fragments. An increased number of HSA mRNA transcripts extending from exon 12 to the end of exon 13 and protein corresponding to the carboxyl terminal of HSA from amino acid residues 490–585 in cultured granulosa cells were detected in the presence of FSH, which might have an important role *in vivo* in stimulating granulosa cells to produce the carboxyl terminal of the HSA gene.

The enhanced expression of this fragment by FSH is an interesting finding compatible with the physiological role of this gonadotrophin to stimulate in the ovaries the production of various steroidal and non-steroidal substances that participate in the feedback mechanisms. GnSAF is such a putative factor that attenuates the LH response to GnRH in women (Messinis and Templeton, 1989). Several studies have suggested the production of GnSAF from the ovaries

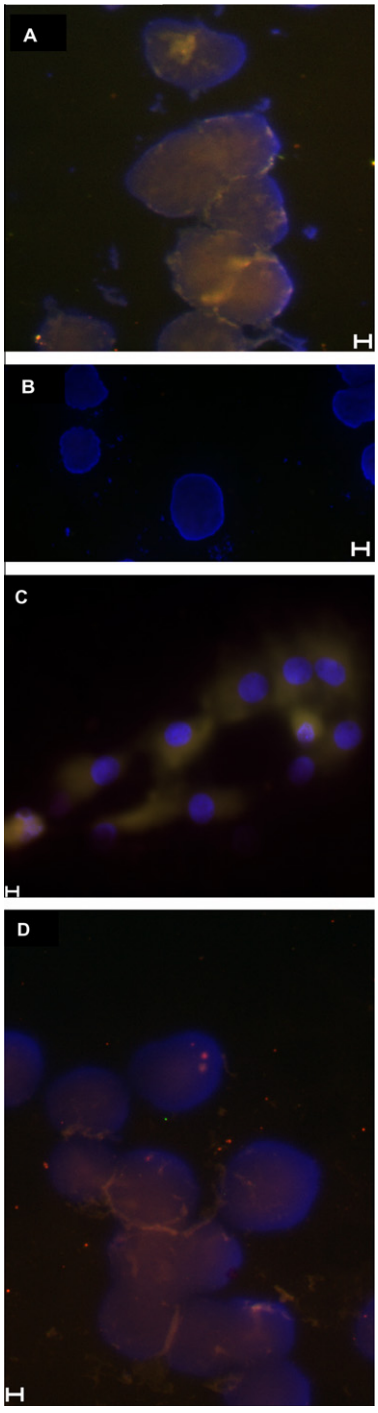

Figure 4 In-situ hybridization and immuncytochemistry of HepG2 cells (positive control) (**A**); HeLa cells (negative control) (**B**); granulosa cells with and without FSH (**C**, **D**, respectively) by using the carboxyl terminal of HSA protein (exons 12–13) as the PCR probe labelled with FastRed (red dots) and a polyclonal HSA carboxyl terminal-specific antibody visualized by a fluorescein isothiocyanate-labelled (green dots) secondary antibody. Cells were coloured with blue (4,6-diamidino-2-phenylindole) dye. Bars = 100 μm (**A**, **B** and **D**) and 800 μm (**C**). HSA = human serum albumin.

especially in FSH-superovulated women (Messinis et al., 1991, 1993, 1994a,b). It has been found that the higher the degree of superovulation the greater the in-vivo bioactivity of GnSAF (Messinis et al., 1986). FSH was added to the culture at a standard concentration of 10 ng/ml, used in similar experiments with granulosa cell cultures (Hillier et al., 1991). The level of expression recorded was similar to that seen for HepG2 cells.

One of the main aims of this research was to evaluate qualitatively the simultaneous expression of both RNA and protein expression from granulosa cells. In accordance, it was determined that FSH could be connected to the HSA carboxyl terminal production by granulosa cells, as it promoted both the mRNA and the protein expression. In future studies, a quantitative approach could provide further information on the role of FSH, comparing granulosa cell cultures with the addition of different FSH concentrations. The present findings together with previous data on the expression of this fragment by the granulosa cells (Karligiotou et al., 2006) and the fact that recombinant HSA subdomain IIIB polypeptides corresponding to the carboxyl terminal of HSA were able to express GnSAF bioactivity *in vitro* (Tavoulari et al., 2004) provide further support that this HSA fragment is a GnSAF-bioactive entity.

It is possible, therefore, that the ectopic expression of the albumin gene as the carboxyl terminal of HSA gene may function as the GnSAF gene. The latter can be a splice variant of the albumin gene, as the mRNA precursors enter the cytoplasm, but the possibility of being a different gene that is expressed by a different promoter cannot be excluded (Karligiotou et al., 2006). That luteinized granulosa cells, when cultured in the absence of FSH, show much lower expression of carboxyl terminal of HSA may be because these cells had already been luteinized producing little, if any, GnSAF.

The ISH technique employs the use of non-radioactive-labelled specific probes that show high stringency in the hybridization, giving mRNA transcripts only when there is a high percentage of matching base pairs. This technique can be used to distinguish whether the signals recorded are DNA or RNA, as the image produced is quite different; DNA is seen as two distinct dots, representing the gene loci located on the chromosomes (two copies), while the presence of RNA is depicted as multiple dots, representative of the many copies of mRNA present in the cell.

In this study, there was no signal detection in HeLa cells, which were used as a negative control, indicating that there was no hybridization to mRNA or protein present in these cells. In contrast, many green and red dots, or when combined yellow, were detected in HepG2 cells, which is indicative of the presence of HSA carboxyl terminal and mRNA transcripts both in the nucleus and the cytoplasm. Similarly, multiple green, red and yellow dots were also detected in the nucleus and the cytoplasm of granulosa cells cultured in the presence of FSH. The present findings by ISH are in accordance with existing studies regarding expression of HSA mRNA transcripts in liver tissue (Jochheim et al., 2004; Luk et al., 2005; Theise et al., 2000). In addition, the mRNA transcript expression of the HSA carboxyl terminal from human luteinized granulosa cells (not in culture), at comparable levels with HepG2 cells, was also seen in a previous study with the use of semi-quantitative reverse-transcription PCR (Karligiotou et al., 2006).

Due to the limitations of the ISH method, only the probes up to 500 bp can be used (with optimum length being 200 bp), so that penetration to the cell and correct hybridization to the mRNA in question can be achieved. Therefore, the whole-length HSA mRNA could not be used in these experiments, whereas the use of the specific anti HSA carboxyl terminal antibody resulted in providing evidence of only the carboxyl terminal of albumin being present. The commercially available antibodies do not provide information on whether or not they have an epitope on the carboxyl terminal of HSA. The use of such an antibody though, even if it had an epitope for the carboxyl terminal, could not provide further information on that matter, since a positive signal could not be attributed to the presence of the whole molecule as it could also be derived just from the carboxyl terminal.

The immunocytochemistry technique was employed, apart from the benefit of having a simultaneous image of protein expressed along with the respective mRNA transcripts, as additional evidence on the production site of protein. That was important because, when Western blotting was used, the detection of HSA was questionable as one could argue it may be a mere contaminant from the blood serum. Hence Western blot is a technique that could not provide conclusive evidence. Therefore, ICC was used to provide these data, as the presence of protein could be localized and observed at the site of its production: the cell's cytoplasm. Western blotting was used basically to establish the custom-made antibody and validate its function. Unfortunately due to limitations mentioned above no further quantification experiments could be performed for the blots.

The presence of HSA mRNA transcripts and the carboxyl terminal in the cytoplasm of granulosa cells supports the idea that these transcripts could be the coding region for a precursor HSA molecule which correlates to the GnSAF factor (Karligiotou et al., 2006). Whether a transient overexpression of the studied HSA fragment in the granulosa cells could display the GnSAF biological activities *in vitro* in a way analogous to the recombinant polypeptides (Tavoulari et al., 2004) needs to be investigated. Because, however, GnSAF is produced predominantly by small rather than large growing follicles during the follicular phase of the cycle (Fowler et al., 2001), such experiments might require granulosa cells from small–medium-sized antral follicles as a source, as the luteinized granulosa cells used in this study may not provide adequate amounts of the factor.

In conclusion, the present results demonstrate for the first time that FSH stimulates the expression of the carboxyl terminal fragment of HSA gene and protein in human luteinized granulosa cells. It is suggested that FSH is the hormone or one of the hormones that regulate the promoter responsible for the differential expression of the HSA gene in the ovary. These results support the notion that this HSA fragment is a GnSAF-bioactive entity.

Acknowledgements

The authors wish to thank Georgia Kokka for her technical assistance. This work was supported by a grant (number 3799) from Procter and Gamble, Athens, Greece.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 22 October 2010; refereed 8 June 2011; accepted 8 June 2011.