These molecules are as active in fibrin clot lysis as the native protein. However, it is remarkable that the fibrinogen (Fg) degradation – both in terms of the kinetics as well as that associated with the CL_{50} time-point – associated with the action of the various proteins are quite different. Whereas in case of nSK, extensive degradation of plasma Fg ensued (beginning with the administration of the protein), much lower degradation was seen in case of FBD(4,5)-SK and FBD(4,5)-SK-FBD(4,5), especially the latter (see Table 4). These data clearly establish that PG activation by the two protein engineered derivatives, particularly FBD(4,5)-SK-FBD(4,5), is more fibrin selective (see Fig.39- 41). Thus, if the dose of this agent is not allowed to exceed a lower limit, it is possible that effective fibrin degradation may be consistent with minimal systemic Fg degradation. However, extensive in vivo experiments are needed to establish the toxicity and efficacy characteristics of the new proteins, after which these can be taken up for human trials. The results so far, as presented in this treatise, offer a convincing cause to be optimistic.

Streptokinase is a plasminogen activator widely used in treating circulatory disorders, including myocardial infarction (heart attacks), that are produced as a result of impedence of blood flow by intravascular fibrin clots. The use of plasminogen activators such as streptokinase, tissue plasminogen activator (TPA) and urokinase (UK), has revolutionized the treatment of this modern malady, resulting in the saving of 30-40 percent lives if administered soon after the first onset of symptoms. In developing societies too, heart-related ailments are on a rapid increase, and the current demand in India for clot buster drugs, mainly SK, is estimated to be around Rs 50-60 crore per annum. Asians are now recognized to be particularly vulnerable to cardiac maladies, and nearly 25 lakh deaths annually occur in India currently solely due to heart associated diseases.

Streptokinase, due to its lower much cost compared to TPA and UK, remains the clot buster drug of choice for relatively poor countries like India. It is also recognised, particularly keeping the current 'globalisation' scenario, that there is an urgent need to develop second-generation improved derivatives of streptokinase that have clinically beneficial functional features engineered into them so as to avoid the side-effects of thrombolytic therapy. 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, 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 targeted towards the thrombus but initially pass through the circulation in an inactive and quiet state, the net result 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.

Since structural information of a high resolution nature on the mechanism of action of SK is relatively limited, there was a need to 'utilize' a high degree of empirical logic essentially based on the known biochemical mechanism of action of SK particularly its mode of interaction with both 'partner' and 'substrate' human PG. Fortunately, studies carried out in parallel to the present one in the laboratory (Cf. Nihalani and Sahni, 1995; Nihalani et al., 1997, 1998; Chaudhary et al., 1999; Dhar et al., 2002) revealed early on vital insights as to the 'game plan' one may utilize to design second generation SK molecules with improved fibrin affinity and specificity. Thus, the present study was based on a few key 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. 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 truncated 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. The gene encoding for SK had earlier been cloned in a bacterial expression plasmid in *E. coli* so as to obtain this protein intracellularly in large amounts. Following the successful development of a hyper-expression system for SK, a novel chimeric gene between SK and FBDs of human fibronectin was made by translational in-frame fusion of DNAs encoding SK linked to DNA encoding two of the FBDs (the 4 and 5 domains) through inter-genic sequences encoding flexible polypeptide linkages. Thus, a hybrid FBD-SK was generated that contained a SK portion and the most

important and relevant regions of the FN gene with respect to fibrin interactions at its N-terminal end. Another combinantion was also designed that had, instead of one set of FBD 4 and 5 domains fused at the N-terminal end, another set simultaneously fused at the C-terminal end as well.

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 FBD(4,5)-SK and FBD(4,5)-SK-FBD(4,5) proteins showed a lag of approximately 10 minutes and 25 minutes, respectively. After this period, high (i.e. 'normal') rates of PG activation similar to those seen with native SK were observed.

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 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 seems 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 coincides 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 chimeric 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. When the human fibrin clot dissolution properties of these derivatives were compared with each other and with native SK, it was observed that these were capable of degrading the clots with virtually he same efficiency as the native protein. Remarkably, however, the extent of plasma fibrinogen degradation (a reliable indicator of the 'non-specific' and systemic proteolysis seen with a clot buster agent) was much lower as compared to native SK. These findings clearly establish the derivatives designed, expressed and studied in this work [particularly the 'double' hybrid, FBD(4,5)-SK-FBD(4,5)] to be of potential benefit in clinical settings. However, detailed *in vivo* studies in suitable animal models, toxicity and clinical trials need to be first carried out to derive the full therapeutic benefits of these investigations.