FSH stimulates the expression of the ADAMTS-16 protease in mature human ovarian follicles

Shuping Gao¹, Christian De Geyter¹,²,³, K.Kossowska¹ and Hong Zhang¹,²

¹Department of Research, Women’s Hospital, University of Basel, Switzerland, ²Department of Obstetrics and Gynecology, University Hospital of Basel, Spitalstrasse 21, CH-4031 Basel, Switzerland
³Correspondence address. Tel: +41 61 265 9315; Fax: +41 61 265 9194; E-mail: cdegeyter@uhbs.ch

We report the characterization of full-length human ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs)-16, a novel member of the disintegrin and metalloproteinase with thrombospondin motifs (hence ADAMTS) family. ADAMTS-16 is highly expressed both in the kidney and in the ovary, where it is predominantly expressed in the parietal granulosa cells of pre-ovulatory follicles but only slightly expressed in cells of the cumulus oophorus. In fully differentiated luteinizing granulosa cells, follicle-stimulating hormone and forskolin induces expression of ADAMTS-16, suggesting that it is regulated via the cAMP pathway. Luteinizing hormone had a minor effect on the expression of ADAMTS-16. ADAMTS-16 is capable of cleaving α₂-macroglobulin (MG), a common substrate for proteases, which is present at high concentrations in the follicular fluid of ovarian follicles. These studies provide the first evidence that ADAMTS-16 is an active protease and suggest a physiological role of ADAMTS-16 in ovarian follicles, at least during the pre-ovulatory phase.

Keywords: ADAMTS-16/ovulation/FSH/ovarian follicle/α₂-macroglobulin

Introduction

The ADAMTs (a disintegrin and metalloproteinase with thrombospondin motifs) are a family of 19 structurally related proteases, which are secreted in various organ systems and which can bind to specific proteins of the extracellular matrix (ECM). Since their first description (Kuno et al., 1997) studies on ADAMTS have recently been rapidly expanding, because they have been implicated in a number of diseases including cancer (Coussens et al., 2002). In addition to their involvement in ECM remodeling, the ADAMTs are involved in the functional regulation of a large number of cytokines, hormones, growth factors and proteases. Consequently, various members of the ADAMTS family are involved in the pathogenesis of apparently unrelated diseases such as osteoarthritis (Kevorkian et al., 2004), bleeding disorders (Vazquez et al., 1999), endothelial defects (Zheng et al., 2001) and cancer (e.g. breast cancer, Porter et al., 2004). Additionally, various members of the ADAMTS family play important roles in normal ovarian physiology. It has been demonstrated that ADAMTS-1 mRNA and protein were induced in granulosa cells of periovulatory follicles by luteinizing hormone (LH) in wild type but not in progesterone receptor knockout mice (PRKO), indicating that ADAMTS-1 is a transcriptional target of the progesterone receptor around the time of ovulation (Robket et al., 2000; Doyle et al., 2004). Studies in ADAMTS-1 (-/-) knockout mice demonstrated that the number of ovulated oocytes is significantly reduced as compared with heterozygous controls (Shindo et al., 2000; Shozu et al., 2005). The presence of oocytes within the ovary is significant and can be reduced, in ADAMTS-1 (-/-) knockout mice and the involvement of other members of the ADAMTS family of proteases in the ovulatory process demonstrates a large degree of redundancy among the various members of this family (Richards et al., 2005).

Two proteases structurally and functionally closely related to ADAMTS-1, ADAMTS-4 and ADAMTS-5 are present in granulosa cells of small ovarian follicles, indicating that various members of the ADAMTS family also have functions beyond ovulation and the formation of the luteal body (Richards et al., 2005).

Searching for novel markers of granulosa cell function, we identified ADAMTS-16 during the analysis of our ovary-specific gene expression database based on its high expression level in the ovary. In this report, novel data concerning both the regulation and the function of ADAMTS-16 in the ovary are presented.

Materials and Methods

Cloning of human ADAMTS-16 variants and chromosomal location of ADAMTS-16

OR0021 was identified based on its high level of expression in the ovary in our ovary-enriched gene expression database and OR0021 was found to be identical to ADAMTS-1 (Cal et al., 2002). Searching the GenBank database of human expressed sequence tags (ESTs) with the ADAMTS-16 cDNA sequence, and another EST sequence (GenBank accession no. AK122980) was detected and sequenced; its gene was named ADAMTS-16s. The search of the GenBank database for STSs using ADAMTS-16 as a template revealed that a human STS WI-6822 (accession number G06277) matches 100% to the nucleotides +941 to +1229 of the ADAMTS-16 sequence. ADAMTS-16 was found to be located on chromosome 5 by using the NCBI Unigene programme. The GenBank accession number for ADAMTS-16s is DQ266647.

Dragon Gene Start Finder was used to predict the transcriptional start site of ADAMTS-16 upstream of the translational initiation codon (ATG). The programme PromotorInspector was used to analyse the genomic sequences at the 5’ end of the ADAMTS-16 gene for putative promoter regions.
Tissue expression pattern analysis by RT–PCR

In order to determine the expression patterns of ADAMTS-16 mRNA in different human tissues, RT–PCR was performed, using the primers 5′-AACT CAGCCTGCGAGTCTGCAG-3′ and 5′-CAGTGGCCATCTGAGTAC-3′ on a panel of first-strand cDNAs from various human tissues (Origene, MD, USA). The PCR conditions were as described in the manufacturer’s protocol with 30 cycles performed on the β-actin control employing Taq polymerase. Microarray data of mouse testis, generated by the GNF Mouse Atlas v2 project (Su et al., 2004), were obtained from the hgFixed database of the UCSC Genome Browser (Karolchik et al., 2003).

Hormonal treatment of ovarian cells in vitro

Human luteinizing granulosa cells were obtained by follicular aspiration from women undergoing oocyte retrieval for assisted reproduction. The study was approved by the local research ethics committee. The age range of the women was 24–41 years. For each experimental cell culture, the granulosa cells obtained from one to three patients collected during the same day were pooled, enzymatically dispersed with 0.1% hyaluronidase (Sigma Chemical Co., St Louis, MO, USA) and separated from red blood cells by centrifugation through Ficoll-Paque (Pharmacia Biotech, Sweden). The cells were plated at a density of 2–5 × 10⁵ cells/well on 35 mm six-well dishes and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), 2 mmol/l L-glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) at 37°C in a 95% air–5% CO2 humidified environment. Cell culture media were changed every other day. On day 7, the cells were stimulated for 24 h with forskolin (10 μM) or with recombinant human follicle-stimulating hormone (rhFSH, 500 ng/ml, Gonal F, Serono, Zug, Switzerland) or with rhLH (100 IU/l, Luveris, Serono). Each experiment was repeated three times.

Fluorescence activated cell sorter and sorting of granulosa cells

Follicular fluid aspirates containing luteinizing granulosa cells were collected from infertile patients treated with exogenous gonadotrophins for assisted reproduction. The freshly collected clumps of granulosa cells were mechanically dissociated by nylon filters and stored frozen at −80°C. After thawing, the isolated granulosa cells were identified by the simultaneous presence of two specific markers, FSH receptor (FSHR) and LH receptor (LHR), on their surface and subsequently sorted using a fluorescence activated cell sorter (FACS). In order to identify and sort granulosa cells, a dual labelling technique was introduced, where granulosa cells were identified as CD3-negative cells, distinguishing them from leukocytes, which are CD3-positive (De Neubourg et al., 1998). The first polyclonal goat antibody rose against a peptide mapping either the FSHR or LHR of human origin (Santa-Cruz Biotechnology) or with anti-LHR antibody (sc-25828, Santa Cruz Biotechnology), respectively. As a negative control, granulosa cells were cultured in the presence of both anti-FSH and anti-LHR antibodies (as above).

Quantitative real-time PCR

Total RNA was extracted from GCs using a commercially available RNAeasy Total RNA kit from Qiagen (Hilden, Germany). The quantity of RNA was assessed by measuring the optical density at 260 nm. Total RNA (1 μg) from GCs was reversed transcribed into single strand cDNA using the cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). Primers for real-time PCR were synthesized by Microsynth, Balgath, Switzerland: hA16 (forward) 5′ AAACCTCTCACTGCGGCTCCTTC 3′ and R (reversed) 5′ CGTAT CATGCCCTGACAGCCCT 3′. Power SYBR Green PCR Master Mix (AB Applied Biosystems) for real-time PCR and TaqMan GAPDH Control Reagent (PE Applied Biosystems) as internal control were used. cDNA was subjected to 40 cycles of real-time PCR amplification carried out using ABI PRISM 7000 Sequence Detector System (AB Applied Biosystems). The difference between the results was assessed statistically with the Mann–Whitney U-test.

Plasmid constructs

Pblue-script-k-Kiaa029 (ADAMTS-16) was kindly provided by Kazusa DNA Research Institute (Kikuno et al., 2004). Full-length ADAMTS-16 was cloned in-frame into pGEX-4T-2 Vector with Sal I /Not I sites. ADAMTS-16s was generated by PCR and cloned using the primers 5′-CGTGGATCCCATTTCTCGTGAAGACGGC-3′ and 5′-CCGGAATTCCTGCAATTCG-3′. The PCR-product was digested with BamHI and EcoRI and cloned in-frame into the EcoRI/BamHI I sites of pGEX-4T-1 or pcDNA3-HisC (Invitrogen, Groningen, The Netherlands). All constructs were confirmed by sequencing.

Purification of ADAMTS-16 GST-fusion and His-tagged proteins

In brief, pGEX-4T-2-ADAMTS-16 and pGEX-4T-1-ADAMTS-16 pep were transformed to DE3 bacteria (BL20). The positive clones were cultured in 250 ml LB (100 mg/ml ampicillin) for 3–4 h at 30°C in a shaker until OD.600 nm reached 0.6–0.8. Then, IPTG was added to a final concentration of 0.1 mM (final concentration: 350 ng/ml), and the bacteria were cultured for an additional 4 h. Cells were collected by centrifugation and the pellets were stored at −70°C. Subsequently, the pelleted cells were lysed in 3 ml cold lysis buffer (50 mM Na2HPO4, pH 8.0, 0.3 M NaCl: 1 mM PMSF with protease inhibitors) containing freshly added lysozyme (100 μg/ml) and sonicated 5 × 20 s until the sample cleared and was no longer viscous. Upon centrifugation, samples were boiled at 95°C for 5 min and loaded on 10% SDS–PAGE. His-tagged proteins were produced by transfection of pcDNA3-HisC-ADAMTS-16 in HEK293 cells. Protein concentration was determined by BioRad mini kit.

Cleavage of α2-macroglobulin

About 45 μl of buffer (100 mM Tris–HCl, 20 mM CaCl₂ and 200 mM NaCl, pH 7.5) were added to 45 μl 50% GST beads (containing 5 mM Tris, 10 mM NaCl, pH 7.5, GE) and/or His Bead (Roche). ADAMTS-16 and ADAMTS-16s at a concentration of 20 μg/ml were incubated with 5 μl of 10 mM CaCl₂ and 5 μl of 100 ng α₂-macroglobulin (MG) at 37°C for 2 h in 1.5 ml Eppendorf tubes. Subsequently, the products were analysed for total protein by SDS–PAGE on 8–10% gels followed by staining with Coomassie Brilliant Blue R-250 to analyse for cleavage of MG.

Results

Molecular cloning of human ADAMTS-16 and its variant ADAMTS-16s

During the search of our ovarian-rich gene expression database, OR0021 was identified based on its high expression levels in the ovary (see below). This gene has been designated as ADAMTS-16, a member of the family of proteases, jointly denoted as ADAMTSs. By screening the EST database, followed by PCR, we identified the full-length cDNA (Fig. 1), including the 3′ sequence, which was missing in an earlier report (Cal et al., 2002). In addition, we identified a novel splicing variant (ABB70405) and termed it ADAMTS-16s for the short splicing form of the ADAMTS-16 transcript (Fig. 1). Sequence analysis revealed that the full-length ADAMTS-16 exhibits the typical ADAMTS modular structure (containing signal sequence, propeptide, metalloproteinase domain, disintegrin-like domain, central TS1 motif, cysteine-rich region and a C-terminal module with several TS1 submotifs) and that ADAMTS-16s encoded an open reading frame of 570 amino acids, containing only the peptide domain (Fig. 1). Sequencing of AK031314 from NCBI provided an ORF encoding the full-length mouse ADAMTS-16. The human
mouse nucleotide and predicted amino acid sequences have an overall identity of 86% and 91%, respectively (Fig. 1).

The full-length cDNA sequence of human ADAMTS-16 was mapped by a BLAST comparison to the BAC clones CTD-2297D10 (GenBank accession no. AC022424) and CTC-485I21 (AC010269) on human chromosome 5p15. ADAMTS-16 appears to consist of 23 exons spanning 180 kb of genomic DNA (Table 1). Analysis of the genomic region at the 5' end of ADAMTS-16 indicated that it has a CpG island (AC022424, nt 28391–27251; %GC = 66.6; O/E = 0.848; number of CpG = 112), suggesting that it is expressed in a tissue-specific manner (Fig. 2B). A single transcriptional start site was predicted at the upstream position of 560 bp relative to the translational codon (ATG). The programme PromotorInspector was used to analyse the genomic sequences at the 5' end of the ADAMTS-16 gene for putative promotor regions. The presence of one strong promotor region of ADAMTS-16 was predicted at nt 27728–27478 of AC002244. Two spl sites and one egr-1 site are present in the promotor (Fig. 2C), suggesting that they mediate the expression of ADAMTS-16 during development.

Expression of ADAMTS-16 mRNA in the ovary
To determine the tissue-specific expression of ADAMTS-16 in the human, semi-quantitative RT–PCR analysis (semi-Q-RT–PCR) was performed by using a pair of primers corresponding to sequences flanking the first intron. As shown in Fig. 2A, ADAMTS-16 was expressed mainly in adult kidney, pancreas and ovary. We subsequently analysed the expression of the ADAMTS-16 transcript in the UniGene (Build #184). Expression of ADAMTS-16 was also present in ESTs from brain medulloblastoma, brain amygdala, lung focal fibrosis, lung large cell carcinoma, endometrial adenocarcinoma and retinoblastoma (Wheeler et al., 2003). These results obtained from

Figure 1: Isolation of cDNAs encoding the human gene ADAMTS-16 and its variant ADAMTS-16s
Alignment of the amino acid sequence of ADAMTS-16 and its splice variant ADAMTS-16s from human and mouse using the multiple alignment programme (Multalin). Letters in red indicate 100% identity within 3 sequences, letters in blue are those identical between two sequences, letters in black indicate low consensus value
ADAMTS-16 analysis, the increase in the granulosa cells and cumulus cells induction of Hormonal regulation of ADAMTS-16 expression is also expressed to some extent in other tissues. RT–PCR analyses were also supported by microarray expression analyses collected from GNF (Genomics Institute of the Novartis Research Foundation) Expression Atlas Chips U133A, GNF1H and Affy U95 (data not shown, http://symatlas.gnf.org/SymAtlas), confirming that ADAMTS-16 is highly expressed in the ovary, but that it is also expressed to some extent in other tissues.

**Hormonal regulation of ADAMTS-16 expression**

We subsequently examined the effects of both FSH and LH on the induction of ADAMTS-16 mRNA expression in both luteinizing granulosa cells and cumulus cells in vitro, collected from patients treated with exogenous gonadotrophins for assisted reproduction. As shown in Fig. 3A, ADAMTS-16 mRNA was predominantly detected in luteinizing granulosa cells with little expression in cumulus cells. A weak but distinct expression pattern was also found in cells of an immortalized granulosa cell line developed from a granulosa cell tumour (KGN, Nishi et al., 2001). By semi-quantitative RT–PCR analysis, the increase in the ADAMTS-16 expression level was detected in the presence of FSH and forskolin (Fig. 3B, lane 3–5), whereas LH (at a concentration of 100 IU/l) induced minor changes in the expression level of ADAMTS-16 as compared with FSH (Fig. 3B, lane 1).

In order to further dissect the effects of FSH and LH on the expression of ADAMTS-16 in granulosa, luteinizing granulosa cells were collected from infertile patients treated with exogenous gonadotrophins for assisted reproduction and sorted with FACS based on the concomitant presence of both the FSHR and the LHR. These sorted granulosa cells were then cultured in the presence of recombinant FSH together with anti-LHR-antibodies or in the presence of recombinant LH together with anti-FSHR-antibodies, respectively. As shown in Fig. 3C, under these conditions, the expression level of ADAMTS-16 in granulosa cells cultured in the presence of recombinant FSH and anti-LHR antibody was significantly higher as compared with that of granulosa cells cultured in the presence of recombinant LH and anti-FSHR-antibodies ($P = 0.02$, Mann–Whitney U-test).

**Table 1: Human ADAMTS-16 gene structure**

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**MG is a substrate of ADAMTS-16**

Several sequence features of the human ADAMTS-16 are very similar to those of other members of the ADAMTSs family of proteases including the number of cysteine residues (Porter et al., 2005). Thus, this ‘trapping’ mechanism induces a delayed migration of MG in SDS–PAGE upon incubation with a protease and is considered an experimental evidence of its proteolytic activity, which is different from the cleavage pattern induced by trypsin (Kuno et al., 1999). As shown in Fig. 4A and B, in the presence of ADAMTS-16 produced either by *Escherichia coli* (Fig. 4A, lane 1 and 2) or by HEK293 (Fig. 4B), migration of MG on SDS–polyacrylamide gel was altered. Conversion of intact MG to the 90 kDa species was also observed, indicating the formation of MG-ADAMTS-16 complexes, together with proteolysis of MG by ADAMTS-16. MG-ADAMTS-16 complexes were mainly detected in HEK293 cells (Fig. 4B, lane 1). In contrast, high levels of MG could not form high order complexes in the absence of ADAMTS-16 (Fig. 4B), migration of MG on SDS–polyacrylamide gel was altered. Conversion of intact MG to the 90 kDa species was also observed, indicating the formation of MG-ADAMTS-16 complexes, together with proteolysis of MG by ADAMTS-16. MG-ADAMTS-16 complexes were mainly detected in HEK293 cells (Fig. 4B, lane 1). In the control experiments, trypsin attack sites were different to those affected by ADAMTS-16 (Fig. 4C). These studies demonstrate that ADAMTS-16 was able to cleave MG.

**Discussion**

Various members of the ADAMTS protease family have been implicated in the process of ovulation (Espey et al., 2000; Robker et al., 2000; Madan et al., 2003; Doyle et al., 2004; Mittaz et al., 2004; Shozu et al., 2005; Richards et al., 2005). The liberation of an oocyte from its mature follicle during ovulation is the climax of complex remodelling processes occurring both in the follicular wall and in the cumulus–oocyte complex. This involves remodelling of various components of the ECM through the action of a multitude of proteases. The efficacy of each of these processes results from the equilibrium between the specific components of the ECM and each of the proteases. Both the amount of various components of the ECM and the expression of specific proteases seem to be hormonally regulated during the entire process of follicular development and

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ovulation. Due to the dependency of the expression and secretion of each component on a particular endocrine signal, which may be conveyed to the tissue both locally and during particular time intervals, the whole process becomes tightly regulated. The stringent control of these processes and the specificity of the various substances involved carries the risk of making ovulation—and with this also the ultimate success of reproduction—crucially dependent on the exact functioning of each individual process. Therefore, the involvement of a large number of substrates and proteases adds an aspect of redundancy to this system (Richards et al., 2005), permitting the ongoing of successful ovulations even in the absence of one of the players (e.g. ADAMTS-1, Mittaz et al., 2004; Shozu et al., 2005). One of the components of the ECM of the cumulus oophorus, versican, is induced in the granulosa cells under the influence of LH (Russell et al., 2003a) and is a substrate for various members of the ADAMTS protease family, mainly ADAMTS-1 (Russell et al., 2003b). In the PRKO mouse, which fails to ovulate in response to LH, the expression of ADAMTS-1 is significantly reduced (Robker et al., 2000), suggesting an important role of the ADAMTS protease family in the downstream pathway leading to ovulation.

ADAMTS-16 is another member of this family and has not yet been studied in detail. Phylogenetically, it is most related to ADAMTS-18 (Cal et al., 2002) and has been implicated in the pathogenesis of osteoarthritis (Kevorkian et al., 2004). We identified high-expression levels of ADAMTS-16 in the ovary during our search of an ovary-specific gene expression database. The ADAMTS-16 gene was shown to consist of 23 exons spanning 180 kb of genomic DNA and is located on the human chromosome 5p15. ADAMTS-16 was expressed only in the parietal granulosa cells of pre-ovulatory follicles, not in the cumulus oophorus, both previously collected from patients treated with exogenous gonadotrophins for assisted reproduction. The increase in ADAMTS-16 expression in luteinizing granulosa cells was only detected in the presence of FSH and forskolin. The change in the expression levels of ADAMTS-16 in sorted granulosa cells provoked by recombinant LH was significantly lower than those induced by recombinant FSH (P = 0.02, Fig. 3C). LH considerably reduced ADAMTS-1 expression in human granulosa cells and was modestly repressed after treatment with FSH (Freimann et al., 2005). Most dramatically, forskolin highly suppressed ADAMTS-1 expression as compared with non-treated controls (Freimann et al., 2005). These results...

Figure 2: Both the expression level and the distribution of ADAMTS-16 mRNA species vary in different tissues (A) RT–PCR was performed on a panel of first-strand cDNAs prepared from different human tissues: (1) brain, (2) heart, (3) placenta, (4) lung, (5) liver, (6) smooth muscle, (7) kidney, (8) pancreas, (9) spleen, (10) thymus, (11) prostate, (12) testis, (13) ovary, (14) small intestine, (15) colon and (16) peripheral blood leukocytes. (B) Analysis of the genomic region at the 5' end of ADAMTS-16 using CpGplot. (C) The programme PromoterInspector was used to analyse the genomic sequences at the 5' end of the ADAMTS-16 gene for putative promoter regions.
suggest a distinct role of ADAMTS-16 in ovarian function as compared with that of other members of the ADAMTS protease family such as ADAMTS-1. Since the present studies were carried out with parietal granulosa cells aspirated from follicles under the influence of FSH, may thus be involved in regulating the local concentration of MG, thereby regulating the development of perifollicular capillaries. These have been demonstrated to be important for follicular development and for the provision of competent oocytes (Van Blerkom et al., 1997). Other members of the ADAMTS family have also been implicated in modulating the formation of intraovarian capillaries, such as ADAMTS-1 (Shozu et al., 2005).

Our studies provide the first evidence that ADAMTS-16 is expressed in parietal granulosa cells of ovarian follicles under the influence of FSH and that it is an active protease of MG, which is present at high concentrations in follicular fluid. We therefore suggest a physiological role of ADAMTS-16 in ovarian follicles, at least during the pre-ovulatory phase.

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References


