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Molecular characterization of p.Asp77Gly and the novel p.Ala163Val and p.Ala163Glu mutations causing protein C deficiency

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ABSTRACT

Introduction: Protein C (PC) is a major anticoagulant and numerous distinct mutations in its coding gene result in quantitative or qualitative PC deficiency with high thrombosis risk. Homozygous or compound heterozygous PC deficiency usually leads to life-threatening thrombosis in neonates.
Patients and Methods: The molecular consequences of 3 different missense mutations of two patients have been investigated. The first patient suffered from neonatal purpura fulminans and was a compound heterozygote for p.Asp77Gly and p.Ala163Glu mutations. The second patient had severe deep venous thrombosis in young adulthood and carried the p.Ala163Val mutation. The fate of mutant proteins expressed in HEK cells was monitored by ELISA, by Western blotting, by investigation of polyubiquitination and by functional assays. Their intracellular localization was examined by immunostaining and confocal laser scanning microscopy. Molecular modeling and dynamics simulations were also carried out.
Results and Conclusions: The 163Val and 163Glu mutants had undetectable levels in the culture media, showed intracellular co-localization with the 26S proteasome and were polyubiquitinated. The 77Gly mutant was secreted to the media showing similar activity as the wild type. There was no difference among intracellular PC levels of wild type and mutant proteins. The 163Val and 163Glu mutations caused significant changes in the relative positions of the EGF2 domains suggesting misfolding with the consequence of secretion defect. No major structural alteration was observed in case of 77Gly mutant; it might influence the stability of protein complexes in which PC participates and may have an impact on the clearance of PC requiring further research.

Introduction

Protein C (PC) is a zymogen; its activated form (APC) plays an important role in the regulation of blood coagulation [1,2]. APC inactivates activated factor V (FVa) and activated factor VIII (FVIIIa) by cleaving them at specific arginine residues. Free protein S (PS) and free PS together with cleaved native factor V (FV) serve as cofactors in FVa and FVIIIa inactivation, respectively. PC is a vitamin K-dependent glycoprotein, synthesized by the liver as a single chain protein. The gene encoding human PC (PROC) is located at the 2q13–q14 position, it contains nine exons and eight introns [3]. The plasma concentration of PC is 3–5 mg/L; it has a short half-life, approximately 8 hours, in the circulation. The mature 62 kDa protein is composed of a heavy (41 kDa) and a light (21 kDa) chain held together by a single disulfide bond. The domain structure of PC shows high similarity to that of other vitamin K-dependent coagulation factors. It has a pre-pro leader sequence, a gamma-carboxyglutamic acid (Gla) domain, a short amphipathic helix, two epidermal growth factor (EGF) domains, an activation

Abbreviations: PC, Protein C; APC, Activated protein C; FVa, activated factor V; FVIIIa, activated factor VIII; PS, protein S; FV, native factor V; PROC, gene for human PC; Gla, gamma-carboxyglutamic acid; EGF, epidermal growth factor; SP, serine protease; VTE, venous thromboembolism; MRI, magnetic resonance imaging; PS, protein S; Lp(a), lipoprotein a; ACA, anticardiolipin antibody; B2GPI, beta2glycoprotein I; APTT, activated partial thromboplastin time; TT, thrombin time; AT, antithrombin; LA, Lupus anticoagulant; PCR, Polymerase Chain Reaction; DNA, Dezoxyribonucleic acid; dNTP, desoxynucleotide triphosphate; DMSO, Dimethylsulphoxyde; HEK, Human embryonic kidney cells; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; CLSM, confocal laser scanning microscopy; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; DAB, 3,3′-diaminobenzidine; ER, endoplasmic reticulum; PBS, phosphate buffered saline; PPA, Protein Proximity Analyser; PPI, protein proximity index; FIXa, activated factor IX; FVII, factor VII; PME, particle mesh Ewald; DVT, deep venous thrombosis.

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peptide domain containing the Lys–Arg dipeptide released upon maturation and a catalytic or serine protease (SP) domain. To our knowledge no resolved atomic structure is available for the full zymogen PC or its activated form. A theoretical model, however has been proposed for the zymogen PC by Perera et al. [4].

The deficiency of PC is associated with increased thrombotic risk and recurrent venous thromboembolism (VTE) [5]. PC deficiency is classified as type I (quantitative, with equally decreased PC activity and antigen concentration) and type II (qualitative, with decreased activity and normal antigen level) deficiencies. Type I deficiencies mainly result from defective synthesis or secretion of the protein, while type II deficiencies are associated with impaired substrate, calcium-ion or receptor binding. In heterozygous patients PC activity values are usually between 30% and 65% and VTE often appears in early adulthood. Cases with arterial thrombosis, intracardial thrombus formation, pregnancy-associated thrombosis have also been reported [6–9]. Homozygosity or compound heterozygosity usually results in undetectable or very low PC activity, which is regularly associated with disseminated thrombosis, in the form of purpura fulminans, in newborns [10].

Most of the mutations cause type I deficiency; type II deficiency is diagnosed in approximately 10%–15% of all cases. About 250 causative mutations have been found so far; they are collected in databases, like the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk) or the ProCMD, a database and 3D web resource for protein C mutants (http://www.itb.cnr.it/procmd). In spite of the high number of PC deficiencies with accurate molecular genetic diagnosis further investigations concerning the molecular consequences of missense mutations have been carried out only in a few cases.

In our present study a newborn with purpura fulminans and a patient with deep vein thrombosis at the age of 27 were investigated. The first patient was a compound heterozygote possessing the p.Asp77Gly mutation and the novel p.Ala163Glu mutation. The second patient was heterozygous for the novel mutation p.Ala163Val. The fate of the mutant proteins was explored by in vitro expression experiments and the structural consequences of the mutations were investigated by molecular modeling and molecular dynamics simulations.

Patients/Materials and Methods

Patient Characteristics

Patient 1

A female baby was born by cesarean section at the gestational age of 36 weeks. Hydrocephalus was detected by intrauterine ultrasonography on the 33th week of gestation. The occlusion of the Sylvian aqueduct was confirmed by MRI after birth and ventriculosubgaleal shunt was inserted on the 15th day of life. Shortly after birth the newborn presented severe cyanosis and swelling of both feet. Laboratory evaluation excluded perinatal infection. Based on the coagulation tests consumption coagulopathy was diagnosed. Liver and renal functional tests were normal. On the second day of life purpura fulminans appeared on her feet, and severe PC deficiency was confirmed by laboratory methods (see details below). Fresh frozen plasma treatment was promptly initiated and was continued then, as PC concentrate was not available, warfarin therapy was introduced overlapping with low molecular weight heparin [11]. The severe skin lesions were transiently regressed; however later on dermal vascular thrombosis developed several times requiring necrectomy and her 5th right toe was resected due to ischemic necrosis. Ophthalmological examination revealed retinal detachment on both sides. At the age of 3 months renal failure and acute respiratory distress syndrome developed, atrophy of the left kidney and decreased renal vascularization at both sides were observed. The respiratory failure could not be controlled even by mechanical ventilation and the patient died. This case resembled a published PC deficiency raising the hypothesis on Sylvian duct occlusion that thrombophilia may play a role in congenital hydrocephalus [12]. The patient's parents were not consanguineous and neither the mother (23) nor the father (25) had a personal or family history of thrombosis.

Patient 2

A 50-year-old woman was sent by her general practitioner to the Outpatient Service of our University for thrombophilia screening, because of her positive history of thrombosis and suspected familiar occurrence. She had left femoral vein thrombosis during pregnancy at the age of 27. Thrombophilia screening at the age of 42 revealed FV Leiden heterozygous mutation, low level of PC activity and antigen (please see details below) elevated Lp(a) and transient positivity for anticardiolipin (ACA) and anti beta2glycoprotein I (B2GPI) antibodies. At the age of 44 years ulcerative colitis was diagnosed. She has been smoking since the age of 16 (10–15 cigarettes/day). The patient has been on vitamin K antagonist (acenocumarol) treatment since the diagnosis of PC deficiency. The patient's mother had calf vein thrombosis at the age of 38, her brother had left femoropopliteal vein thrombosis at the age of 35 and a right femoral vein thrombosis at the age of 44, the latter was complicated by pulmonary embolism. Her two daughters (30 and 33) are symptom free.

Routine Laboratory Methods

Nine volume blood was collected into evacuated tubes containing 1 volume 3.2% Na-citrate and was centrifuged at 2500 rpm, 25 °C for 20 minutes. DNA was extracted from peripheral white blood cells using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Plasma and DNA were stored at −80 °C until further use. Prothrombin time, activated partial thromboplastin time (APTT), thrombin time (TT) were performed by commercial reagents on Siemens BCS-XP coagulometer (Siemens, Marburg, Germany). Fibrinogen concentration was determined by the Clauss assay. FVIII activity was measured by APTT-based clotting assay. Thrombophilia testing was performed on the same instrument using Siemens reagents for antithrombin (AT) activity (Berichrom ATIII), for PS activity (Protein S Ac) and for PC activity (Protein C clotting reagent). Free PS antigen was determined by Liatest PS (Diagnostica Stago, Asnieres, France). PC antigen was determined by commercial ELISA (Asserachrom PC, Diagnostica Stago). Lupus anticoagulant (LA) screening was executed by the APTT-LA reagent (Diagnostica Stago), ACAs and anti-beta2-glycoprotein I antibodies were detected by Quanta Lite reagents (Innova Diagnostics Inc., USA), FV Leiden and Prothrombin 20210A polymorphisms were detected by real-time PCR and melting curve analysis on a Light Cycler 2.0 (Roche Molecular Diagnostics, Pleasanton, CA). Plasma homocysteine concentration, lipid levels, liver and kidney function tests were performed by routine laboratory methods.

Polymerase Chain Reaction (PCR) and Sequencing of the PROC Gene

All the 9 exons, exon-intron borders and 5'-flanking part of the PROC were amplified using primer sets designed at our laboratory (available on request along with the detailed protocol). Fluorescent direct sequencing was carried out in ABI PRISM 3130-Avant Genetic Analyzer (Applied Biosystems), for the evaluation Sequencing Analysis 5.4 software was used.

According to the recent guidelines amino acid numbering of PC starts at the first initiator methionine [13], however, traditional numbering started at an alanine residue, which is the first amino acid at the N-terminus of the mature protein. The pre-pro leader sequence, which is cleaved off upon maturation used to be considered as −42 to −1. Here we follow the numbering recommended by the Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis throughout the manuscript and 42 should be subtracted to convert to the traditional amino acid numbering form.