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ORIGINAL ARTICLE

Intracellular readthrough of nonsense mutations by aminoglycosides in coagulation factor VII

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Summary. *Background:* Nonsense mutations in coagulation factor (F) VII potentially cause a lethal hemorrhagic diathesis. Readthrough of nonsense mutations by aminoglycosides has been studied in a few human disease models with variable results. *Objectives:* We investigated the K316X and W364X FVII mutations, associated with intracranial hemorrhage, and their correction by aminoglycosides. The rare nonsense mutations in FVII represent favorite models to test this strategy, because even tiny increases in the amount of functional full-length protein in patients could ameliorate hemorrhagic phenotypes. *Results:* A FVII–green fluorescent protein (GFP) chimaera provided us with a fluorescent model of FVII expression in living cells. Appreciable fluorescence in cells transfected with nonsense FVII–GFP mutants was detected upon geneticin treatment, thus demonstrating suppression of premature translation termination. To investigate the rescue of FVII function, nonsense variants of the native FVII without GFP (p316X–FVII and p364X–FVII) were transfected and found to secrete low amounts of FVII (~1% of Wt–FVII activity), thus suggesting a spontaneous stop codon readthrough. Geneticin treatment of cells resulted in a significant and dose-dependent increase of secreted FVII molecules (p316X–FVII, 24 ± 12 ng mL^{-1}, 3.6 ± 0.8% of Wt–FVII activity; p364X–FVII, 26 ± 10 ng mL^{-1}, 3.7 ± 0.6%) characterized by reduced specific activity, thus indicating the synthesis of dysfunctional proteins. Similar results were observed with gentamicin, a commonly used aminoglycoside of potential interest for patient treatment. *Conclusions:* Our approach, extendable to other coagulation factors, represents an effective tool for a systematic study of the effects of aminoglycosides and neighboring sequences on nonsense codon readthrough. These results provide the rationale for a mutation-specific therapeutic approach in FVII deficiency.

Keywords: aminoglycosides, coagulation factor VII deficiency, FVII–GFP, *in vitro* expression, neonatal intracranial bleeding, nonsense mutations.

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Introduction

Factor (F) VII is a plasma vitamin K-dependent serine-protease that in association with tissue factor, an integral membrane protein exposed in the vascular lumen upon injury, triggers blood coagulation [1]. FVII shows high sequence identity with other members of the family of coagulation serine-proteases, and particularly with FIX, FX and protein C [2].

FVII deficiency is a rare hemorrhagic disorder with an autosomal recessive inheritance pattern [3]. Deficiency of FVII is associated with a variable bleeding diathesis and, as suggested by the absence of homozygous large gene deletions in humans [4,5] and knock-out experiments in mice [6], its complete absence is virtually lethal.

A large number of molecular defects has been described in FVII-deficient patients [4,5]. Those associated with the most life-threatening symptom [3], neonatal central nervous system bleeding, impair FVII biosynthesis and/or function [4,5,7–11]. Elucidation of the molecular mechanisms underlying severe FVII deficiency would favor the design of new therapeutic strategies, currently characterized by frequent administration of fresh frozen plasma or recombinant activated FVII, because of the short half-life of FVII molecules in plasma [12].

Aminoglycosides have been exploited to suppress premature termination of translation by nonsense mutations in the cystic

fibrosis transmembrane regulator [13–15], dystrophin [16], α-L-iduronidase [17], V2 vasopressin receptor [18], ATM [19], FVIII and FIX [20]. However, these studies produced extremely variable results, partially explained by differences in nonsense triplets and their sequence context, which are determinants of the spontaneous and aminoglycoside-induced ribosome readthrough [21]. The wide heterogeneity of the investigated genes and proteins, and of the experimental methodologies used to assess suppression of translation termination and rescue of protein function, further complicate the comparison of results so far produced.

In this study, through functional assays and a fluorescence-based approach, we investigated the spontaneous and aminoglycoside-mediated readthrough of nonsense mutations in FVII. These mutations, relatively frequent in severe FVIII (http://europium.csc.mrc.ac.uk) and IX (http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html) deficiencies, are virtually unique in FVII and represent favorite models to address this issue, as even tiny increases in the amount of functional full-length FVII can be quantitatively evaluated and could ameliorate hemorrhagic phenotypes in patients.

Methods

Patients and genetic analysis

Patients PFVII-type (T)1 (male, 2 years old) and PFVII-T2 (male, 14 years old) manifested intracranial bleeding at 11 days and 2 months of age respectively, and have been receiving fresh frozen plasma prophylaxis as the diagnosis of FVII deficiency was made. FVII activity and antigen levels in plasma from both patients, assayed after a 4 days wash-out replacement therapy period, were undistinguishable from those in the FVII-depleted plasma, used as a negative control.

Mutation search in the FVII gene was conducted by PCR amplification and sequencing of all coding regions, the exon/intron boundaries and the promoter region as described previously [22,23].

Expression vectors

To make the pWt–FVII–green fluorescent protein (GFP) vector, the FVII coding sequence originally cloned into pCDNA3–FVII [23] was amplified with PfuTurbo™ DNA polymerase (Stratagene, La Jolla, CA, USA) using the forward primer in the CMV promoter (5′-GGCGTGTACGGTGG-GAGGTC-3′) and the reverse primer designed to abrogate the FVII stop codon and to introduce a *Bam*HI site (5′-GTGAATGGATCCGGGAAATGGGCTCGCAG-3′). The amplified fragment was digested with *Bam*HI and cloned in frame with the coding sequence of the enhanced GFP, inserted in the pCDNA3 vector [24].

FVII mutations were introduced into pWt–FVII–GFP, pCDNA3–FVII and pCMV5–FVII using the Quick-Change™ mutagenesis kit (Stratagene) with the 5′-GCA-GTCACGGTAG GTGGGAGACTCC-3′ (K316X) and the 5′-CGGGCATCGTCAGCTGAGGCCAGGGC-3′ (W364X) forward oligonucleotides.

The plasmid expressing the cytosolic protein kinase Cβ–GFP (pPKCβ–GFP) was available in our laboratory [24].

Cell culture, transfection and treatment with aminoglycosides

BHK (baby hamster kidney) cells, known to correctly process FVII, were transiently transfected in the presence of serum-free medium (OptiMEM®; Gibco BRL, Gaithersburg, MD, USA) into 30 mm 6-well plates using FuGENE™6 (Boehringer, Mannheim, Germany) according to the manufacturer's instructions.

To test the suppression of nonsense mutations by aminoglycosides, transfected cells were cultured for 48–72 h in the presence of 0–1200 μg mL^{-1} geneticin (G418 sulfate, Gibco, Life Technologies, Gaithersburg, MD, USA), gentamicin (Gentalyn®80, Schering-Plough, Kenilworth, NJ, USA) or netilmicin (Nettacin®100, Schering-Plough, Kenilworth, NJ, USA).

Fluorescence microscopy

BHK cells were cultured on 24 mm glass coverslips and transfected with pFVII–GFP vectors as reported above. Forty-eight hours post-transfection, cells on coverslips were observed using an Axiovert 200 M fluorescence microscope (Zeiss, Oberkochen, Germany), 470 nm excitation/505 nm emission. The images were digitized using the Metamorph/Metafluor 4.5 Software (Universal Imaging Corporation, Downingtown, PA, USA). Transfected cells were treated with 200 μM digitonin in medium mimicking intracellular ion composition (140 mM KCl, 10 mM NaCl, 1 mM K_3PO_4, 5.5 mM glucose, 2 mM $MgSO_4$, 1 mM ATP, 2 mM sodium succinate, 20 mM HEPES, pH 7.05) and monitored every 5 s for 5 min using the Metamorph/Metafluor system, which provides the average cell fluorescence over time given as a percentage of the fluorescence at time 0.

In the study with aminoglycosides, untreated transfected cells were investigated in parallel with transfected cells cultured in the presence of geneticin, added at the time of transfection.

Cells transfected with pCDNA3–FVII, expressing Wt–FVII without GFP, were considered as a negative control.

Determination of FVII levels in plasma and in conditioned medium

FVII antigen FVII protein levels in plasma and in conditioned medium were measured using a commercially available ELISA kit (Asserachrom VII:Ag; Diagnostica Stago, Asnieres-sur-Seine, France). To quantify antigen levels of recombinant FVII, besides the standard curve with a serial dilution of pooled normal plasma (PNP), a reference curve with a serial dilution of recombinant Wt–FVII in conditioned medium was made. In our hands, the detection limit of the ELISA was 4 ng mL^{-1} FVII (~1% of PNP).

FVII activity FVII coagulant activity in plasma was assessed by conventional prothrombin time (PT)-based assays using FVII-depleted plasma and Thromborel (Dade Behring, Marlburg, Germany). As determined by a standard curve with a serial dilution of PNP in FVII-depleted plasma, the detection limit of this assay was ~1% of PNP.

The ability of FVII to activate its physiologic substrate FX was assessed as described previously [25]. Generation of activated FX (FXa) was monitored over time by exploiting a specific FXa fluorogenic substrate ($MeSO_2$–D–CHA–Gly–Arg–AMC-AcOH; American Diagnostica Inc., Greenwich, CT, USA).

FXa generation assays in plasma were standardized using serial dilution of PNP in FVII-depleted plasma. The assays in media were standardized using a serial dilution of recombinant Wt–FVII in medium from cells transfected with the gutted pCDNA3 or pCMV5, not encoding FVII, used as a negative control. In medium, the fluorogenic FXa generation assay was able to detect the activity produced by as low as 0.15 ng mL^{-1} Wt–FVII.

To establish the specific activity, FVII in conditioned medium was concentrated using Microcon centrifugal filters (50 kDa cut-off; Millipore, Bedford, MA, USA).

All assays have been conducted in duplicate in each sample obtained from at least five independent experiments.

Results

Mutation detection

Direct sequencing of all exons, exon–intron junctions and promoter regions in the FVII gene (Fig. 1) identified in PFVII–T1, the A10863T transversion (FVII gene numbering) [26] in homozygous condition, resulting in the K316X nonsense mutation. In PFVII–T2, the G11009A transition producing the W364X mutation and the G to T transversion at position +1 of intron 6 (IVS6 + 1G/T), predicted to abolish correct splicing, were identified.

Fluorescent model of FVII expression

To study FVII biosynthesis within living cells, a fluorescent FVII–GFP chimaera was created by expressing FVII as a fusion protein with GFP in the pCDNA3 vector (Fig. 1). Cells expressing the Wt–FVII–GFP chimaera (Fig. 1A, time 0) showed a bright fluorescence comparable to that observed (Fig. 1B, time 0) for the well-known PKCβ–GFP chimaera [24], used as a positive control. Upon treatment of cells with digitonin, which selectively perforates plasma membrane, the fluorescence of the Wt–FVII–GFP was maintained over time (Fig. 1A, time points), whereas that of the cytosolic PKCβ–GFP completely disappeared (Fig. 1B, time points).

Readthrough of nonsense mutations by geneticin in living cells

The K316X and W364X nonsense mutations were then introduced into pWt–FVII–GFP. In cells transfected with the

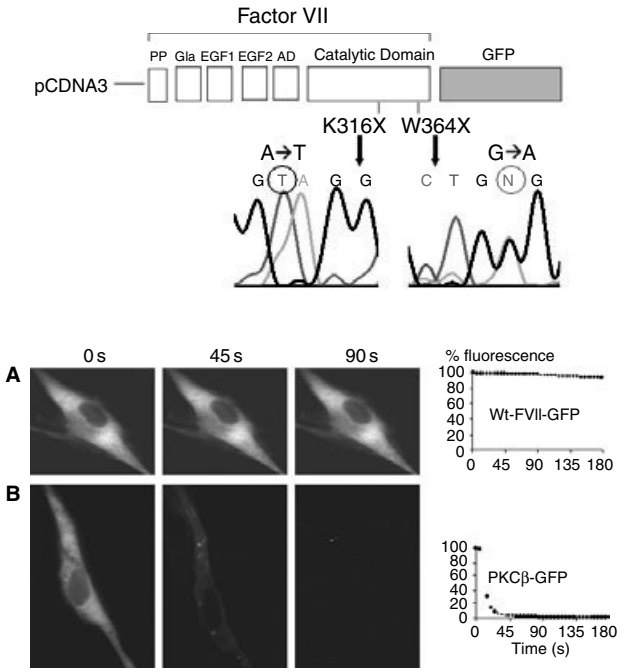

Fig. 1. Expression of factor (F) VIII–green fluorescent protein (FVII–GFP). Upper section. Schematic representation of the pFVII–GFP vector and sequence of mutations. Nucleotide changes are reported above chromatograms. P_{CMV}, cytomegalovirus promoter; PP, preproleader; GLA, γ-carboxyglutamic rich domain; EGF1 and EGF2, epidermal growth factor-like domains 1 and 2; AD, activation domain; GFP, green fluorescence protein. Lower section. Fluorescence microscopy images of cells transfected with GFP variants. Fluorescence images of cells expressing the Wt–FVII–GFP (A) and PKCβ–GFP (B) before (time 0) and 45 or 90 s after treatment with digitonin. For each construct, the cell shown is representative of at least 50 cells examined in three independent experiments. Time-lapse movies are available on request. Graphics report the average cell fluorescence over time given as a percentage of the fluorescence at time 0.

p316X–FVII–GFP and p364X–FVII–GFP variants, the fluorescence was undetectable, as in cells transfected with the non-chimaeric pWt–FVII construct (Fig. 2). Treatment of transfected cells with the aminoglycoside geneticin (400 μg mL^{-1}) induced an appreciable fluorescence for both variants (Fig. 2).

Rescue of FVII function in conditioned medium

To investigate the ability of geneticin to rescue FVII function, nonsense variants of the native FVII, without GFP, were expressed in the pCDNA3 expression system used for fluorescent studies. While the mean FVII protein levels of Wt–FVII were 120 ± 35 ng mL^{-1}, those of the 316X–FVII and 364X–FVII were below the sensitivity limit of the FVII ELISA assay (Fig. 3A). The activity of FVII in conditioned medium was investigated through fluorogenic FXa generation assays. Using this assay, the FVII activity in medium from cells transfected with pWt–FVII was appreciable whereas that from cells transfected with the p316X–FVII and p364X–FVII variants was barely detectable (Fig. 3B).

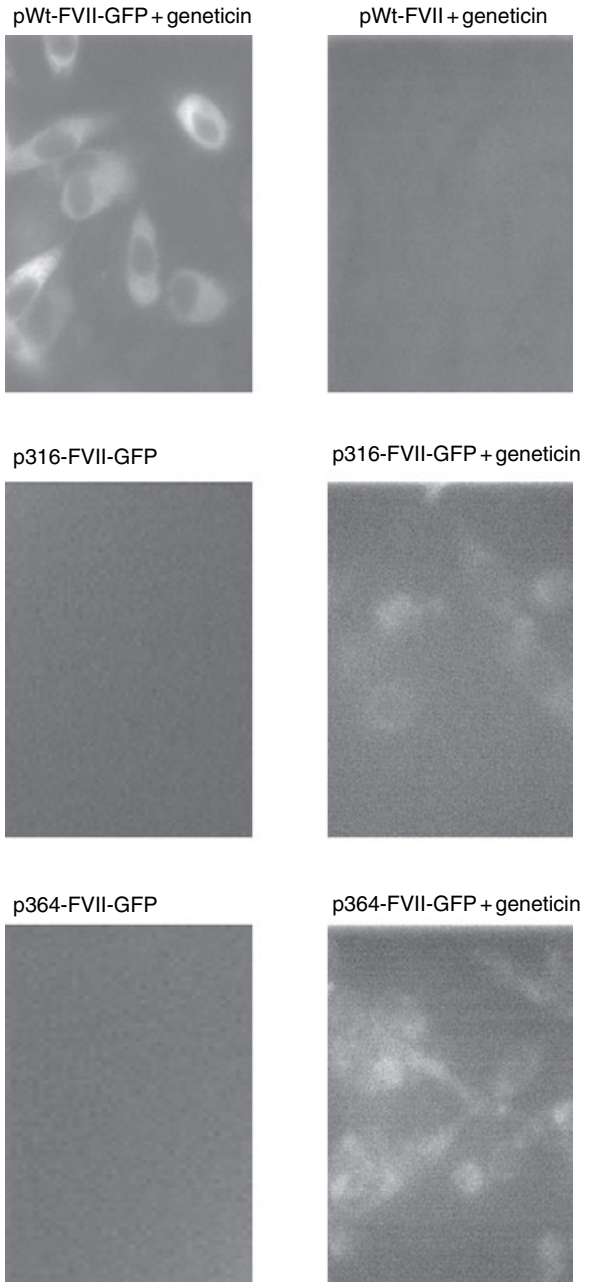

Fig. 2. Expression of FVII–GFP nonsense variants and effect of geneticin. Fluorescence microscopy images of cells transfected with pFVII–GFP variants before and after treatment with geneticin. Cells transfected with pWt–FVII–GFP and pWt–FVII treated with geneticin were used as positive and negative controls, respectively.

Treatment of transfected cells for 48 h with 400 μg mL^{-1} geneticin, which did not produce variations in expression levels of Wt–FVII, induced an increase in protein levels in conditioned medium for both 316X–FVII (24 ± 12 ng mL^{-1}) and 364X–FVII (26 ± 10 ng mL^{-1}) variants (Fig. 3A). In these conditions, FVII activity of the 316X–FVII and 364X–FVII variants was 3.6% ± 0.8% and 3.7% ± 0.6% of Wt–FVII, respectively (Fig. 3B). The addition of 1000 μg mL^{-1} geneticin did not result in a further increase in FVII activity levels.

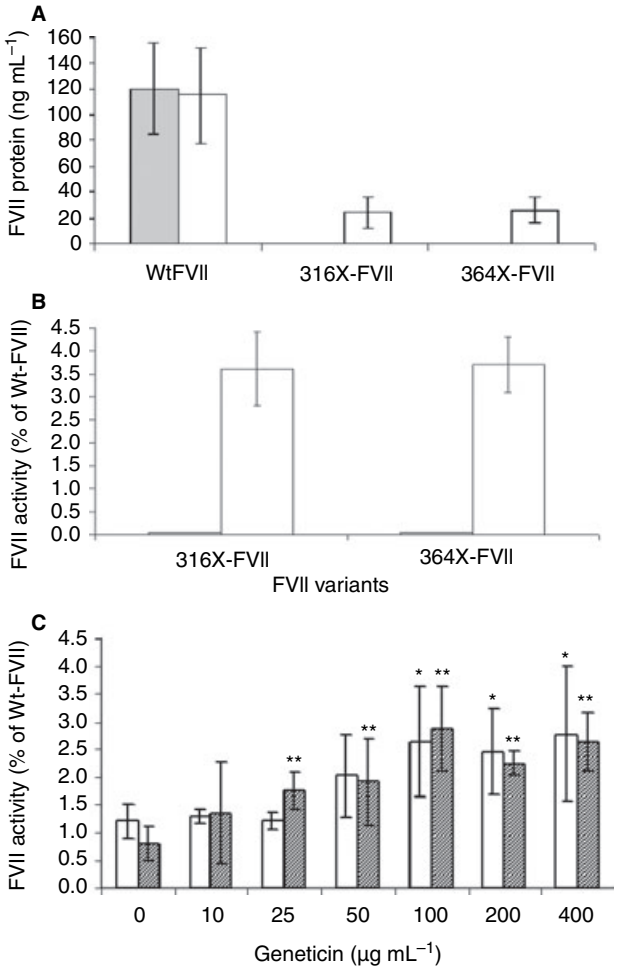

Fig. 3. Expression levels of FVII variants and effects of geneticin. FVII protein (A) and activity (B) levels of nonsense variants in conditioned medium in the pCDNA3 expression system. Mean and standard deviation of FVII levels before (grey) and upon (white) treatment with 400 μg mL^{-1} geneticin are reported. (C) Dose-dependent effect of geneticin on expression levels of 316X–FVII (white) and 364X–FVII (striped black) in the pCMV5 expression system after incubation with 0–400 μg mL^{-1} geneticin. Mean and standard deviation are reported. Statistical comparison with activities in the absence of geneticin, *=$P < 0.05$, **=$P < 0.01$.

To evaluate the dose-dependent effect of geneticin on FVII function, the nonsense variants were expressed by exploiting the pCMV5 vector that, differently from pCDNA3, is devoid of the aminoglycoside resistance cassette. In medium from cells transfected with p316X–FVII and p364X–FVII, a low FVII activity was observed (1.2% ± 0.3% and 0.8% ± 0.3% of Wt–FVII, respectively, Fig. 3C). While the mean FVII protein levels of Wt–FVII were 130.9 ± 6.8 ng mL^{-1}, those of 316X–FVII and 364X–FVII were below the sensitivity limit of the FVII ELISA assay.

Treatment of transfected cells with increasing concentrations of geneticin (0–400 μg mL^{-1}) for 48 h induced a statistically significant and dose-dependent increase in activity for both variants (Fig. 3C). The highest activity for 316X–FVII (2.6% ± 1% of Wt–FVII) and 364X–FVII (2.9% ± 0.8% of Wt–FVII) was reached at 100 μg mL^{-1} geneticin. The increase of FVII activity in these conditions was paralleled by

that in protein levels of 316X–FVII (17.7 ± 3.0 ng mL^{-1}) and 364X–FVII (8.6 ± 2.6 ng mL^{-1}).

These results prompted us to investigate in the pCMV5 system the dose-dependent correction efficacy of gentamicin and netilmicin, commercially available aminoglycoside antibiotics of potential interest for treatment of patients. Gentamicin appeared to increase, in a dose-dependent manner, FVII levels of both variants (Fig. 4). The highest activity and protein levels in medium from cells transfected with p316X–FVII (2% ± 0.6% of Wt–FVII; 10.6 ± 3.4 ng mL^{-1}) and p364X–FVII (2% ± 0.3% of Wt–FVII; 11.6 ± 0.4 ng mL^{-1}) were observed at 800 μg mL^{-1} gentamicin, and were significantly higher than those before the addition of aminoglycoside.

Netilmicin did not produce a significant effect on secreted FVII levels, at least in the range of the concentrations tested (0–1200 μg mL^{-1}).

Specific activity of FVII variants

FVII variants in conditioned medium showing the highest activity were concentrated approximately 10-fold by exploiting Millipore devices to better evaluate FVII protein and activity levels, and to permit a more reliable estimate of the specific activity [relative fluorescence units (RFU)/min/nMFVII].

The specific activity of variants was remarkably reduced as compared with that of Wt–FVII (32 916.3 ± 2066.2 RFU/min/nMFVII). In particular, the specific activity of FVII in cells transfected with p316X–FVII and p364X–FVII was 9981.2 ± 1716.2 RFU/min/nMFVII and 18 895.7 ± 3997.6 RFU/min/nMFVII upon treatment with geneticin, and 11 860.6 ± 4397.7 RFU/min/nMFVII and 7648.8 ± 1055.2 RFU/min/nMFVII upon treatment with gentamicin.

The concentration of media also permitted the evaluation of the antigen levels and of the specific activity of FVII variants in the absence of aminoglycoside treatment. In cells transfected with p316X–FVII and p364X–FVII, we estimated the presence of a low amount of FVII protein (3.4 ± 1.5 ng mL^{-1} and 3.7 ± 0.8 ng mL^{-1}) with reduced specific activity (8756.9 ± 2741.8 RFU/min/nMFVII and 8034.5 ± 2056.1 RFU/min/nMFVII, respectively).

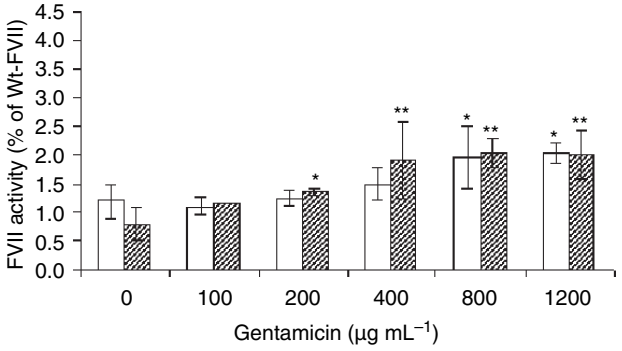

Fig. 4. Effect of gentamicin on FVII expression levels. Dose-dependent effect of gentamicin on expression levels of 316X–FVII (white) and 364X–FVII (striped black) in the pCMV5 expression system after incubation with 0–1200 μg mL^{-1} gentamicin. Mean and standard deviation are reported. Statistical comparison with activities in the absence of gentamicin, *=$P < 0.05$, **=$P < 0.01$.

Discussion

The detection of the nonsense K316X and W364X mutations in FVII-deficient patients with the most severe life-threatening hemorrhagic symptom, central nervous system bleeding, prompted us to investigate *in vitro* the aminoglycoside-mediated correction in FVII.

Nonsense changes might be associated with nonsense-mediated decay, which would vanish any correction attempt at the translation level. However, the late localization of the K316X and W364X changes makes the occurrence of this adverse event very unlikely [27].

Termination of translation at positions 316 and 364 would give rise to FVII molecules lacking the carboxyl-terminus region that is essential for secretion [28] and formation of the active site of this serine protease. Based on previous studies of nonsense codons and their gene sequence context [21], the UAGG and UGAG sequences in FVII, introduced by the naturally occurring nucleotide changes at codons 316 and 364, are predicted to undergo a spontaneous readthrough and to be relatively prone to aminoglycoside-mediated correction. However, the aminoglycoside-mediated introduction of aminoacids other than the native ones at these positions could produce proteins with altered function and/or stability, whose features are difficult to predict by inspection of the FVII structure [29,30] or molecular modeling. K316 is a surface-exposed residue belonging to the highly flexible 170s loop in the catalytic domain [31,32], involved in the allosteric conformational changes leading to the active form FVIIa. Non-conservative substitutions of the adjacent R315 by a W residue produced stable FVII molecules that partially maintained their activity (60%) [33]. On the other hand, W364 takes part in the formation of the catalytic active site and its substitution by a C residue produced a stable but inactive molecule [34].

For regulated or constitutively secreted proteins, such as FVII, the first step to reveal suppression of premature termination of translation is the investigation in the endoplasmic reticulum compartment (ER).

Expression of FVII fused with the GFP provided us with a novel fluorescent model of FVII expression within living cells. The bright and punctate fluorescence, which was maintained upon plasma membrane permeabilization with digitonin, indicated that Wt–FVII–GFP correctly entered the ER, the initial step of the secretory pathway.

While cells transfected with p316X–FVII–GFP and p364X–FVII–GFP did not show measurable fluorescence, thus indicating that the mutations prevented the GFP synthesis, the appearance of cell fluorescence after geneticin treatment demonstrated that this aminoglycoside was able to overcome both premature stop codons in living cells.

To provide a more complete picture of the premature translation termination of FVII and its suppression, the

investigation was addressed at the extracellular protein and functional levels. We exploited sensitive assays able to efficiently evaluate FVII function, an approach hardly feasible for most of the human disease proteins so far studied with aminoglycosides [13–19]. This methodological approach enabled us to reveal low but detectable FVII levels in medium from cells transfected with the native 316X–FVII and 364X–FVII variants. Taking into account that the 316X–FVII lacks most of the active site including the catalytic serine 344, this observation suggests the occurrence of spontaneous stop codon readthrough. The extent of readthrough was similar for both variants (~1%), and was consistent with that obtained with a reporter gene (~1%) bearing identical nonsense triplets [21].

Results from both pCDNA3 and pCMV5 expression systems indicated that treatment of transfected cells with the aminoglycosides geneticin or gentamicin induces a significant increase in extracellular FVII protein levels. However, the specific activity of FVII variants resulted in being remarkably reduced as compared with Wt-FVII, thus indicating the synthesis of dysfunctional FVII molecules. Although our data would suggest differences between variants, the fine evaluation of the influence of mRNA and protein features at positions 316 and 364 would require further extensive investigations by combinatorial mutagenesis.

The observed spontaneous and induced suppression of translation termination might have an implication in human pathophysiology. In patients homozygous for nonsense mutations in FVII [35], this process would explain residual FVII levels in plasma able to guarantee a minimal hemostatic function and to prevent the lethal consequences associated with this genetic condition. Rescue of FVII function by geneticin and also by the commonly used aminoglycoside antibiotic gentamicin, if obtained in patient's plasma, could significantly ameliorate the hemorrhagic phenotype [36].

In conclusion, our approach, extendable to other coagulation factors, represents an effective tool for the systematic study of the effects of aminoglycosides and neighboring sequences on nonsense codon readthrough. These results also provide the rationale for a mutation-specific therapeutic approach in FVII deficiency.

Addendum

M. Pinotti and F. Bernardi conceived the study and were responsible of the interpretation of results and the final drafting of the paper.

A. Chuansumrit, P. Charoenkwan and G. Mariani were the physicians responsible for recruitment of patients and collection of laboratory and clinical data. P. Ferraresi was responsible for the mutation detection. L. Rizzotto created the vectors, carried out the expression studies and performed functional assays. P. Pinton and R. Rizzuto gave important contribution to the design of the fluorescent model of FVII expression and were responsible for imaging and interpretation of these data. G. Marchetti critically revised the paper and gave important intellectual contribution. All authors were involved in the final revision of the article, giving contribution to the final interpretation of the data and final approval.

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