SUMMARY

Construction of Novel Streptokinase Derivatives with Enhanced Fibrin Specificity.

Fibrinolysis is currently the most promising method used for the treatment of myocardial infarction. One of the agents commonly used in fibrinolytic therapy is the bacterial protein, Streptokinase. Although many studies have clearly established SK as a very effective clot dissolving agent clinically, it has a few unwelcome side-effects. Fibrinolytic therapy with this agent often induces systemic fibrinogen breakdown and haemorrhagic diathesis. The major cause of this side effect is that SK starts activating plasminogen (i.e plasmin generation) immediately after entering the blood stream. Since SK lacks any intrinsic fibrin affinity, during thrombolytic therapy the plasminogen activation is not sufficiently limited to the locale of the fibrin clot. Hence, one of the challenges in the development of therapeutically improved, second-generation SK derivatives by protein engineering is to design and produce streptokinas(es) with intrinsically high fibrin specificity. Thus, the coveted property of fibrin affinity would be conferred on this 'humble' clot dissolver drug, a property that is one of the most clinically advantageous features of TPA the clot-buster drug of choice but one that is roughly 10-15 times more expensive than SK. In addition to improved clot specificity, if one could also engineer in the second-generation SK molecule an initial delay in the rates of plasminogen activation i.e. a 'latent PG activation' property, so that these streptokinase molecules are now not only targetted towards the thrombus but initially pass through the circulation in an inactive and quiet state, the net result, it hardly needs to be spelt out, will be a more sustained and localised thrombolysis with minimal systemic side-effects. This was the principal aim of the study compiled in this treatise.

The present study was based on a few semi-empirical premises that were utilised for the design of the SK-FBD chimeras so as to achieve the simultaneous incorporation into the constructs of the two desired properties viz. fibrin affinity and altered kinetics of activation of PG. By the latter attribute is meant that the virtually immediate PG activation displayed by SK upon addition into excess PG is so altered that for at least several minutes post-addition to PG, there is a lag, or delay (wherein little PG activation is observed), in the initiation of the PG activation reaction catalysed by the engineered activator protein. As briefly explained above, the direct beneficial implication of this property would be that upon injection into the body, the protein could then traverse in an inactive state through the circulatory system and bind to the pathological clot by virtue of its fibrin affinity, thereby obviating or minimizing sytemic PG activation. To achieve this 'functional objective', the design utilizes the fusion of 'minimal' regions of the fibrin bindin domains (FBDs) of human fibronectin (FN) with SK (or its partially truncated form/s) at 'strategically useful points' so as to kinetically hinder the initial interaction of SK with PG that is necessary to form the 1:1 stoichiometric activator complex.

It is known that of the 414 residues constituting native SK, only the first 15 residues and the last 31 residues are expendable, with the resultant truncated

polypeptide being nearly as active as the native full-length protein in terms of PG activation ability. Further truncation at either end results in drastic decrease in the activity associated with the molecule. It has been demonstrated that SK interacts with PG through at least two major loci, mapped between residues 16-51 and 230-290, and probably also the region in and around residues 331-332. In addition, the sequences at the C-terminal ends, especially those before the last 30-32 residues of the native sequence are important in generating the activator activity associated with the complex. Since a primary consideration in designing the SK-FBD chimeras was the engineering of a decreased, or kinetically slowed, initial PG activation rate, we reasoned that the C-terminal end could be utilized to bear the FBDs in the hybrid structures, and that the presence of such 'extra' domains in SK, already truncted to the 'most permissible limits' would suitably retard and/or delay the PG activation rates observed normally with native SK. Further, if the polypeptide in between these two distinct (heterologous) parts constituting the chimera were sufficiently flexible, proteolytic scission in this region would then result in the removal of the retarding portion from the SK and lead to a burst of PG activation after an initial delay. As discussed above, if the delay is sufficient to allow the thrombolytic agent to traverse the circulation prior to this activation, the fibrin affinity in the chimera would allow it to bind to the clot, thereby localizing the PG activation to the immediate vicinity of the thrombus.

The amino acid sequence of human FN is known to be composed of three types of homologous repeats (termed type-1, type-2 and type-3), of which the FBDs at the amino terminus of FN are made of five type-1 repeats, each approximately 50 residues long and containing two disulfide bridges. The C-terminus of FN also has three type-1 homology repeats that are involved in fibrin-FN interactions. Therefore, altogether, a large portion of the FN molecule, representing the several N- and C-terminally located FBDs, could be linked with SK if all of the fibrin-interacting regions need to be incorporated into the contemplated SK-FBD chimeras. However, such a design would produce a chimeric protein that is not only too bulky, but also decreases the probability for the polypeptide to fold into a biologically active conformation due to the presence of a large number of S-S bridges that may form non-native, intra- and inter-molecular disulfide bonds. Instead, a potentially more worthwhile proposition was considered to seek miniaturised but, nevertheless, functionally active combinations of selectively truncated regions of SK and the FBDs of human FN.

The DNA sequences encoding for the fibrin binding domains of human fibronectin were selectively amplified from known plasmids containing cloned cDNA for the FN gene. Kornblihtt et al., 1983 have cloned the cDNA encoding for the human fibronectin (FN) gene in a plasmid vector in *E. coli* (pFH1). This cDNA extends approx. 2.1 kb from the poly-A tail of the mRNA of fibronectin, around one-fourth of the estimated size of the human FN message (approx. 7900 nucleotides). By further mRNA "walking" type of experiments, these investigators

carried out the construction of longer cDNA clones using synthetic oligonucleotides complementary the DNA of clone pFH1. By this method, cDNAs corresponding to the complete FN mRNA were prepared and cloned in several vectors. One such plasmid (pFH6) contained the entire sequences coding for the FBDs of the N-terminal region of human FN. Plasmid pFH6 served as the source for these sequences in the construction of the SK-FBD hybrids. The fibrin binding domains located in the N-terminal region of human FN gene were selectively amplified by PCR using specially designed oligonucleotide primers that hybridized with DNA sequences flanking the FBD DNA segments to be amplified. These primers also contained non-hybridizing sequences at their 5'-ends that provided the intergenic sequence (i,e. between the SK and FBD DNA) as well as a restriction site through which the amplified DNA could be ligated with the SK gene in-frame in an appropriate plasmid vector.

The gene encoding for SK had been cloned in a bacterial plasmid in *E. coli* after isolation from the wild-type *S. equisimilis* genomic DNA according to the procedures earlier disclosed in Indian patent application No. DEL/94) and in recent research publications from this institute (see Pratap et at., 1996) Briefly, the complete structural gene of SK, including its promoter and upstream secretory signal sequence, was first cloned in the well-known cloning vehicle, plasmid pBR 322, by first constructing a genomic library of *S. equisimilis* H46A strain DNA (Accession No. MTCC 389) in the cosmid vector pHC 79, employing established recombinant DNA procedures. The clones so obtained were then screened by the plasminogen-overlay method to identify SK-producer clones, and the plasmid DNAs from such clones were then analyzed for the presence of heterologous, *S. equisimilis* -derived DNA (inserts). The inserts from a few positive clones were then digested with restriction enzymes Hind III and Pst I separately (to isolate smaller fragments carrying the SK gene), and the restriction fragments were ligated with pBR 322 at the respective R.E. sites. The transformants were again screened for SK-producer clones using the plasminogen-overlay method, and positive clones were isolated, and DNA from these were analyzed with respect to insert size as for the cosmid clones previously. The plasmid carrying the smallest insert from the SK-secreting clones identified by the plasminogen overlay procedure was taken up for further genetic manipulation. This plasmid, designated pJKD-8, carried the SK gene under its own promoter and secretory signals. The SK gene encoding for mature SK i.e. devoid of its 5'- regulatory and secretory sequences was then selectively amplified by PCR and cloned in a pBR-based plasmid vector; details of this procedure have been recently published (Pratap et al., 1996). In the present study, attempts were made to develop an *E. coli* -based hyper-expression system for SK using a number of well-known plasmid vector systems based on strong promoters viz., trc and phage T7 RNA polymerase. In both systems, however, relatively low levels of expression of SK were observed. In order to optimise the SK expression, one of these (T7 RNA polymerase based plasmid vector) was further taken up for more rigorous analysis. When the DNA corresponding to the ORF of

SK was modified in a translationally silent manner in order to reduce the stability of its transcript particularly at its 5'-coding end, followed by the cloning of this modified SK gene under the control of the T7 RNA polymerase promoter in plasmid vector pET23(d), the expression of large quantities of biologically active SK intracellularly in *E. coli* was observed.

Following the successful development of a hyper-expression system for SK in the present study, a novel chimeric gene between SK and FBDs of human fibronectin was made by translational in-frame fusion of DNAs encoding SK devoid of its 31 C-terminal amino acid residues, linked to DNA encoding two of the FBDs (the 4 and 5 domains) through inter-genic sequences encoding flexible polypeptide linkages. Thus, a hybrid SK-FBD was generated that contained a C-terminally truncated SK portion and the most important and relevant regions of the FN gene with respect to fibrin interactions. Another combinantion was also designed that had, instead of the FBD 4 and 5 domains, the FBD 1 and 2 domains fused at the C-terminal end of the SK gene.

The cloned hybrid genes were then expressed in *E. coli* under the strong T7 RNA polymerase promoter so as to produce large quantities of the chimeric polypeptides intracellularly. The proteins were then isolated from the *E. coli* cells and subjected to a process of purification and oxidative refolding to a biologically active form. Both polypeptides could refold to forms that could bind to fibrin with high affinity. On the other hand, native SK, or rSK expressed in *E. coli*, had no discernable binding with fibrin under these conditions. Detailed analysis of the properties of these chimeric proteins was then carried out. These studies established that these chimeras indeed possessed the functional properties expected from their design i.e. plasminogen activation ability as well as fibrin affinity. Remarkably, kinetic analyses showed that they also displayed the additional desired property of a very slow initial kinetics of PG activation. Unlike native SK or *E. coli* - expressed rSK, which showed normal PG activation kinetics marked by immediate initiation of the conversion of PG to PN upon addition of SK to the former, the chimeric SK-FBD(1,2) and SK-FBD(4,5) proteins showed a lag of approximately 10 minutes. After this period, high (i.e. 'normal') rates of PG activation similar to those seen with native SK were observed. The time-course of actual fibrin clot dissolution by the chimeric constructs in plasma mileaus also clearly demonstrated the presence of a distinct 10-12 minutes' lag. However, after this period, clot dissolution proceeded as in the case of native/rSK controls.

The mechanism underlying the altered PG activation kinetics was also investigated. It was observed that the lag period in PG activation by the SK-FBD chimeras could be progressively shortened by gradually increasing the free PN content of the PG used for the activation assays. Conversely, the lag period could be increased (to nearly 35 min from 10-12 min) by decreasing the PN content of PG by passage of the latter through soybean trypsin inhibitor-agarose, an affinity matrix that selectively absorbs free plasmin. Thus, free PN sems to mediate the activation

of the SK-FBD chimeras from their initially inactive forms that have little or no PG activation ability of their own. That the abolishment of lag, and the consequent activation of the chimeric polypeptides co-incides with the proteolytic cleavage of these proteins was clearly revealed by SDS-PAGE analyses of samples withdrawn during the time-course of activation of equimolar mixtures of SK-FBDs and PG.

Our studies unequivocally establish that both the SK-FBD constructs indeed possessed the functional properties expected from their design i.e. plasminogen activation ability as well as fibrin affinity together with the property of latent activation of PG. However, detailed *in vivo* studies in suitable animal models need to be first carried out to derive the full clinical benefits of these investigations.
