INTRACELLULAR TRAFFICKING ANALYSIS OF C111Y AND C111S MUTATIONS IDENTIFIED IN FACTOR IX FROM MEXICAN PATIENTS WITH SEVERE HEMOPHILIA B

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INTRODUCTION AND OBJECTIVE

There were characterized different mutations in the clotting factor IX gene *(FIX)* causing a severe phenotype of hemophilia B in nine Mexican patients[1]. We identified a group of punctual recurrent mutations out of CpG sites (typically "hotspots" of mutations in *FIX* gene). These mutations were relevant in terms of a *non-random mutagenesis process*, demonstrated for Latin-American populations[1]. The mutations have *functional relevance* since they involve cysteine residues, which forms a disulfide bond in a domain of the FIX protein with a high level of structural commitment to develop its normal function[1-2]. In light of this findings, the functional analysis of this group of mutations was carried out. The present study focuses on the analysis of two mutations located at the 17,747 nucleotide in the second-like epidermal growth factor (EGF2) domain of the FIX gene[3]. Both mutations change the cisteine 111 by serine (C111S) and tyrosine (C111Y).
The objective of this work is to identify the effect of these mutations in the structure-function relationship of the FIX protein through the study of their intracellular trafficking.

METHODS

For the study of the intracellular dynamics, C111 wild-type and the mutations C111S and C111Y were inserted by directed-site mutagenesis into an expression vector (pcDNA 3.1®) containing the *FIX* wild-type (wt) gene[2]. The correct insertion in the vector of C111wt and the two mutations were verified by restriction analysis and sequencing. The vectors were transfected by the liposomal-agent Fugene6® into mammalian adherent-secretory cell lines. The transfection efficency was tested by a control plasmid, which contained the green fluorescence protein (pGFP) in the cell lines CV1, PT-K2 and Cos-7. The Cos-7 cells were selected by the highest transfection efficiency (64.5%), which was evaluated by a flux-cytometry cell counter (Figure 1). Optimal transfection conditions were obtained after 48 hrs using 1µg of vector and 3µl of Fugene6®. The intracellular FIX production and its secretion to the media in the transfected cells by the vectors with C111 wild-type and the mutations C111S and C111Y were quantified by ELISA assay and also, this parameter was used to test the inhibitor effects of the intracellular transit and protein degradation[2]. The tested inhibitors were ***Brefeldin A***, which blocks protein transport from endoplasmic reticulum (ER) to the Golgi complex and causes translocation of Golgi components back to the ER; ***Cicloheximide***, which arrests the protein synthesis; ***N-Acetyl-Leu-Leu-Norleucinal (ALLN)*** **or** ***Calpain*** and ***Clasto-lactacystin β-lactone***, proteosomal inhibitors, and ***NH_4Cl*** and ***Leupeptin***, lisosomal inhibitors[2].

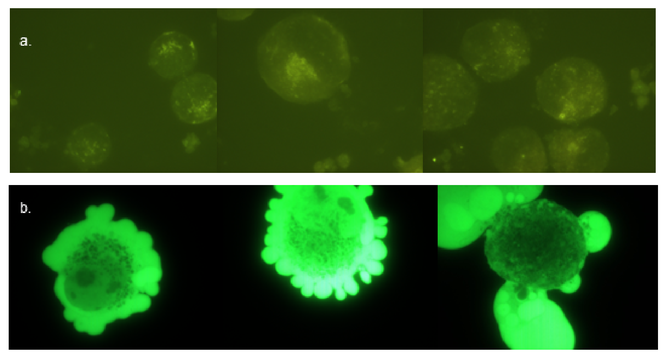

Figure 1. Photograph of COS-7 cells in fluorescence microscopy (40X). a) COS-7 cells negative control transfections without vector b) COS-7 cells transfected by a vector contanining fluorescent green protein like marker (pGFP).

RESULTS

The mutants showed a decreased FIX secretion (21%) and intracellular accumulation of 138% (C111Y) and 166% (C111S) with respect to wt factor IX. Some inhibitors caused higher intracellular accumulation, which evidenced degradation primarily in lysosomes (NH4Cl) of both mutants. The effects of the inhibitors of intracellular transit showed that the disruption of disulfide bond at C111 caused an increment of degradation of mutant proteins, mainly at lysosomes and secondly at proteosomal level. Different effects were observed on the mutants: C111S mutation showed a strong effect of Brefeldin A, suggesting an adequate transport from ER to Golgi complex in contrast to C111Y, which showed higher proteasomal degradation, evidenced by the effect of ALLN (Figure 2). We had previously presented the results of 3 trials.Here we present the final results of trials for 5 repetitions.

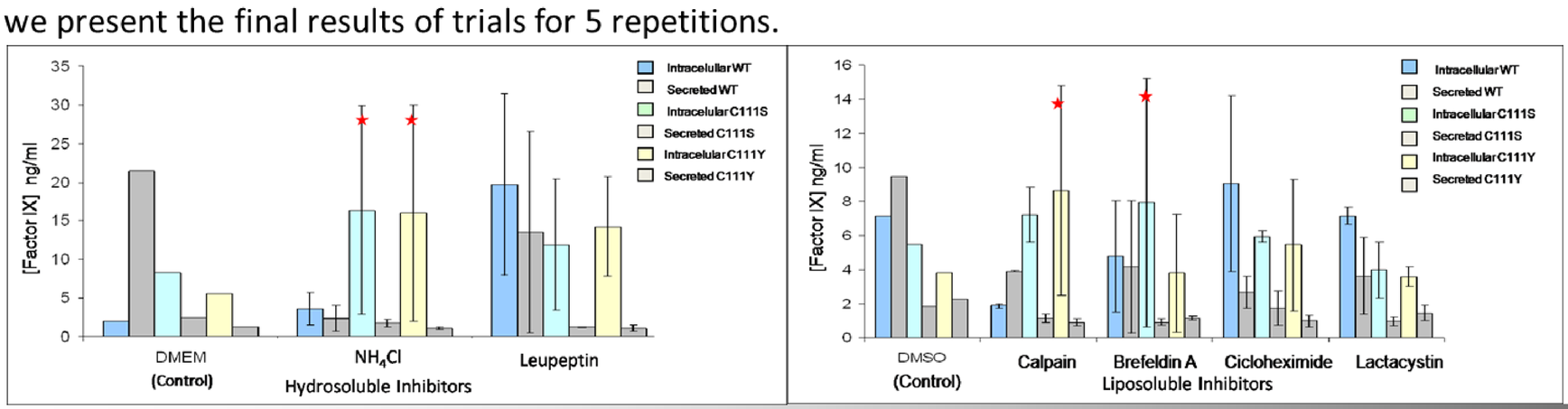

Figure 2. Effect of inhibitors of cellular trafficking on factor IX production (intracellular and secretion) of C111S and C111Y mutations of *factor IX gene. Significant effect is highlighted* by red stars.

CONCLUSIONS

The study of the effects of the inhibitors of intracellular transit through the model of transitory transfection allowed us to obtain important information related to the structure-function relationship of the mutated FIX proteins. Our findings suggest that the mutations at C111 have an important effect, mainly by the disruption of the disulfide bond at EGF2 domain, which was evident by the accumulation of the mutant proteins at intracellular level and their decreased secretion of mature FIX protein. The disruption of the disulfide bond in the mutants had an important effect on the native folding of the FIX protein and its alteration was evident by the effects on its transport through ER[4]. We suggest that a higher effect is showed when the change in the nature of the amino-acid (aa) substitution is greater respect to the polarity of SH- of Cysteine aa.

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