Matrix Biology 28 (2009) 416–424

Contents lists available at ScienceDirect

Matrix Biology

journal homepage: www.elsevier.com/locate/matbio

Characterization and regulation of ADAMTS-16

Alison K Surridge [a,1], Ursula R Rodgers [a], Tracey E Swingler [a], Rose K Davidson [a], Lara Kevorkian [a,2], Rosemary Norton [a], Jasmine G Waters [a], Mary B Goldring [b], Andrew E Parker [c,3], Ian M Clark [a,c,*]

[a] School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK
[b] Hospital for Special Surgery, 535 East 70th Street, New York, USA
[c] Respiratory and Inflammation Department, AstraZeneca Pharmaceuticals, Cheshire, UK

ARTICLE INFO

Article history:
Received 11 March 2009
Received in revised form 26 June 2009
Accepted 17 July 2009

Keywords:
ADAMTS
Metalloproteinase
Chondrocyte
Cartilage
Promoter
Transcription

ABSTRACT

The ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) family includes 19 secreted proteinases in man. *ADAMTS16* is a recently cloned gene expressed at high levels in fetal lung and kidney and adult brain and ovary. The ADAMTS-16 protein currently has no known function. *ADAMTS16* is also expressed in human cartilage and synovium where its expression is increased in tissues from osteoarthritis patients compared to normal tissues. In this study, we ascertained that the full length *ADAMTS16* mRNA was expressed in chondrocytes and cloned the appropriate cDNA. Stable over-expression of *ADAMTS16* in chondrosarcoma cells led to a decrease in cell proliferation and migration, though not adhesion, as well as a decrease in the expression of matrix metalloproteinase-13 (*MMP13*). The transcription start point of the human *ADAMTS16* gene was experimentally identified as 138 bp upstream of the translation start ATG and the basal promoter was mapped out to −1802 bp. Overexpression of Egr1 induced *ADAMTS16* promoter constructs of −157/+138 or longer whilst Sp1 induced all *ADAMTS16* promoter constructs. Transforming growth factor beta (TGFβ) stimulated expression of endogenous *ADAMTS16* gene expression in chondrocyte cell lines.

1. Introduction

The ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) family includes 19 secreted proteinases in man. These enzymes have a complex domain structure consisting of at least a signal peptide, pro domain, metalloproteinase domain, disintegrin domain, thrombospondin type I motif and cysteine rich domain (Porter et al., 2005). Phylogenetically, the enzyme family separates into eight clades which to some extent correlate with function where assigned (Huxley-Jones et al., 2005). ADAMTS-1, -4, -5, -8, -9 and -15 are all capable of degrading aggrecan at a specific set of loci with ADAMTS-5 being the major aggrecanase in cartilage destruction, at least in mice (Clark and Parker, 2003; Collins-Racie et al., 2004; Glasson et al., 2005; Stanton et al., 2005). At least three ADAMTS enzymes (ADAMTS-4, -7 and -12) can degrade cartilage oligomeric matrix protein, another component of cartilage ECM (Dickinson et al., 2003; Liu et al., 2006a,b). Three ADAMTS enzymes, ADAMTS-2, -3 and -14, are procollagen N-propeptidases (PCNPs), which have roles in collagen biosynthesis. ADAMTS-2 acts preferentially on type I collagen and ADAMTS-3 on type II collagen, whereas the preference of ADAMTS-14 is currently unknown (Colige et al., 2002). Mutation of the *ADAMTS2* gene causes Ehlers–Danlos syndrome Type VIIC in man (Colige et al., 1999). ADAMTS-13 cleaves von Willebrand factor with mutations in this enzyme leading to an inherited thrombotic thrombocytopenia (Tsai, 2007). A form of Weill–Marchesani syndrome is caused by mutation in the *ADAMTS10* gene (Dagoneau et al., 2004).

ADAMTS-16 is a recently described member of the *ADAMTS* gene family (Porter et al., 2005). The cDNA was cloned using a combination of bioinformatics and degenerate RT-PCR, which also identified ADAMTS-13, -14, -15, -17, -18 and -19 (Cal et al., 2002). Amino acid sequence alignment showed a significant percentage of identity between ADAMTS-16 and ADAMTS-18 (overall identity, 57%), particularly in the catalytic domain where identity reaches 85% (Porter et al., 2005). Indeed, the zinc-binding motif is identical between these two proteinases (HESGHNFGMIHD) (Somerville et al., 2003a). ADAMTS-16 and -18 form a phylogenetic clade, with the nearest evolutionary neighbours being ADAMTS-6, -7, -10 and -12.

Whilst the substrates for ADAMTS-16 are currently unknown, a recombinant truncated form of ADAMTS-16 shows weak aggrecanase

Abbreviations: ADAMTS, a disintegrin and metalloproteinase domain with thrombospondin motif; MMP, matrix metalloproteinase; RACE, rapid amplification of cDNA ends; TGFβ, transforming growth factor beta; TIMP, tissue inhibitor of metalloproteinases.

* Corresponding author. Cellular Protease Group, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK. Tel.: +44 1603 592760; fax: +44 1603 592250.
E-mail address: i.clark@uea.ac.uk (I.M. Clark).
[1] Current address: Department of Zoology, University of Cambridge, UK.
[2] Current address: UCB Celltech, Slough, Berkshire, UK.
[3] Current address: OPSONA Therapeutics Ltd, Dublin, Ireland.

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doi:10.1016/j.matbio.2009.07.001

activity (Zeng et al., 2006) and full length recombinant ADAMTS-16 is capable of cleaving the proteinase inhibitor α_2-macroglobulin (Gao et al., 2007).

An initial expression analysis in a selection of human tissues shows high expression of *ADAMTS16* mRNA in fetal lung and kidney, and adult brain and ovary (Cal et al., 2002). In this latter tissue, *ADAMTS16* is expressed predominantly in the parietal granulosa cells of pre-ovulatory follicles. Expression of the gene can be induced by follicle-stimulating hormone and forskolin in granulosa cells, suggesting that the cAMP pathway may be involved in its regulation in this system (Gao et al., 2007). *ADAMTS16* has also recently been genetically linked to inherited hypertension (Joe et al., 2009).

We undertook the expression profiling of all *ADAMTS* genes in cartilage and synovium. We compared expression in patients undergoing hip replacement for osteoarthritis (OA) to phenotypically normal tissues from patients undergoing hip replacement following fracture to the neck of femur. In cartilage, the expression of *ADAMTS16* increased in the OA samples with a significance of $p<0.001$, comparable to the increase in expression of *MMP13*, a collagenase whose activity is pathognomic with cartilage destruction in OA. This was backed up with a preliminary study in the knee using cartilage from OA patients compared to normal cartilage from post-mortem, where a similar increase in expression of these two genes was measured (Kevorkian et al., 2004). Similarly, in synovium, the expression of *ADAMTS16* was significantly increased in OA with the absolute level of *ADAMTS16* mRNA approximately 10-fold higher in synovium than in cartilage (Davidson et al., 2006).

In order to provide some insight into the regulation and function of ADAMTS-16 in the joint, the current study examines the expression of ADAMTS-16 by chondrocytes, the regulation of the gene and the consequence of stable over-expression of the gene in chondrosarcoma and immortalized chondrocyte cell lines.

2. Results

2.1. Characterisation of ADAMTS16 and creation of stably expressing cell lines

Using PCR primer pairs to amplify overlapping sections of the *ADAMTS16* gene, the entire transcript was amplified from cDNA reverse transcribed from mRNA purified from an immortalized human chondrocyte cell line, C28/I2 (Loeser et al., 2000) (data not shown). No evidence for expression of splice variants was found (though it is possible that some truncated forms of the transcript might not have been detected if they were not amplified at all by selected primers). Based on these data, a full length cDNA was cloned and the final cDNA verified by sequencing in both directions. It should be noted that, despite using high fidelity polymerases and low numbers of amplifications cycles, frequent mutations were introduced by the PCR. The correct version of *ADAMTS16* could only be created by amplifying sections of the gene (up to approximately 1000 bp), verifying sequence, then assembling via restriction digestion and ligation into an error-free cDNA.

Initially, both immortalized chondrocytes (C28/I2) and chondrosarcoma (SW1353) cells were transfected with the full length *ADAMTS16* construct (with a C-terminal FLAG tag). However, the C28/I2 transfectants did not grow robustly after selection and experiments were pursued in the SW1353 line alone. Following cloning by limiting dilution, four different clonal populations expressing *ADAMTS16* (TS16) were compared to two clonal populations transfected with vector only control constructs (VO). Expression of *ADAMTS16* at the mRNA level was confirmed using qRT-PCR (Fig. 1a). The *ADAMTS16* expressing clones had an average of approximately 95-fold higher expression than the vector only controls. Western blot using both an anti-ADAMTS-16 antibody and an anti-FLAG antibody showed expression of a high molecular weight (~130 kDa) protein predominantly in the extracellular matrix fraction of the ADAMTS16 transfectants (Fig. 1b). Two bands of similar size were also detected in the conditioned medium and cell lysate fractions (Fig. 1c).

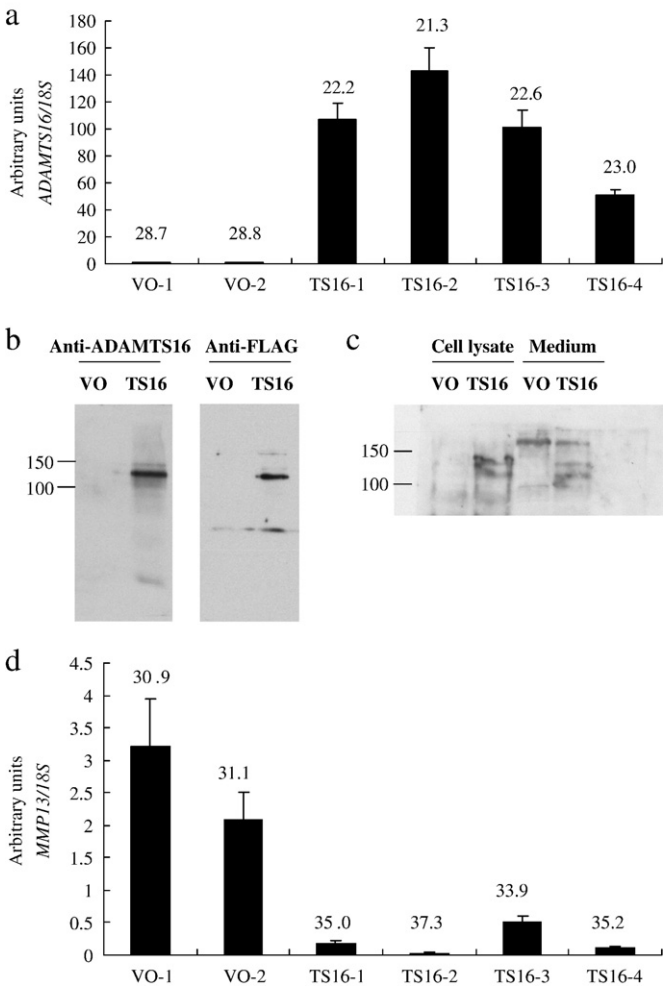

Fig. 1. Stable over-expression of *ADAMTS16* in SW1353 cells. SW1353 cells were stably transfected with either vector only (VO) or ADAMTS16 (TS16) expression constructs. Cells were harvested and total RNA isolated and subjected to qRT-PCR for expression of (a) *ADAMTS16* or (d) *MMP13*. Data are normalized to 18 S and expressed as mean ± s.e.m. Mean threshold cycle (Ct) is given above each bar. Extracellular matrix (b) or cell lysate and conditioned medium (c) were harvested (VO vs. TS16) and subjected to western blot analysis using anti-ADAMTS-16 and/or anti-FLAG primary antibodies.

2.2. Influence of ADAMTS-16 on expression levels of other metzincin genes

Expression levels of all 19 members of the ADAMTS family, all four members of the TIMP gene family and MMP2, MMP9, MMP13 and MMP28 were determined in cells stably over-expressing ADAMTS16 compared to vector only controls using qRT-PCR. The representatives of the MMP family were chosen because of their expression pattern in tissues of the OA joint (Davidson et al., 2006; Kevorkian et al., 2004). MMP13 expression levels were significantly reduced in ADAMTS16-expressing clones compared to vector only controls ($p<0.005$; Fig. 1d) with a mean of approximately 9-fold lower expression. Interestingly, the level of MMP13 expression was inversely correlated with that of ADAMTS16 (compare Fig. 1a and d). Expression levels of the other genes analysed did not change significantly between over-expressing cells and vector only controls (data not shown).

2.3. Effects of ADAMTS16 expression on cell phenotype

Expression of ADAMTS-16 by stably expressing clones was confirmed by immunocytochemistry using anti-FLAG antibodies