Thrombosis Research 147 (2016) 16–23

Contents lists available at ScienceDirect

Thrombosis Research

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

Full Length Article

Degradation of two novel congenital TTP ADAMTS13 mutants by the cell proteasome prevents ADAMTS13 secretion

Mary Underwood [a,b,*,1], Flora Peyvandi [a,b,c], Isabella Garagiola [c], Samuel Machin [a], Ian Mackie [a]

[a] Haemostasis Research Unit, University College London, London, England
[b] Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy
[c] Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

ARTICLE INFO

Article history:
Received 21 June 2016
Received in revised form 13 September 2016
Accepted 14 September 2016
Available online 15 September 2016

Keywords:
ADAMTS13
Von Willebrand factor-cleaving protease
Thrombotic thrombocytopenic purpura
Mutation
Congenital
Proteasome

ABSTRACT

Introduction: Over 150 mutations have been identified in the *ADAMTS13* gene in patients with congenital thrombotic thrombocytopenic purpura (TTP). The majority of these (86%), lead to reduced (<50%) secretion of mutant recombinant ADAMTS13. The mechanism by which this occurs has not been investigated *in vitro*. Two novel ADAMTS13 mutations (p.I143T and p.Y570C) identified in two congenital adolescence onset TTP patients were studied, to investigate their effects on ADAMTS13 secretion and subcellular localisation.
Materials and Methods: HEK293T cells were transiently transfected with wild type or mutant ADAMTS13 cDNA. Immunofluorescence and confocal microscopy were used to study localisation within the endoplasmic reticulum (ER) and Golgi. The cell proteasome and lysosomes were inhibited in cells stably expressing ADAMTS13 to investigate degradation of ADAMTS13 by either organelle.
Results: Both mutations severely impaired secretion and both mutants localised within the ER and Golgi. Proteasome inhibition led to the intracellular accumulation of both mutants, suggesting proteasome degradation. Lysosome inhibition on the other hand did not lead to increased intracellular accumulation of the mutants.
Conclusions: Proteasome degradation of these ADAMTS13 mutants contributed to their reduced secretion.

1. Introduction

Thrombotic thrombocytopenic purpura (TTP) patients have an acquired or congenital deficiency in ADAMTS13 activity [1–3]. ADAMTS13 processes and cleaves Von Willebrand factor (VWF) at the Tyr1605-Met1606 peptide bond in the A2 domain [4,5]. Its deficiency leads to the accumulation of ultra-large VWF (ULVWF) multimers within the circulation, leading to the uncontrolled formation of platelet-rich thrombi in the microcirculation and subsequent vessel occlusion.

Congenital TTP patients are presently treated with plasma infusion/exchange which provides a source of ADAMTS13. Despite these patients typically having severely reduced ADAMTS13 activity, the clinical presentation and response to treatment is highly variable. Evidence suggests that the *ADAMTS13* genotype may account for some of this heterogeneity. Over 150 mutations have been identified throughout the *ADAMTS13* gene in patients with congenital TTP [1,6–19] and relationships appear to exist between *ADAMTS13* genotype, age of disease onset [20] and residual ADAMTS13 activity [11]. Residual ADAMTS13 activity in turn appears to be associated with the annual rate of TTP episodes and with the requirements for fresh frozen plasma prophylaxis [11].

As residual ADAMTS13 activity appears to be associated with disease severity and with *ADAMTS13* genotype, it is important to understand how different *ADAMTS13* mutations affect its function. Of the mutations identified, approximately 30% have been expressed *in vitro* [7,20–22]. These mutations affect to various degrees the secretion and activity of ADAMTS13 [20]. The majority (86%), lead to reduced (<50% of wild type [WT]) secretion of mutant recombinant ADAMTS13, suggesting misfolding of ADAMTS13 and subsequent retention and degradation by the cell. The mechanism by which this may occur however has not been investigated *in vitro*.

With this in mind we studied two novel previously uncharacterised mutations (p.I143T, p.Y570C) present in a homozygous form in two different congenital TTP patients [17,23]. Both of these mutations are located within the N-terminal region of ADAMTS13. The p.I143T mutation is located within the metalloprotease domain which contains the catalytic site necessary for VWF cleavage [24]. The p.Y570C mutation localises within the spacer domain which is important for VWF binding [25–27]. The clinical history of both patients has been described previously [17], but in brief both presented with acute TTP during adolescence with undetectable ADAMTS13 activity and antigen and both receive prophylactic plasma infusion every three to four weeks. The effect of

* Corresponding author at: Haemostasis Research Unit, University College London, England.
E-mail address: m.underwood@ucl.ac.uk (M. Underwood).
[1] Present address: Haematology Department, Imperial College London, 5th Floor Commonwealth Building, Du Cane Road, W12 0NN.

http://dx.doi.org/10.1016/j.thromres.2016.09.014
0049-3848/

these mutations on ADAMTS13 secretion was assessed along with their effect on the intracellular localisation of ADAMTS13.

2. Materials and Methods

All reagents were obtained from Sigma Aldrich Chemical Company Ltd. (Poole, UK) unless stated otherwise.

2.1. Site Directed Mutagenesis

The p.I143T (c.428 T > C) and p.Y570C (c.1709 A > G) *ADAMTS13* mutations were introduced by site directed mutagenesis into a pcDNA 3.1/V5-His TOPO® vector (Life technologies, Paisley, UK) containing the complete *ADAMTS13* cDNA as previously described [28], primers are available upon request.

2.2. Transient and Stable Line Expression

HEK 293T cells were transiently transfected with WT or mutant *ADAMTS13* cDNA as previously described [21], along with 1 μl of pRL-TK plasmid (Promega, Southampton, UK) to normalize the transfection efficiency. The pRL-TK vector expresses Renilla luciferase which produces luminescence in the presence of its substrate (luminol). The Renilla luciferase assay system (Promega) was used to measure luminescence in cell lysate samples according to the manufacturer's recommendations. The luminescence values were used to normalize antigen and activity results. DMEM media was exchanged for OptiMEM media just before transfection. Supernatant and cell lysate samples were harvested four days after transfection as previously described [28].

Stable lines expressing WT or mutant ADAMTS13 were created as previously described [28]. WT and mutant supernatant from transiently transfected cells was concentrated ~20 fold, whereas WT and mutant supernatant from stable lines were concentrated ~100 fold using centrifugal filter devices with a 100 kDa cut-off (Millipore UK Ltd., Livingston, UK).

2.3. Western Blotting

For SDS-PAGE on transient transfection samples, the volumes loaded were normalized according to the transfection efficiency. Samples were loaded onto 4–12% Bis-Tris polyacrylamide gels (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and protein was transferred onto 0.45 μm nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). A primary mouse anti-V5 monoclonal antibody (Life technologies) against the C-terminal V5 tag expressed by the recombinant protein was used, followed by an ECL anti-mouse IgG HRP secondary antibody (GE Healthcare) for supernatant samples. For cell lysate samples membranes were also incubated with an anti β actin antibody loading control (Abcam, Cambridge, UK) after proteasome and lysosome inhibition. Membranes were developed using supersignal west pico chemiluminescent substrate (Fisher Scientific, Loughborough, UK) followed by exposure onto Amersham Hyperfilm ECL (GE Healthcare).

2.4. ADAMTS13 Antigen and Activity

ADAMTS13 antigen in supernatant samples from transiently transfected cells was measured using an in house ADAMTS13 antigen ELISA [29] (detection limit 3% of pooled normal plasma (PNP). ADAMTS13 antigen in supernatant samples from proteasome and lysosome inhibition experiments was measured using an Imubind ADAMTS13 antigen ELISA (Sekisui Diagnostics, Stamford, USA) (detection limit <60 ng/ml, ~6% PNP) as one of the antibodies for the in house ELISA is no longer commercially available. ADAMTS13 activity in supernatant samples was measured using FRETS-VWF73 as substrate [30] with minor modifications [31] (detection limit 5% of PNP).

2.5. Confocal Microscopy

ADAMTS13 localisation within the cell endoplasmic reticulum (ER) and Golgi was investigated using immunofluorescent labelling and confocal microscopy, with antibodies directed against ADAMTS13 and either the ER or Golgi. HEK 293T cells transiently transfected with ADAMTS13 cDNA were fixed with 4% paraformaldehyde, permeabilised with 0.2% Triton and blocked overnight with 3% BSA containing 0.3 M glycine. A chicken anti-V5 primary antibody (Cambridge Biosciences Ltd., Cambridge, UK) and either a mouse anti-GM130 (BD Biosciences, Oxford, UK, *cis* Golgi marker) or a mouse anti-PDI (protein disulphide isomerase) primary antibody (RL90 Abcam, Cambridge, UK, ER marker) was used. A rabbit FITC conjugated anti-chicken antibody (Life technologies) was used to detect the primary anti-V5 antibody. A goat Cy3 conjugated anti-mouse antibody (Millipore UK Ltd) was used to detect the ER/Golgi primary antibody. Cells were mounted with Vectashield® mounting media containing DAPI to stain for nuclei (Vector Laboratories Ltd., Peterborough, UK). Four to six slides were imaged per experiment, over an area of 4.84cm^2.

Cells were viewed at room temperature using a spinning disk confocal microscope (Perkin Elmer, Massachusetts, USA) with a Volocity acquisition system, built on a Zeiss inverted microscope. Images were captured using either a ×63 or ×100 immersion oil objective both with a 1.4 numerical aperture. Wavelengths for excitation vs. emission were as follows: DAPI, 405 nm vs. 455/460 nm; FITC, 488 nm vs. 527/555 nm and Cy3 568 nm vs. 615/670 nm.

2.6. Quantitation of Cis Golgi Colocalisation

The degree of colocalisation between ADAMTS13 and the *cis* Golgi was quantified using Volocity 3D analysis software (Perkin Elmer). Clearly defined cells containing unsaturated voxels (throughout the Z-stack) were used for analysis. The threshold Pearson Correlation Coefficient (TPCC) [32] and M_1 Manders overlap colocalisation coefficient (M_1) [32,33] were measured. For both types of analysis a 'threshold' was set to exclude background voxels, only voxels above this threshold were used for analysis. This was set by selecting a region containing no cells, the average voxel intensities within this region of interest plus 3 standard deviations was then calculated by the software. Threshold values for each channel (FITC: 527/555 nm, Cy3:515/670 nm) were calculated separately. The same threshold values were used throughout the analysis. Images from three separate experiments were used for analysis. Analysis was restricted to regions containing ADAMTS13 expressing cells. The numbers of cells analysed were as follows: WT (n = 35), p.I143T (n = 29) and p.Y570C (n = 52). Values are expressed as mean ± SEM.

The TPCC gives a value for the correlation between the intensity distributions of the components (Golgi and ADAMTS13) whereas the overlap between the intensities can be described by the coefficient M_1. Here M_1 represents the sum of the intensities of the 'red' Golgi expressing voxels which are colocalised with the green ADAMTS13 voxels divided by the sum of the intensities of all the red (Golgi) voxels [32,33].

2.7. Proteasome and Lysosome Inhibition

Proteasome and lysosomes were inhibited initially in HEK 293T cells transiently transfected with WT or mutant ADAMTS13. However the results from this were unreliable due to differences in the transfection efficiency which could not be normalized using the pRL-TK vector. Instead HEK 293 cells stably expressing WT or mutant ADAMTS13 cDNA were incubated with either 10 μM MG132 (Merck Chemicals, Nottingham UK), 6 μM ALLN (Merck chemicals, prepared in DMSO), 0.1 μM Bafilomycin A1 (prepared in DMSO), or 0.1% DMSO (volume added equivalent to greatest volume of drug added). DMEM media was exchanged for OptiMEM media prior to inhibitor incubation. After 5 h cell lysate and supernatant samples were harvested and analysed. The