Molecular and clinical spectrum of type I plasminogen deficiency: a series of 50 patients

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Severe type I plasminogen (PLG) deficiency has been causally linked to a rare chronic inflammatory disease of the mucous membranes that may be life threatening. Here we report clinical manifestations, PLG plasma levels, and molecular genetic status of the *PLG* gene of 50 patients. The most common clinical manifestations among these patients were ligneous conjunctivitis (80%) and ligneous gingivitis (34%), followed by less common manifestations such as ligneous vaginitis (8%), and involvement of the respiratory tract (16%), the ears (14%), or the gastrointestinal tract (2%). Four patients showed congenital occlusive hydrocephalus, 2 with Dandy-Walker malformation of cerebellum. Venous thrombosis was not observed. In all patients, plasma PLG levels were markedly reduced. In 38 patients, distinct mutations in the PLG gene were identified. The most common genetic alteration was a K19E mutation found in 34% of patients. Transient in vitro expression of PLG mutants R134K, delK212, R216H, P285T, P285A, T319_N320insN, and R776H in transfected COS-7 cells revealed significantly impaired secretion and increased degradation of PLG. These results demonstrate impaired secretion of mutant PLG proteins as a common molecular pathomechanism in type I PLG deficiency. (Blood. 2006;108:3021-3026)

Introduction

Plasminogen (PLG) plays an important role in intra- and extravascular fibrinolysis, wound healing, cell migration, angiogenesis, and embryogenesis.[1] Plg is primarily synthesized by liver tissue.[2] However, other minor sources identified in mice include adrenal gland, kidney, brain, testis, heart, lung, uterus, spleen, thymus, and gut tissue.[3] In humans, the cornea has been described as an extrahepatic site of PLG synthesis.[4] Inherited PLG deficiency in humans can be divided into 2 types: true PLG deficiency (type I, or hypoplasminogenemia) and dysplasminogenemia (type II). In the former, both immunoreactive PLG level and functional activity are reduced, while the latter shows a normal or slightly reduced level of immunoreactive PLG while functional activity is significantly decreased. It has been shown by several authors since 1995 that homozygous or compound-heterozygous type I PLG deficiency is a major cause of a rare inflammatory disease affecting mainly mucous membranes in different body sites.[5,6] The most common clinical manifestation is ligneous conjunctivitis, characterized by development of fibrin-rich, woodlike ("ligneous") pseudomembranous lesions. Involvement of the cornea may result in blindness. Other, less common manifestations are ligneous gingivitis, otitis media, ligneous bronchitis and pneumonia, involvement of the gastrointestinal or female genital tract, juvenile colloid milium of the skin, and congenital occlusive hydrocephalus.[6] In severely affected patients, prognosis is poor and treatment options are few. Worldwide, more than 150 patients with this disease have been reported since 1847, the date of first description.[6,7]

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Submitted December 14, 2005; accepted June 7, 2006. Prepublished online as *Blood* First Edition Paper, July 18, 2006; DOI 10.1182/blood-2006-04-017350.

Supported in part by the Deutsche Forschungsgemeinschaft (grant Schu 560/4-3 to V.S.) and by the Medical Faculty, University of Leipzig (formel.1-59 to K.T.).

The online version of this article contains a data supplement.

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Here we report clinical findings and *PLG* gene molecular status in 50 new patients with severe type I PLG deficiency. In order to evaluate the molecular pathogenesis of type I PLG deficiency, we investigated the secretion kinetics of 9 naturally occurring PLG mutants transiently expressed in vitro in COS-7 cells.

Materials and methods

Patients, measurement of PLG levels, and molecular genetic analysis of the PLG gene

Fifty patients from 44 families with severe type I PLG deficiency were studied. Clinical data of 3 patients have already been published elsewhere.[8-10] Informed consent to sequence DNA and analyze PLG protein in plasma was obtained from 49 patients and their relatives. One patient, for whom only a 20-year-old paraffin-embedded histologic specimen of ligneous pseudomembrane was available,[9] was lost for follow-up. The protocol of the studies was approved by the local ethics committee of the University Medical School of Leipzig (registration number 025/99) and abides by the tenets of the Helsinki protocol.

PLG antigen concentration in citrated plasma was determined by enzyme-linked immunosorbent assay (ELISA, Glu-PLG-antigen ELISA-kit; Technoclone, Vienna, Austria) or by nephelometry (Dade Behring Diagnostics, Marburg, Germany). Reference range for PLG antigen in plasma was 60 to 250 mg/L (6-25 mg/dL) for both assays.

PLG functional activity in citrated plasma was determined by a chromogenic assay (Berichrom Plasminogen; Dade Behring Diagnostics; reference range, 75%-140%) or in a Hitachi 904 analyzer (Roche Diagnostics, Mannheim, Germany) using reagents from Roche Diagnostics (reference range, 70%-120%).

Genomic DNA was prepared from peripheral blood samples, or, in one case from a paraffin-embedded histologic specimen of ligneous pseudomembrane, and studied for mutations and polymorphisms in the *PLG* gene by polymerase chain reaction (PCR), single-strand conformation polymorphism (SSCP) analysis, and direct sequencing, as described previously.[11,12] 5′-Regulatory sequences of the *PLG* gene[13,14] were amplified full length by PCR and digested with *Drd*I (New England BioLabs, Frankfurt, Germany) to remove homologous sequences of PLG-related genes (*PRG-A* and *PRG-B*), followed by nested PCR and direct sequencing. Information concerning primer sequences and PCR conditions is available from the authors on request.

DNA constructs

Full-length wild-type *PLG* cDNA, a kind gift of Prof Dr T. E. Petersen, University of Aarhus,[14] Denmark, was cloned into pcDNA3 expression vector (Invitrogen, Karlsruhe, Germany). Using the Quick-Change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany), 9 different naturally occurring PLG mutations (Figure 1) identified in patients with type I PLG deficiency were introduced into the human full-length *PLG* cDNA: K19E, R134K, K212del, R216H, P285T, R285A, T319_N320insN (insertion of an asparagine between threonine-319 and asparagine-320), R471X, and R776H. Furthermore, type II PLG mutant A601T was generated similarly. The presence of the expected mutations was confirmed by direct DNA sequencing analysis using the BigDye Terminator Reaction kit (PE Applied Biosystems, Weiterstadt, Germany). Primer sequences and PCR conditions are available from the authors on request.

Cell culture and transient expression of recombinant PLG mutants

Monkey kidney COS-7 cells (LGC Promochem, Wesel, Germany) were cultured in Dulbecco minimal essential medium (DMEM; GIBCO/Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, grown at 37°C in a humidified atmosphere of 5% CO_2. Dishes (35 mm) containing nearly confluent COS-7 cells were transiently transfected with 3 μg pcDNA3 vector containing wild-type or mutant *PLG* cDNA or with vector containing no cDNA, using Lipofectamine2000 (Invitrogen). Transfection efficiency was estimated by measuring green fluorescence signal of cotransfected pEGFPc1 (0.5 μg; Clontech, Heidelberg, Germany) using a Coulter Epics XL FACS Analyzer (Beckman Coulter, Krefeld, Germany). The presence of the expected *PLG* mutations within the transfected cells was confirmed by RT-PCR and direct DNA sequencing analysis.

Pulse-chase labeling and immunoprecipitation

Twenty-four hours after transfection, cells were starved for 1 hour in methionine-cysteine–free DMEM (GIBCO/Invitrogen) containing 10% fetal calf serum, 1 mM sodium pyruvate, and 25 mM Hepes, followed by metabolic "pulse" labeling for 60 minutes with 100 μCi (3.7 MBq)/well [^{35}S]-methionine-cysteine (TRAN^{35}S-LABEL; MP Biomedicals, Eschwege, Germany). Cells were then either harvested directly or chased for the indicated times in the media described under "Cell culture and transient expression of recombinant PLG mutants," containing in addition unlabeled 10 mM methionine and 10 mM cysteine. Culture media were centrifuged to remove cell debris followed by adding 1% Triton X-100 and a protease inhibitor mixture (Complete protease inhibitor cocktail tablets; Roche Diagnostics). Labeled cells were washed with PBS and lysed for 30 minutes by shaking at 4°C in modified radioimmunoprecipitation assay (RIPA) lysis buffer containing 150 mM NaCl, 65 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% NaDOC, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, and a protease inhibitor mixture (Complete protease inhibitor cocktail tablets; Roche Diagnostics) followed by centrifugation at 18 000 *g* at 4°C for 30 minutes.

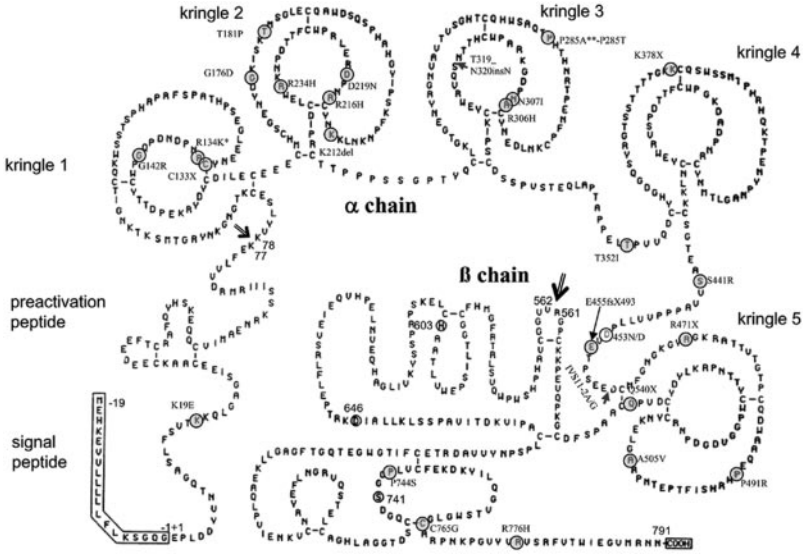

Figure 1. Structure of the PLG protein and the localization of mutations reported in this study. (Reprinted from Petersen et al[14] in modified form with permission of the American Society for Biochimistry and Molecular Biology and the Journal of Biological Chemistry.) Light gray circles indicate the position of mutations (missense and nonsense mutations, deletions); black triangles (insertions, splice-site mutations), *PLG* gene mutations; and a white circle, polymorphism 453N/D. *Mutant R134K has already been identified in a patient with ligneous conjunctivitis and vaginitis[12] and was studied here by transient in vitro expression. **Mutant P285A has been identified recently in a patient with ligneous cervicitis[15] and was studied here by transient in vitro expression.

Cell extracts and metabolically labeled culture media were immunoprecipitated by adding 5 μL of a rabbit anti–human PLG antibody (Dako, Hamburg, Germany) and 30 μL protein A–agarose (Santa Cruz Biotechnology, Heidelberg, Germany) for 20 hours at 4°C. Pellets were collected by centrifugation for 5 minutes at 500 *g*, and washed 3 times with immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% DOC, and 1% NP40, and one time with PBS. The immunoprecipitated proteins were released from protein A–agarose by boiling for 5 minutes in 30 μL 2 × SDS sample buffer and centrifuged at 500 *g* for 5 minutes. Supernatants were separated using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, followed by drying the gels for 2 hours at 80°C in a gel dryer (Bio-Rad, München, Germany), and were visualized with a Fluorescent Image Analyzer FLA-3000 (Fujifilm, Düsseldorf, Germany).

Results

Clinical findings in 50 subjects with severe type I PLG deficiency

The most common clinical manifestation was ligneous conjunctivitis (40 [80%] of 50 patients), followed by ligneous gingivitis (17 [34%] of 50 patients) (Figure 2 and Table S1, which is available on the *Blood* website; see the Supplemental Table link at the top of the online article). Seven patients suffered from both. Further manifestations included involvement of the upper and lower respiratory tract (ears, sinus, larynx, bronchi, lungs; 15 [30%] of 50 patients), the female genital tract (4 [8%] of 50 patients), the gastrointestinal tract (duodenal ulcer, n = 1), and the skin (juvenile colloid milium, n = 1), and congenital occlusive hydrocephalus (4 [8%] of 50 patients). Two of the patients exhibited Dandy-Walker malformation, a congenital hypoplasia and upward rotation of the cerebellar vermis and cystic dilation of the fourth ventricle.

The median age of first clinical manifestation was 9.75 months (range, 3 days to 61 years). The female-male ratio was 1.27:1. Venous thrombosis did not occur in any of 45 patients; in 5 patients history was unknown. One patient coincidently suffered from Ehlers-Danlos syndrome.

Seven of 31 patients from whom information was available had a body weight in the fifth percentile or lower; in 5 of 31 cases the body mass index (BMI) was of fifth percentile or lower. Four patients exhibited a body weight and BMI of 97th percentile or higher.

Patients were mainly of Turkish origin (n = 21), but also came from Germany (n = 6; 4 of them of Turkish descent), United States (n = 7), Sweden (n = 2), United Kingdom (n = 3; 2 of them of Turkish descent), Brazil (n = 6), Australia (n = 1), Switzerland (n = 1), the Netherlands (n = 1), Hungary (n = 1), and Czech Republic (n = 1). In 21 cases the parents were consanguineous; 19 of them were of Turkish descent.

Treatment of ligneous conjunctivitis included topical solutions containing corticosteroids, heparin, fresh frozen plasma, PLG, chymotrypsin, mitomycin C, and cyclosporine A, and systemic immunosuppressive drugs such as corticosteroids, azathioprine, or cyclosporine A, each of these with varying success. Surgical excision of pseudomembranes was often followed by relapse. Gingivectomy was unsuccessful in 6 patients with ligneous gingivitis, followed by a complete loss of teeth in at least 2 patients. Congenital occlusive hydrocephalus required placement of a ventriculoperitoneal (VP) shunt in 3 of 4 patients.

PLG plasma values in patients with type I PLG deficiency and in their heterozygous family members

In plasma samples available from 43 patients, PLG antigen and PLG functional activity was markedly decreased. Values ranged from less than 10 to 90 mg/L (1-9 mg/dL) for PLG antigen plasma level and 4% to 51% for functional PLG activity. Thirty-five parents and 4 heterozygous siblings, whose plasma was available, exhibited PLG antigen values between 50 to 160 mg/L (5-16 mg/dL) and functional PLG activity values between 29% to 83%, respectively (Table S1).

PLG gene mutations and polymorphisms in 50 subjects with severe type I PLG deficiency

In 31 patients, we identified homozygous or compound-heterozygous mutations in the *PLG* gene. Of these 31 patients, 1 was found to have 3 concurrent mutations. In 7 patients, only a heterozygous mutation could be detected. In 3 of these 7 patients, a combination of 3 common *PLG* gene polymorphisms has been found on the second allele. In 12 patients of Turkish origin, partly with severe clinical manifestations, no true mutation was identified within the *PLG* gene including the 5′-regulatory regions. In 9 of these cases, a homozygous combination of 3 common *PLG* gene polymorphisms, 406T>C (91N), 1490A>G (453N/D), and 2362G>T (743G), was identified, suggesting a founder effect (Table S1).

The most common genetic defect found in the *PLG* gene was the K19E mutation (17 [34%] of 50 patients), which was identified in a homozygous (6 patients) and in a compound-heterozygous (9 patients) state, as well as in 2 patients missing a second mutation. The 6 patients with homozygous K19E mutation in this series had a milder clinical course and higher residual PLG antigen and activity levels than patients with other *PLG* gene mutations. Furthermore, a homozygous K19E mutation (and similarly decreased PLG values) was found also in a currently healthy brother of one patient. Other mutations that were found in a homozygous and/or compound-heterozygous state were as follows: missense mutations, G142R (n = 1), G176D (n = 1), T181P (n = 1), R216H (n = 2), D219N (n = 1), R234H (n = 1), P285T (n = 1), R306H (n = 1), N307I (n = 1), T352I (n = 2), P491R (n = 2), A505V (n = 2), P744S (n = 1), C765G (n = 1), and R776H (n = 2); nonsense mutations, C133X (n = 1), K378X (n = 1), R471X (n = 4), and Q540X (n = 1); deletion, K212del (n = 3); insertion, T319_N320insN

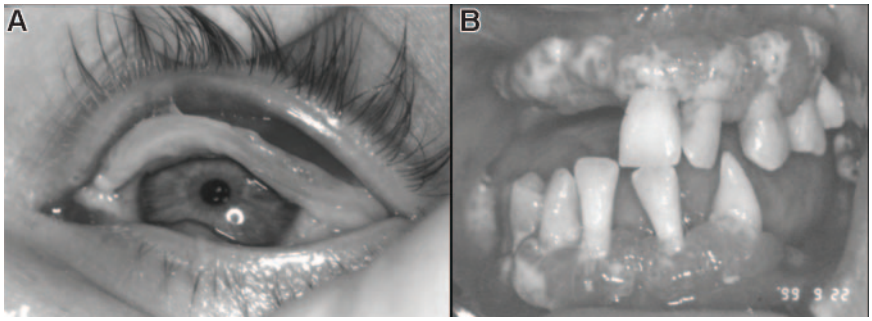

Figure 2. Clinical manifestations of type I PLG deficiency. (A) Ligneous conjunctivitis of the left upper eyelid in one of our patients. (B) Ligneous gingivitis with partial loss of teeth in one of our patients.

(n = 1); frameshift mutation, E455fsX493 (n = 2); and splice site mutation, IVS11-2A/G (n = 1). The locations of the detected mutations in the PLG molecule are shown in Figure 1. Numbering of nucleotides and amino acids were rendered according to Forsgren et al[16] and Petersen et al,[14] respectively.

Unexpectedly, 2 currently healthy sisters (11 and 14 years old) of a patient (9 years old) were also compound-heterozygous for the PLG gene mutations K378X/T319_N320insN and also had significantly decreased PLG plasma levels (patient: 3% PLG activity, healthy sisters: < 1% PLG activity of normal). Both are still clinically healthy but may eventually develop symptoms later in life.

In vitro protein expression and secretion of recombinant PLG mutants

To investigate the mechanism of reduced PLG plasma levels in PLG-deficient individuals, a pulse-chase labeling study of 9 different type I PLG mutants was performed in transiently transfected COS-7 cells. A *PLG* wild-type cDNA containing a known polymorphism was genetically engineered substituting aspartic acid (453D) with asparagine (453N) at position 453 to exclude differences in secretion due to this natural polymorphism.

Western blot analysis showed that both wild-type and mutant PLG proteins were expressed in transfected cells. In cells transfected with pcDNA3 vector alone, no material cross-reacting specifically with the PLG antibody was found. Furthermore, no band specific for plasmin has been detected in SDS-PAGE analysis. Morphologic examination of the cells showed no increased cell death.

Wild-type PLG molecules were detected in the culture medium of cells transfected with pcDNA3/wild type, and the intensity of the bands increased rapidly over chase time (Figure 3). Intracellular wild-type PLG protein gradually decreased over the chase period. Secretion kinetics of PLG wild-type protein containing the polymorphism 453N showed no difference (Figure 3). In contrast, only small amounts of PLG mutants R134K, delK212, R216H, P285T, P285A, T319_N320insN, and R776H were secreted into the culture medium during the 24-hour chase period (Figure 3B). Analysis of cell lysates showed that after 4 hours of chase time, high amounts of mutant protein could still be detected inside the transfected cells (Figure 3A). After 24 hours, the amount of intracellular mutant protein was similar to the amount of intracellular wild-type PLG protein. The total amount of radioactivity incorporated into these mutant PLG proteins at 24 hours was decreased to 32% in case of R216H, 42% in case of P285A, 46% in case of P285T, 51% in case of R134K, 57% in case of delK212, and 60% in case of R776H of the initial radioactivity, indicating instability and degradation of these proteins (Figure 3). Secretion of PLG mutant T319_N320insN was also significantly diminished after 4 hours. However, after 24 hours, no significant difference was found when compared with wild-type PLG protein secretion.

Secretion kinetics of PLG mutants K19E and R471X appeared to be normal (Figure 3). However, protein derived from PLG mutant R471X was detectable as a 62-kDa band (data not shown) due to a lack of the PLG β chain and parts of kringle 5 (Figure 1).

In vitro secretion kinetics of PLG type II mutant A601T that is associated with dysplasminogenemia in vivo were comparable with wild-type PLG protein (Figure 3).

Results of the pulse-chase experiment are not affected by transfection, transcription, and translation efficiency as they refer at each time point to the PLG protein detected at the time of the 60-minute pulse. In addition, similar amounts of coexpressed GFP protein indicate that differences in transfection efficiency are not responsible for the observed differences of wild-type and mutant PLG. Thus, these results confirm that differences in secretion between wild-type and mutant proteins are causally linked to their respective mutation.

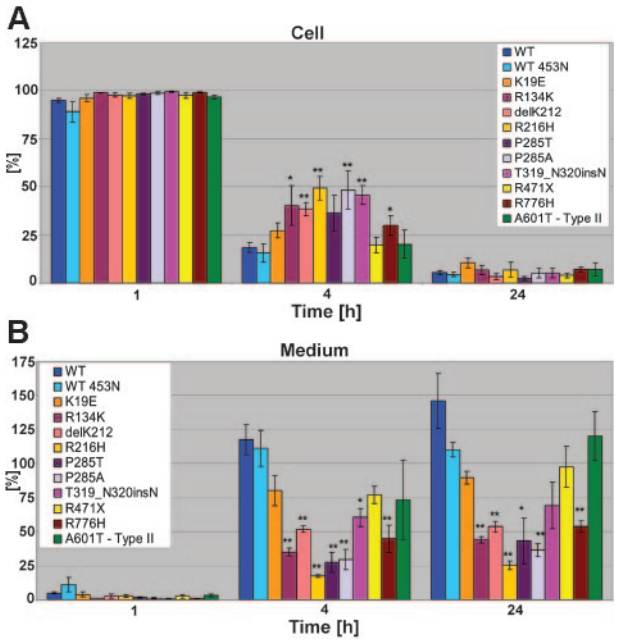

Figure 3. Quantitative analysis of pulse-chase experiments using transient expression in COS-7 cells. Radiolabeled media and cell lysates were immunoprecipitated and electrophoresed on SDS-PAGE. Radioactivity of PLG protein bands on dried gels was measured using an image analyzer. The amount of radioactive PLG protein at the beginning of the experiment was assigned a value of 100%. The graphs represent the radioactivity recovered from cell lysates (A) and medium (B) at each time point. The values represent the mean ± SEM (standard error of the mean) of 3 or 4 independent experiments. The *P* value is given as * (significant, $P < .05$) and ** (highly significant, $P < .01$).

Discussion

We present the clinical and molecular data of a series of 50 clinically symptomatic individuals with severe type I PLG deficiency. Since its original description in 1847, more than 150 cases of ligneous conjunctivitis have been reported worldwide.[6,7] In 1995, severe type I PLG deficiency was shown to be the most common cause of ligneous conjunctivitis and its associated complications.[6]

The median age of first clinical manifestation of ligneous conjunctivitis and/or other complications was 9.75 months (range, 3 days to 61 years) in our patients. Similar to the description in the literature, the disease mostly affects young children, but may also manifest later in life at any age.[6] Although type I PLG deficiency shows autosomal-recessive inheritance,[11,12,17,18] we observed a female-male ratio of 1.27:1. A female preponderance has also been reported by others[6] and might be attributed to additional factors still unknown.

In our series of patients with type I PLG deficiency, ligneous conjunctivitis was the most common clinical manifestation (80%). The second most common manifestation was ligneous gingivitis (34%). In severe cases, this can lead to complete loss of teeth.

Twenty-one patients experienced ligneous conjunctivitis alone; 19 patients had additional symptoms (ligneous gingivitis, ligneous vaginitis, otitis media, or involvement of the respiratory or gastrointestinal tract, kidneys, or skin). The reason for the clinical heterogeneity, which also occurs within the same family, is still unknown. Since it has been shown that external triggers such as injuries, lime burns, local infections, or foreign bodies, alone or in

combination with infections, may induce formation of pseudomembranes in predisposed subjects,[6] we can assume that certain physical or chemical alterations of mucous membranes may trigger the development of ligneous conjunctivitis (local irritations of conjunctivae, eg, by dust, rubbing of the eyes, or local infection), ligneous gingivitis (possibly hot and spicy food), and ligneous inflammation of the female genital tract (sexual activity).[6,15]

In general, clinical symptoms in homozygous Plg-deficient ($Plg^{-/-}$) mice are more severe compared with patients with severe type I PLG deficiency. These differences may be due to complete absence of PLG in the $Plg^{-/-}$ mice, and possibly due to other still-unknown factors. Up to 100% of $Plg^{-/-}$ mice develop ligneous conjunctivitis.[19] Lesions are indistinguishable from human ligneous conjunctivitis.[20] Additional clinical manifestations in these animals are fibrin-rich lesions in the liver (100%); the urogenital, respiratory (8%-41%), and gastrointestinal (gastric ulcers in up to 59%, ulcers of the colon in up to 24%, rectal ulcerations in 77%) tracts; and the vagina (31%).[19,21,22] Rectal prolapse develops in approximately 19% of $Plg^{-/-}$ mice.[22] Healing of skin wounds is severely impaired only in $Plg^{-/-}$ mice, but not in humans with type I PLG deficiency.[23] Furthermore, $Plg^{-/-}$ mice experience widespread thrombotic occlusions within terminal vessels and wasting.[19,21]

$Plg^{-/-}$ mice show reduced body weight due to a decreased rate of adipose tissue and whole-body fat accumulation during growth.[24] In contrast, only 7 of 31 patients of this report had a body weight lower than the fifth percentile; in 5 of 31 cases the BMI was lower than the fifth percentile. Four patients exhibited a body weight and BMI of above the 97th percentile. With regard to these parameters, the human phenotype varies considerably from the mouse model.

Four of our patients also suffered from congenital occlusive hydrocephalus and 2 revealed Dandy-Walker malformation, a hypoplasia and upward rotation of the cerebellar vermis and cystic dilation of the fourth ventricle. Congenital occlusive hydrocephalus has already been reported in at least 18 patients with ligneous conjunctivitis and type I PLG deficiency[6,25,26] as well as in $Plg^{-/-}$ mice.[20] Dandy-Walker malformation has previously been reported in 2 patients with type I PLG deficiency,[17,18] suggesting that these 2 rare complications may be causally linked to type I PLG deficiency. The pathological mechanisms remain unclear at present.

Of at least 45 of our patients with severe type I PLG deficiency, as well as at least 30 patients with homozygous or compound-heterozygous type I PLG deficiency described previously[6] and their heterozygous parents or siblings, none have developed venous thrombosis. Furthermore, 4 epidemiologic studies suggest that heterozygous type I PLG deficiency by itself is not a risk factor for venous thrombosis.[27-30] There is no explanation yet why patients with low or undetectable PLG values do not exhibit an increased risk for venous thrombosis. In $Plg^{-/-}$ mice, deficient intravascular plasmin-dependent fibrinolysis may be compensated in part by other fibrinolytic enzymes released from polymorphonuclear leukocytes.[31] This suggests that severe type I PLG deficiency impairs mainly extravascular but not intravascular fibrinolysis.

In this study, the K19E mutation was found to be the most common genetic cause (34%) of PLG type I deficiency. In an epidemiologic study of a Scottish population, heterozygous K19E mutation was also the most common genetic defect described, with a prevalence of 0.14%.[32] In general, a homozygous K19E mutation was associated with less reduced PLG plasma values, while still below normal levels, when compared with other PLG gene mutations and with a milder clinical course and may also be found in healthy subjects. Furthermore, clinical manifestations in subjects with homozygous K19E mutation may be quite heterogeneous: 4 patients suffered from ligneous conjunctivitis, and 1 patient developed isolated ligneous gingivitis. Another patient suffered from ligneous conjunctivitis and otitis media, but is healthy at present.

In 38 patients of this study, we identified a number of different mutations. To the best of our knowledge, the following PLG gene mutations are newly described: G142R, G176D, T181P, D219N, R234H, P285T, R306H, N307I, T319_N320insN, T352I, K378X, S441R, E455fsX493, IVS11-2A/G, P491R, A505V, Q540X, L650fsX652, P744S, C765G, and R776H.

In 12 Turkish patients with severe type I PLG deficiency, no *PLG* gene mutation was identified. However, 9 of these patients were homozygous for 3 PLG gene polymorphism loci (91N, 453D, 743G), suggesting a founder effect. In 6 of these cases studied so far, characterization of patient's PLG protein in plasma by isoelectric focusing (IEF) gel electrophoresis revealed a normal type A IEF pattern,[33] indicating that PLG structure is not altered (K.T. and M.G., unpublished data, 2004-2005). The cause for hypoplasminogenemia in these patients might be due to still-unidentified mutations or aberrations affecting RNA transcription in additional regulatory regions that are not amplified by our PCR primers.

The molecular basis of type I PLG deficiency is unknown for most PLG gene mutations. To date, only the missense mutation S572P has been characterized by in vitro genetic engineering techniques showing significant reduced secretion of the mutant protein by transfected cells.[34] Here, we show that other naturally occurring PLG mutants such as R134K, delK212, R216H, P285T, P285A, T319_N320insN, and R776H also exhibit significantly decreased and decelerated secretion from transfected cells in vitro. Furthermore, after a 24-hour chase period, the total amount of radioactivity incorporated in these mutant PLG proteins was less than 50% of that incorporated in wild-type protein at the same time point, indicating protein instability and degradation. PLG gene mutations R134K, delK212, T319_N310insN, and R216H are located in the vicinity of disulfide bridges located within PLG kringles 1, 2, and 3. This might lead to destabilization of the mutant PLG and impair proper kringle folding.[35] For all these mutations, we observed a good correlation between in vitro secretion patterns and plasma PLG values in patients having the same mutations in homozygous fashion.

In contrast, secretion kinetics of crippled PLG mutant R471X from transfected cells was *not* impaired. Possibly, the half-life of this protein is shortened, leading to type I PLG deficiency in vivo.

Similarly, in vitro secretion of mutant K19E protein from transfected cells was *not* significantly reduced. One might speculate that PLG mutant K19E exhibits a more open and "loose" "R"-conformation similar to Lys78-PLG (half-life, approximately 5 hours),[17] compared with the closed T conformation of Glu1-PLG (half-life, approximately 2 days),[36] due to loss of a lysine residue at position 19 in the preactivation peptide.[37-39] Possibly, this change of conformation might reduce the half-life of PLG mutant K19E when compared with Glu1-PLG, leading to decreased PLG levels in the plasma of affected patients.

In contrast, in dysplasminogenemia (type II PLG deficiency), in vitro secretion of recombinant PLG mutant A601T was shown to be normal. In accordance with these results, IEF analysis of a heterozygous subject has previously shown equal amounts of mutant A601T and wild-type PLG levels in plasma.[33]

The presence of endogenous PLG activators in mammalian cell lines, such as u-PA, converting newly synthesized PLG to plasmin is discussed controversially in the literature,[40,41] with obvious

differences between the mammalian cell lines used, and has clearly not been observed in our experiments. In addition to consistent detection of intact full-length PLG in cells and media, activation-resistant (R561S)[34] PLG molecules (WT, K19E, R216H) did not show any difference in secretion kinetics compared with their corresponding (non–activation-resistant) mutants or wild type (data not shown).

Severe type I PLG deficiency is a rare disease that occurs worldwide. Impaired secretion and increased degradation seem to be a main pathogenic mechanism in type I PLG deficiency. Current treatment options are still frustrating. Elucidation of the molecular basis of PLG deficiency, as attempted here, remains a cornerstone for research and a prerequisite for developing efficient therapy.

Acknowledgments

Full-length wild-type PLG cDNA was a kind gift of Prof Dr T. E. Petersen, Denmark.

We also wish to thank for further evaluation, diagnosis, treatment, and provision of material of their patients: Dr Erdal Ince, Dr Ulker Dogru, Dr Nejat Akar, Prof Dr Ayfer Kanpolat, Prof Dr Ergül Tuncbilek, Dr Banu Bozkurt, Prof Dr Mehmet Orhon, Prof Serdar Cintan, Prof Ahmet Efeoglu, Fabian Schmidt, Jenny Thomson, Chris Bennett, Patti Noblet, Amy Shapiro, Dr Meral Günhan, Dr Elif Ünsal, Dr Timuçin Baykul, Stephen Kaye, and Paula Bolton-Maggs.

We are grateful to Dr Sabine Heger for revision of the paper.

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