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A novel variant of t-PA resistant to plasminogen activator inhibitor-1; expression in CHO cells based on *In Silico* experiments

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Resistance to PAI-1 is a factor which confers clinical benefits in thrombolytic therapy. The only US FDA approved PAI-1 resistant drug is Tenecteplase®. Deletion variants of t-PA have the advantage of fewer disulfide bonds in addition to higher plasma half lives. A new variant was developed by deletion of the first three domains in t-PA in addition to substitution of KHRR 128-131 amino acids with AAAA in truncated t-PA. The specific activity of this new variant, 570 IU/μg, was found to be similar to those found in full length t-PA (Alteplase®), 580 IU/μg. A 65% and 85% residual activity after inhibition by rPAI-1 was observed for full length and truncated-mutant form, respectively. This new variant as the first PAI-1 resistant truncated t-PA may offer more advantages in clinical conditions in which high PAI-1 levels makes the thrombolytic system prone to re-occlusion. [BMB reports 2011; 44(1): 34-39]

INTRODUCTION

Coronary heart diseases including myocardial infarction have a significant portion (52%) on percentage of death caused by cardiovascular diseases (1). Accordingly, the treatment of ischemic stroke is one of the most challenging areas in medicine today. Plasminogen activators are of great clinical significance as thrombolytic agents for management of stroke and myocardial infarction. Tissue-type plasminogen activator (t-PA) is generally preferred for its more efficacy and safety compared to urokinase and streptokinase. Tissue- type plasminogen activator (t-PA) is a glycoprotein consisting of 527 amino acid residues (72 KDa) with seventeen disulfide bonds and approximate 7% carbohydrate in total molecular weight. Enhanced activity in the presence of fibrin; i.e. fibrin-specific plasminogen activation is the major advantage of t-PA over other thrombolytic agents (2, 3).

Tissue-type plasminogen activator (t-PA), mainly released by endothelial cells, cleaves the zymogen plasminogen into active plasmin. Plasmin degrades fibrin, as the major component of clots, and promotes blood reperfusion. Type-1 plasminogen-activator inhibitor (PAI-1) and a2-antiplasmin (a2-AP) can inhibit this cascade by blocking the proteolytic activity of t-PA and plasmin, respectively (3).

PAI-1 belongs to serpin family which plays its role as an ideal pseudo-substrate for target serine proteases (4, 5). The first source of PAI-1 is synthesized by endothelial cells and/or by hepatocytes. The second pool of PAI-1 is contained within the α-granules of platelets (6).

The interaction between t-PA and PAI-1 bound to fibrin is composed of three sequential steps: (a) Interaction of the catalytic site of t-PA with the reactive center of PAI-1, bound to fibrin (7, 8). (b) Conformational change in the complex that leads to loss of its affinity for fibrin. (c) Dissociation from the fibrin matrix and rebinding to fibrin subsequently; that would greatly impede t-PA activity (9).

Development of various forms of t-PA (e.g. Alteplase®, Reteplase® and Tenecteplase®) has exploited the activity of t-PA. Since the recognition that residues 296-304 are critical for the interaction of t-PA with PAI-I, several variants of t-PA with mutations or deletions in this domain have been investigated (10-12).

Tenecteplase® is the only FDA approved PAI-1 resistant thrombolytic agent. Tenecteplase® consists of two point mutations at positions 103, 117 that causes prolonged plasma half life. Furthermore, the four amino acids at position 296-299 have been replaced by four alanines which make resistance towards inhibition by PAI-1 (13). Reteplase® is a single-chain non-glycosylated deletion variant of t-PA consisting of only the second kringle and the protease domains. Since finger domain is the responsible domain for fibrin affinity, Reteplase® is characterized by reduced fibrin selectivity and causes more fibrinogen depletion than the full length forms. In the absence of fi-

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brin, Reteplase and Alteplase do not differ with respect to their activity as plasminogen activators, nor do they differ in terms of their inhibition by the PAI-1 (13-15).

In our previous study (16) the first three domains of t-PA were deleted in order to eliminate clearance sites and increase half life, furthermore, a chimeric tetra-peptide Gly-His-Arg-Pro (GHRP) with high fibrin affinity, was added to the upstream of K2S to compensate for reduction of fibrin affinity due to finger domain deletion. Therefore a novel truncated form of t-PA with improved fibrin affinity was expressed in CHO DG44 expression system (16). Aiming at further improvement in pharmacodynamic properties of the protein, a PAI-1 resistant novel form of truncated t-PA was designed based on *In Silico* modeling and then successfully expressed in CHO DG44. Targeting mutations made in this project are intended to increase the resistance of truncated t-PA to inhibition by PAI-1.

RESULTS

In Silico secondary and tertiary structure prediction

Distribution of alpha helix, extended strand, beta turn and random coil percentage in PAI resistant t-PA were 27.41, 26.9, 13.45, 32.23 compared to 26.87, 26.69, 15.84, 30.6 in native full length t-PA. The differences in secondary structure were not significant. The protein model (predicted by Modeller 9V1) was superimposed both on K2 (1TPK) and S (1BDA) PDB templates. The results of superimposing on 1bda, the functional domain of t-PA, showed the RMSD value of 0.3Å which was in acceptable criteria (Å $\leq$ 2). Therefore, the mentioned gene manipulations do not change the main structure drastically. The results of Ramachandran plot analysis also showed 3.1% of residues in outlier region; another confirmation of model validity.

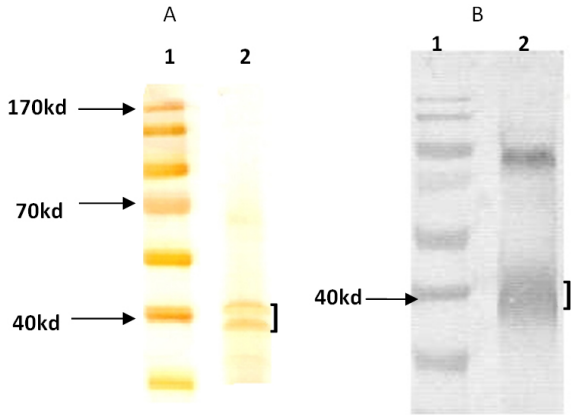

Fig. 1. (A) SDS-PAGE analysis from purified truncated-mutant t-PA, 12% EBT Silver stained, Lane 2: Purified truncated-mutant t-PA, Lane 1 : Protein marker SM0671. (B) Western Blot analysis of purified truncated-mutant t-PA, Lane 2: Truncated-Mutant t-PA Lane 1: Protein marker SM0671.

Preparing desired cassette gene

In accordance with the theoretical calculated lengths, the PCR products with 490 bp, 720 bp and 1,210 bp represent upstream fragment PCR product, downstream fragment PCR product and full length SOEn PCR product respectively. The upstream fragment consists of signal sequence, K2 domain and S domain (up to residue 163).The downstream fragment is composed of, S Domain (residues 161-394); and fused SOEn PCR product includes signal sequence, K2 and mutated S domain. The full length fragment was ligated into the pTracer-SV40 vector (Invitrogen-USA) using EcoRV and BglII restriction enzyme sites on the vector and gene of interest, respectively. Further confirmation was done by restriction analysis and sequencing (Data not shown).

Transient and stable gene transfection

CHO DG44 cells were transfected with circular plasmid. Afterwards the cells were allowed to recover for 48 hours and then assayed for GFP expression by fluorescence microscopy. The transfection efficiency was about 20% (data not shown). For stable expression CHO cells were transfected with linearized plasmid. The positive clones were selected under stringent selection pressure of Zeocin™ for several months. PCR amplification with t-PA specific primers was performed on genomic DNA of CHO in order to trace the integrated t-PA gene during 6 months post transfection. The proper 1,210 bp band related to truncated-mutant t-PA gene was a confirmation for the presence of gene in hosts genomic DNA.

Expression analysis on purified protein

SDS-PAGE and Western Blot analysis using antibody against t-PA, was performed on purified protein. Fig. 1A represents bonds related to truncated-mutant t-PA. The bands are in accordance with theoretically calculated sizes after signal sequence removal; 359 amino acids (39 KDa) and another glycosylated form with 43 KDa. This was confirmed by Western Blot analysis (Fig. 1B). These findings were similar to that reported by Burck *et al.* (17).

Electrophoretic activity analysis

As shown in Fig. 2 clear zones of proteolysis were obvious for both truncated-mutant form and commercial t-PA medicine (Alteplase®). Although, this test is not capable of revealing the exact size of the serine proteases but differences between various sizes of proteins are distinguishable. As seen in Fig. 2 truncated-mutant t-PA and full length t-PA show different places of proteolytic zone on the gel but they are both active.

Quantitative analysis

Expression level of CHO cells was determined during days 3 to 12 post culture in batch culture system with 0.3 $\times$ 10^6 per ml starting cell density. The highest expression level was obtained at day 9 (Fig. 3). It is worth mentioning that the viability reduced from 98% at day first to 65% at day 9. The best ex-

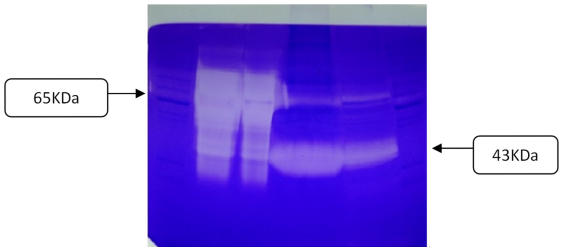

Fig. 2. Gelatin hydrolysis assay for plasminogen activators in 11% polyacrylamide gel containing gelatin and plasminogen. Lane 2 & 3 Actylase® (full length t-PA), Lane 4 & 5: Transfected CHO cells supernatant, Lane 1: Non transfected CHO cells supernatant, Lane 6: protein marker SM0671. After electrophoresis the gels were washed in Triton X-100, incubated in glycine-NaOH buffer at 37°C for 3 h and fixed, stained, and de-stained as described under Method.

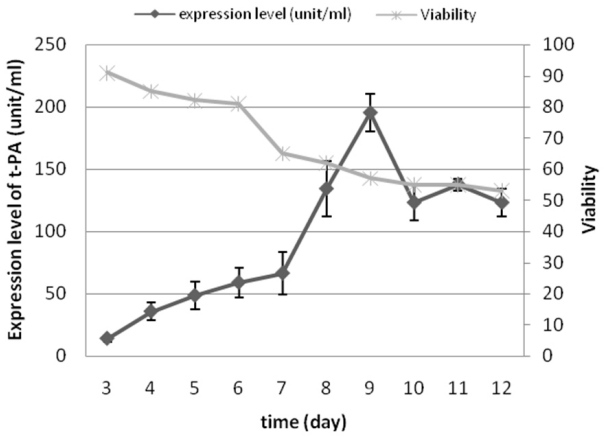

Fig. 3. Expression of truncated-mutant t-PA in CHO DG44 supernatant versus viability; Amidolytic assay to determine the expression kinetic of modified t-PA. Expression level data represent means obtained from three experiments.

pression level was determined to be 750 unit/ml on day 9 of culture in an optimized condition with 0.6 million starting cell density. The estimated specific activity was 570 IU/μg. This activity is comparable to what is reported for full length t-PA: 580 IU/μg and truncated form: 575 IU/μg (18, 19).

PAI-1 resistance assay

The inhibitory activity of human rPAI-l toward full length t-PA and truncated-mutant t-PA was determined *in vitro*. As shown in Fig. 4, PAI-1 resistant t-PA represented partial neutralization of activity (85% residual activity in 128 μM PAI) whereas the full length form was more neutralized in similar concentrations (65% of activity was preserved).

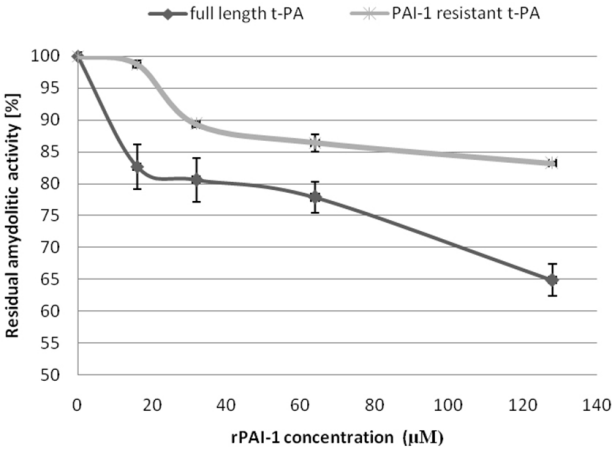

Fig. 4. The residual activity of full length and PAI-1 resistant truncated-mutant t-PA after inhibition by rPAI-1. Full length and truncated-mutant forms (3,000 IU/ml, final) were incubated for 1 hour at 25°C with rPAI-1 (16 to 128 μM).

DISCUSSION

Plasminogen activators play an important role in treating cardiovascular and cerebrovascular obstructions (20). Short plasma half life due to the need for Infusion administration, has complicated full length t-PA's clinical application (21). Improving efficacy by protein engineering is greatly promising. Principal focus of these efforts is eliminating first domains of native t-PA which are involved in hepatic clearance to reduce the size of molecule and increase half life. Deletion-variants of t-PA such as Reteplase® are not resistant to inhibition by PAI-1 (14, 22). On the other side, mutation in amino acids involved in the interaction with PAI-1 from KHRR to AAAA sounds beneficial for generating resistance to PAI-1 (23). Taking advantage of these two factors a truncated-mutant form of t-PA that is resistant to PAI-1 inhibition activity was designed based on *In Silico* analysis.

In Silico analysis showed that the molecule preserves the secondary and 3D structure of active site regions of wild type t-PA. In Ramachandran plot analysis, nearly, 3% of the residues were found to be in outlier region which is favorable compared to similar strategies (24). Furthermore, the best RMSD value (0.3 Å) was the result of superimposing our model on 1 bda template which includes 76% of total protein residues and the active site as well.

Truncated-mutant t-PA was expressed in CHO DG44 cells. The level of expression was found to be 750 IU/ml based on quantitative amidolytic activity assay. This level of expression is promising compared to enzymatic activity of produced t-PA in *E. coli* (3-7 IU/ml) (25), non-modified rCHO (50 IU/ml) (26), Aspergillus nidulans (0.1 μg/ml) (27) or Leishmania tarentolae (70 IU/ml) (28). The specific activity of truncated-mutant t-PA

(570 IU/μg) is comparable to full length t-PA (580 IU/μg) and truncated form (575 IU/μg).

As glycosylation is not necessary for biological activity, t-PA production in bacteria is still desirable and perhaps become feasible with reduction in number of disulfide bonds via protein engineering. The suggested PAI-1 resistant truncated-mutant form is promising due to potential of production in prokaryotic systems similar to its non-glycosylated commercial relative, Reteplase®.

The protein migrated with molecular weights of 39 and 43 KDa on SDS-PAGE which was expected due to glycosylation heterogeneity in CHO expression system (17). An 86kDa bond was obvious while Western Blotting was performed. The conversion of dimer form to monomer form by dissolving the protein in 8 M urea was a confirmatory data to the fact that the doubled molecular mass size was due to partial aggregation of truncated-mutant t-PA.

Biological activity was tested with electrophoretic zymography analysis. The main extracellular protease secreted by the CHO are approximately 92 or 95 KDa (29). Therefore, the presence of an obvious band of almost 43 KDa confirms that the proteolytic activity was exclusively due to truncated-mutant t-PA and not other probably existing serine proteases.

A PAI-1 resistant variant of t-PA is preferred because of increased patency as a fibrinolytic agent especially toward platelet rich thrombi, since high levels of PAI-1 contribute to the re-occlusion (12, 30). The amino acids involved in PAI-1 and t-PA interaction are residues 296 to 304 from t-PA and three acidic residues (Glu^{350}, Glu^{351} and Asp^{355}) from PAI-1. Substitution or deletion in these critical regions has been reported to enhance resistance to PAI-1 inhibition considerably (23, 31).

In the present study, a truncated-mutant variant of t-PA was constructed by mutation of residues 128-131 from KHRR to AAAA performed on truncated form of t-PA from our previous work (16). The biochemical, biological and thrombolytic properties of this protein was confirmed with different qualitative and quantitative analysis.

Purified mutant t-PA mut (K128-R131) was obtained with an amidolytic activity of 570 IU/μg against a chromogenic substrate for t-PA which is comparable to the full length t-PA; Alteplase (Actilyse)® with 580 IU/μg specific activity.

In agreement with previous findings (12, 31), the inhibition rate of mutant t-PA mut (K128-R131), by PAI-1 in purified systems was lower than that of full length t-PA. Reduction of the amidolytic activity to 65% was achieved by the addition of the same concentration of rPAI-1 (128 μM) for full length t-PA, compared to 85% for mutant t-PA confirming the partial resistance of the mutant to PAI-1. These results suggest that, in the presence of PAI-1, the thrombolytic potency of mutant t-PA mut (K128-R131) is superior to that of full length t-PA, as a result of resistance to PAI-1. Whether the clinical use of this PAI-1-resistant t-PA mutant would lead to less PAI-1-mediated re-occlusion after thrombolytic or with a better thrombolytic potency towards clots still has to be investigated.

MATERIALS AND METHODS

In Silico predictions

A dual processor windows based platform workstation was utilized to perform all the molecular modeling and structure visualizations via the Deep-view/Swiss PDB viewer program version 3.7, 2001 (www.expy.org/spdbv), VMD 1.8.7 (http://www.ks.uiuc.edu) and DPM (Double Prediction Method) (32) algorithm for secondary structure prediction. Protein Data Bank (www.rcsb.org/pdb/cgi) was the source for retrieving all atomic coordinates of t-PA domains. The model was designed using auto model package of Modeller 9v1 software. Energy minimization was done by GROMOS96 implementation of Swiss-PDB-viewer (http://iqc.ethz.ch/gromos). RMSD values were calculated by superimposing modeled protein structure on different PDB templates for Kringle 2 and serine protease domains. The Ramachandran plots analysis was performed by RAMPAGE software (http://mordred.bioc.cam.ac.uk/~rapper/rampage) to ensure model validity.

Splicing by overlap extension (SOEing) PCR

Full length human *t-PA* (GenBank accession number I01047) was amplified using the CHO 1-15 cell line (ATCC-CRL 9096) genomic DNA and cloned into pTZ57R during our previous work (28). Amino acid substitution, KHRR to AAAA, at position 128-131 was performed using SOEing PCR via appropriate primer design in a three step reaction utilizing truncated gene previously cloned in a plasmid (16) as DNA template for desired mutations. The primers used to be "SOEn" (B:AGCGA AGATTGCAGCCTGCCAGGGGTGGG and C:CAATCTTCGCT GCTGCAGCTGCCTCGCCAGGAGAAAGGTTC) are made partially complementary to one another and also include nucleotides to be mutated (11) between upstream and downstream fragments. Forward and reverse primers are as follows: A, AG ATCTGCCACCATGGATGCAATG, and D, TGGTCTAGATCA CGGTCGCATGTTG. The PCR products of these first reactions overlap via their homologous sequences at the ends which contain desired mutation (from KHRR to AAAA). The products of the first step are mixed in a SOEing reaction, and in the presence of the appropriate primers ('A' and 'D'), the recombinant product is formed. The first step PCR reactions was done in the following conditions for AB and CD fragments: First reaction: 1 μl t-PA DNA (35.7 ng/μl), 0.2 mM DNTP, 1 μl primer A (0.4 μM), 2 μl primer B (0.4 μM), 21 μl Buffer, 2 mM $MgSO_4$, Second reaction: 1 μl L t-PA DNA (37 ng/μl), 0.2 mM DNTP, 1 μl primer D (0.4 μM), 2 μl primer C (0.4 μM), 21 μl Buffer, 2 mM $MgSO_4$. The SOEing PCR reaction was as follows: Fragment AB as the first template 1 μl (62 ng/μl) and fragment CD as second template 1 μl (58 ng/μl) were combined and the PCR reaction was run in the absence of primers for 3 cycles (94°C, 4 min, 94°C, 1 min, 72°C 1 min). The procedure was completed in 94°C, 1 min, 68°C 1 min, 72°C 1 min for 30 cycles, and a final 5 min 72°C extension time in the presence of A and D primers.

PTracer-SV40-truncated mutant t-PA

The SOEn gene was cloned in EcoRV site in pTracer-SV40's (CHO expression vector). The recombinant pTracer-SV40-mutated t-PA plasmid was purified by EndoFree™ Plasmid Mega kit (Qiagen Germany) and the right orientation of the gene was confirmed by SmaI restriction enzyme digestion and sequencing analysis (data not shown).

Stable expression

Zeocin™ sensitivity of CHO DG44 cells was determined based on pTracer-SV40 manual guide. The pTracer-SV40-mutated t-PA was linearized from BglII restriction site. Linearized plasmid was then transfected to the cells by DG44 transfection kit through lipofection by Lipofectamine 2,000, and stable integrants were selected above the predetermined Zeocin™ sensitivity concentration (500 μg/ml).

Co-polymerized plasminogen polyacrylamide gel electrophoresis

Polyacrylamide gel (11%) was copolymerized with plasminogen and gelatin as previously described by Heussen *et al*. (33). The stacking gel was prepared without plasminogen and gelatin. A constant current of 8 mA was performed at 4°C for electrophoresis. The residual SDS was removed after shaking at room temperature for 1 h in 2.5% Triton X-100. The incubation of gel in 0.1 M glycine-NaOH, pH 8.3 was done for 3 h at 37°C. Finally, the gel was stained and de-stained by standard coomassie brilliant blue (R-250) dying system. In contrast to blue-paint background the location of the proteolytic zone of activity was not stained by dye.

Purification

The HiTrap™ Benzamidine FF (34) columns with specification for the purification of serine proteases were utilized for t-PA purification . The buffers referred to are as follows: 0.05 mM Tris-HCL, 0.5 M NaCl, PH 7.4 was used as binding and wash buffer. Elution buffers were performed using a step gradient of 0.5 M NaCl, 10 mM HCl, PH 2.0 and 1 M NaCl, 10 mM HCl, PH 2.0.

Quantification (amidolytic activity test)

Biopool Chromolize™ t-PA assay Kit with 0.5 unit/ml detection limit was used to measure amidolytic activity quantitatively. The kit is a biofunctional immunosorbent assay based on capturing t-PA by sp-322 monoclonal antibodies coated on the micro test wells. Absorbance at 492 nm is measured and subtracted from 405nm and the amount of developed color by chromogenic substrate S-2251 is proportional to the amount of t-PA activity in the sample.

PAI-1 resistance assay

Resistance of t-PA to inhibition by PAI-1 was assessed by previously reported methods (35, 36). Human rPAI-I in different concentrations from 0 to 128 μM was incubated with full length t-PA or PAI-1 resistant t-PA (in 3,000 IU/ml final concentration) at 25°C and residual activity was measured at the end of 1 hour incubation period. For the activity measurement the rate of the plasmin generated by the residual full length or truncated-mutant t-PA was measured, in different time intervals. The reaction is started by the addition of 100 μl of plasminogen/S-2251 mixture which is prepared immediately prior to use. The absorbance (405 nm) is then measured at different time intervals (32, 37, 38). Furthermore, the residual activity was determined using the quantitative ELISA based Chromolize™ t-PA assay kit.

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