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SITES IN TISSUE-TYPE PLASMINOGEN ACTIVATOR INVOLVED IN THE INTERACTION WITH FIBRIN, PLASMINOGEN AND LOW MOLECULAR WEIGHT LIGANDS. J.H. Verheijen (1), M.P.M. Caspers (2), G.A.W. de Munk (1), B.E. Enger-Valk (2), G.T.G. Chang (1), P.H. Pouwels (2). Gaubius Institute TNO, Leiden, the Netherlands(1) and TNO Medical Biological Laboratory, Rijswijk, the Netherlands (2).

Tissue-type plasminogen activator (t-PA) activates the proenzyme plasminogen to the active protease plasmin which degrades fibrin. The unique properties of t-PA, fibrin binding and stimulation of activity by fibrin make it an interesting molecule for specific thrombolysis. t-PA is thought to consist of five structural regions designated finger (F), growth factor (G), kringle 1 (K1), kringle 2 (K2) and protease (P). Previous studies have shown that the interaction of t-PA with fibrin is mediated by the F and K2 regions.

Mutated t-PA cDNA molecules were expressed in Chinese hamster ovary cells and t-PA analog proteins were purified from serum free culture media using affinity chromatography with immobilized monoclonal antibodies. Besides FGK1K2P (native t-PA) the following analogs were used GK1K2P, k1K2P, K2P, P, FP and FGK1k2P (k1 and k2 have partial deletions of the kringle). All the molecules comprising K2P could be stimulated in plasminogen activation activity by fibrinogen fragments comparable to normal t-PA. The activities of FP and FGK1k2P were only slightly influenced by these fragments. It was shown that the fibrin binding site in K2 was plasminogen dependent whereas that in F was not. K2 was found to contain a binding site for lysine, 6-amino-hexanoic acid but also 6-amino-hexane and thus to differ from the high affinity lysine binding sites in plasminogen.

Chemical modification of lysine and arginine residues in t-PA with citraconic anhydride and cyclohexanedione respectively, revealed no involvement of these residues in interaction with lysine or analogs nor in stimulation of activity by fibrinogen fragments. Arginine modification led to inhibition of plasminogen activation activity, both in the presence and absence of fibrinogen fragments, but the amidolytic activity as measured with a tripeptide paranitroanilide was not changed. The involvement of one or more arginine residues in interaction of t-PA with plasminogen seems likely.

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PHARMACOKINETICS OF GENETICALLY MODIFIED T-PA IN RAT. Glenn R. Larsen, Mark Metzger, Yitzak Blue and Kim Henson. Genetics Institute, 87 CambridgePark Drive, Cambridge, Massachusetts.

To better understand the non-saturable mechanism responsible for the rapid systemic clearance of t-pa, previously demonstrated in all animal species tested so far, we have synthesized a genetically altered form of t-pa and analyzed the pharmacokinetic profile and organ distribution of this mutant. The mutant t-pa has been deleted in the fibronectin finger and epidermal growth factor domain, plus genetically modified to prevent N-linked glycosylation from occurring. Mammalian cells secreting either wild-type or mutant t-pa were radiolabeled with ^{35}S-methionine. The metabolically labeled t-pa was purified to homogeneity from conditioned medium and injected into the tail vein of rats. Multiple plasma samples were taken, post infusion, and analyzed for levels of acid-precipitable radioactivity. All rats were sacrificed fifteen minutes post infusion to determine the distribution of radioactivity in the liver, kidney or lung. The clearance profile of wild-type t-pa (n=8) was biphasic with an initial alpha-phase half-life of 0.8 minutes (r=0.99) for approximately 80% of the protein and a beta-phase half-life of 12.2 minutes (r=0.97). Essentially all of the radioactivity recovered at fifteen minutes post-infusion was found in the liver. The mutant was analyzed (n=3) in identicle fashion and found to have only a single clearance phase with a 15.4 minute half-life (r=.97). Radioactivity determinations performed on organs showed proportionally less protein to be resident in the liver with a substantial increase in radioactivity observed in the kidneys. In conclusion, we have synthesized a genetic variant of t-pa which has a significantly increased half-life in the rat.

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THE ACTIVITY OF A SINGLE CHAIN rt-PA MUTANT IN A PRIMATES AND RABBITS. A. Hotchkiss, G. Knitter*, L. DeGuzman and C. Refino. Genentech, Inc., So. San Francisco, CA USA and *Western Regional Primate Institute, Seattle WA, USA

Recombinant tissue-type plasminogen activator(rt-PA) contains a plasmin sensitive peptide bond domain between amino acids (AAs) 275 and 276. Since the single chain form of rt-PA is rapidly clipped to the two chain form during thrombolysis, one way to determine the relative activities of single chain and two chain rt-PA is to produce a mutant which no longer has this clip site; therefore, a single AA substitution was made at position 275, replacing Glu for Arg (Tate, et al., Biochemistry 1987, in Press). The thrombolytic activity of this mutant (Glu275) was compared to the activity of the wild type rt-PA (Arg275) in a new primate thrombolysis model.

An arterio-venous shunt model (Hotchkiss et al, this meeting) was used. Various doses of the rt-PAs were infused over a 90 min period with 10% of the total dose administered as an initial bolus. The rate and extent of thrombolysis were quantitated by the measurement of radioactivity remaining in the thrombus at 15, 30, 60 and 90 min, using an external probe. Data were collected from 5 cynomolgus monkeys (male 5 to 6 kg). The dose response curves were used to determine the ED_{40} (dose for 40% clot lysis). The ED_{40} for Arg275 and Glu275 were 870 and 680 (ug/kg), respectively. The plasma concentrations of rt-PA were measured at plateau for various doses and the regression of the plasma concentrations (Y,ng/ml) versus the dose rates (X, ug/kg/min) was calculated. The regression equations were: Y=28+51X and Y=39+113X for Arg275 and Glu275, respectively. This indicates that the clearance rate of Glu275 is 2.2 fold slower than Arg275.

These experiments were also performed in rabbits. The ED_{40} for Arg275 and Glu275 were 740 and 660 (ug/kg) respectively. The Glu275 was cleared more slowly, having a 1.7 fold slower clearance rate.

These results support the conclusion, that the "one chain" Glu275 rt-PA is still active in vivo and that it is slightly more potent than the wild type in these animal models. Some of this increase in potency is apparently due to a slower clearance rate for the Glu275 rt-PA.

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EXPRESSION OF A RECOMBINANT ANTIBODY-TARGETED THROMBOLYTIC MOLECULE. T Quertermous, JM Schnee, MS Runge, GR Matsueda, NW Hudson, JG Seidman, and E Haber, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA.

We have recently shown that targeting tissue-type plasminogen activator (t-PA) by covalent linkage to a fibrin-specific monoclonal antibody (59D8) produces a more potent thrombolytic agent which also induces less fibrinogenolysis. A recombinant molecule encoding a t-PA-59D8 fusion protein was constructed to provide a ready source of this agent for further study, and to allow tailoring of the active moities for maximal activity. DNA sequence coding for the 59D8 heavy chain (HC) antigen combining site was cloned from a lambdaphage library by selection with a joining region probe. Gene segments coding for this cloned HC rearrangement, the amino portion of the mouse gamma 2b HC constant region, and the catalytic B chain of t-PA were joined in the pSV2-gpt expression vector. The desired coding sequence was confirmed by nucleotide sequence analysis. The construct was transfected by electroporation into 59D8 hybridoma HC loss variants. Transfectants were screened for antifibrin antibody activity. Positive clones were shown to produce mRNA which hybridized to the human t-PA gene in Northern blot analysis. Supernatants from 5 of these clones were subjected to affinity chromatography on a synthetic fibrin-like peptide-Sepharose column followed by a benzamidine-Sepharose column. Western blots of SDS polyacrylamide gels run under reducing conditions revealed binding to a 60 kd band by a monoclonal anti-human t-PA antibody, consistent with a 59D8 HC-t-PA fusion protein. Also, binding to a 25 kd band by goat anti-mouse Fab indicated the presence of 59D8 light chain. Affinity purified protein was shown to have amidolytic activity of similar potency to t-PA in a chromogenic substrate assay utilizing S-2288. Bifunctionality of the purified protein was demonstrated first by an assay which requires the protein to bind to immobilized fibrin and simultaneously exhibit activity in the S2288 assay, and second by simultaneous fibrin and iodinated anti-t-PA binding.