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Streptokinase—the drug of choice for thrombolytic therapy

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Abstract Thrombosis, the blockage of blood vessels with clots, can lead to acute myocardial infarction and ischemic stroke, both leading causes of death. Other than surgical interventions to remove or by pass the blockage, or the generation of collateral vessels to provide a new blood supply, the only treatment available is the administration of thrombolytic agents to dissolve the blood clot. This article describes a comprehensive review of streptokinase (SK). We discuss the biochemistry and molecular biology of SK, describing the mechanism of action, structures, confirmational properties, immunogenecity, chemical modification, and cloning and expression. The production and physico-chemical properties of this SK are also discussed. In this review, considering the properties and characteristics of SK that make it the drug of choice for thrombolytic therapy.

Keywords Streptokinase (SK) · Plasminogen activators · Fibrinolysis · Thrombolytic therapy

Introduction

In recent years, thrombolytic therapy with fibrinolytic (thrombolytic) agents has revolutionized the treatment of diverse circulatory disorders such as pulmonary embolism, deep-vein thrombosis and myocardial infarction [1]. These circulatory disorders are increasingly becoming the leading causes of mortality in modern societies worldwide. Thrombolytic agents have the unique ability to activate the components intrinsic to the fibrinolytic system, resulting in the degradation of blood clots, which restores blood flow through the occluded vessels [2, 3].

The fibrinolytic agents commonly used in thrombolytic therapy are streptokinase (SK), urokinase (UK) and tissue type plasminogen activator (TPA). These agents are commonly referred to as plasminogen activators, since their mode of action is through the conversion of the enzymatically inert plasminogen (PG) of the fibrinolytic system to an active protease, plasmin (PN), that dissolves the fibrin clots and solubilises degradation products, which can be removed by the phagocytes.

This helps to restore blood flow through the occluded vessel. Unlike UK and TPA, which themselves proteases, SK possesses no enzymatic activity of its own. Rather, it acquires the highly specific PG activating property indirectly, by first forming a high-affinity 1:1 stoichiometric complex with PG or PN. The resultant activator complex is a highly specific protease, which converts other PG molecules to proteolytically active PN through a series of biochemically distinct steps [4].

The choice of a thrombolytic agent during therapy is dictated by a number of factors, which depends essentially upon the relative merits and demerits of individual PG activators. These include the cost of the drug, the side-effects and their severity, in vivo stability and specificity towards fibrin clots and immunological reactivity [5]. The TPA and UK

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despite being a relatively immunologically inert when compared to SK, they possess significantly lower in vivo half-lives. On the other hand, both TPA and UK are considerably more expensive than SK. Therefore, SK is the drug of choice in thrombolytic treatment.

Among the three main thrombolytic agents used clinically, SK has gained wide acceptance, particularly in the developing countries due to its cost effectiveness. In the treatment of myocardial infarction and in terms of therapeutic efficacy, SK is more acceptable over the other two clot dissolvers [6]. In addition, the availability of SK at low cost makes it the drug of choice in most clinical situations, particularly in economically weaker countries. However, the immunogenic nature of SK may occasionally lead to severe allergic reactions, including urticaria, itching, flushing, nausea etc, thus restricting the administration of multiple doses of SK in thrombolytic therapy [2, 5].

To overcome some of the limitations associated with the thrombolytic agents, particularly with SK, the second generation derivatives of SK are expected to develop [7].

Fibrinolytic system

Extensive studies have been made over the last 25 years to understand the physiology of the fibrin-clot formation [8–10].

The fibrinolytic system and its constituents directly responsible for the dissolution of the fibrin clot [9, 11] are briefly described in Fig. 1.

Fibrinolysis

Fibrinolysis refers to the dissolution of the fibrin blood clot by an enzyme system present in the blood of all mammalian species [4]. The fibrinolytic system consists of the plasma zymogen, plasminogen; its activated product, the proteolytic enzyme, plasmin; activators of plasminogen; inhibitors of both plasmin and plasminogen activators and fibrinogen and fibrin.

The basic reaction of the plasma fibrinolytic system is the conversion of a plasminogen to the active proteolytic enzyme plasmin, by a limited proteolytic cleavage mediated by different plasminogen activators [12]. The plasminogen activators are synthesized and released from endothelial cells and other tissues. Plasmin has the capacity to hydrolyse fibrin and various plasma coagulation proteins, including fibrinogen. The activitiy of the fibrinolytic system is modulated by inhibitors that inhibit both plasminogen activators and the proteolytic effect of plasmin. The main players of the fibrinolytic system are plasminogen itself, the zymogen of a trypsin like serine protease, two activators of plasminogen and three protease inhibitors [13].

The major two activators that occur in the circulating blood are: the tissue TPA and the urinary type plasminogen activator (U-PA), also called urokinase.

Plasminogen

Human plasminogen (Fibrinolysin) is a single chain glycoprotein consisting of 790 amino acids and exists in

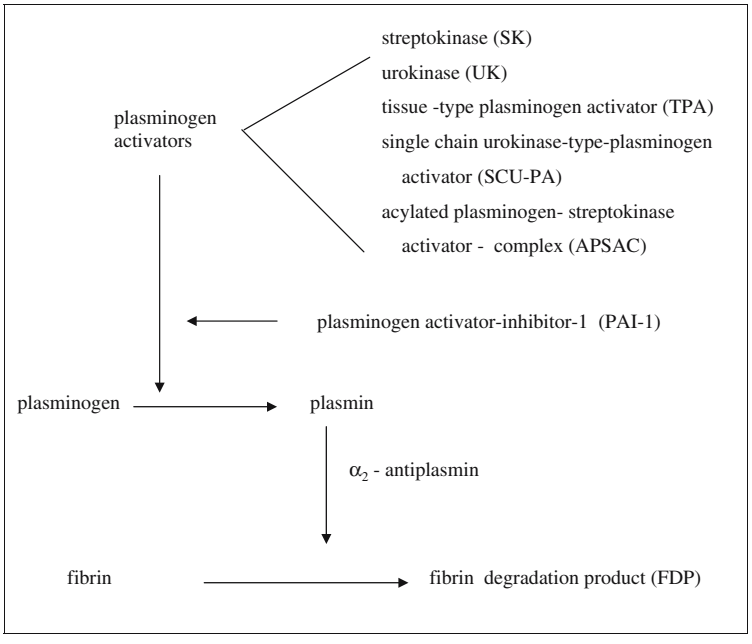


Fig. 1 Schematic representation of the fibrinolytic system

two major forms having molecular weights 92 and 94 kDa [14, 15]. These two molecular forms of HPG differ in their chemical as well as physical properties. Both the forms have Glu at their N-terminal and Asn at the C-terminal amino acid [14, 16]. It is produced primarily in liver but also present in other cells and in the extra vascular space of most tissues.

Plasminogen is comprised of 5 ''Kringle'' domains which are present between the residues Tyr_{79} and Arg_{560} which on the basis of amino acid sequence homology with kringle structures of prothrombin [17]. Each of these kringle domains is stabilized by three disulfide bonds and are involved in the binding of HPG to fibrin as well as antiplasmin [4]. It spans 53.5 kb and therefore, it is the largest gene among the constituent of the fibrinolytic system. N-terminal position of the molecule, on which five kringle domains are located is known as A-chain and the C-terminal carries the catalytic domain, known as B-chain.

Plasmin (PN) is a two-chain molecule (comprising of a heavy and a light chain), with molecular weight 85 kDa stabilized by two disulfide bonds [4]. Native plasmin is formed as a consequence of the cleavage of at least two peptide bonds in the PG molecule. The cleavage at Arg_{560}–Val_{561} is essential for the catalytic activity to be expressed. Here, no peptides are released, since the PN molecular is stabilized by disulfide linkages at Cys_{557}–Lys_{565} and Cys_{547}–Cys_{665}. As a consequence of PN formation, autolysis of Lys_{76}–Lys_{77} occurs, with covalent liberation of the amino terminal 76 residues [4]. This PN is termed as Lys_{77} PN [15]. Summaria and Robbins [14] have shown that the light chain region of this PN is responsible for the complex formation with SK. In general, the active site of PN possesses trypsin like specificity and thus hydrolyses proteins and peptides at the lysyl and arginyl bonds. In the fibrinolytic system, PN, a serine protease is responsible for the degradation of both fibrinogen as well as fibrin [18]. It may also degrade other plasma proteins such as factors V, VIII, IX, XI and XII, insulin, growth hormones and many others [2, 11]. This indeed is one of the unwanted results of the generalized PG activation seen consequent to the thrombolytic therapy, which accounts for the systemic depletion of several plasma proteins in the aftermath of the administration of clot dissolver drugs [9].

Fibrinogen is composed of three chains, A-α, B-β and gamma, stabilized by disulfide bonds. The conversion of human fibrinogen to fibrin is catalyzed by the serine protease thrombin. Thrombin catalyzes cleavage of fibrinopeptides A (16 residues) and B (14 residues) from the amino terminus of the A-α and B-β chains, respectively, yielding the α and β chains of the fibrin monomer [19]. As a result of liberation of these small peptides the fibrin monomer polymerizes, through non-covalent interactions, to yield a form of fibrin. Thrombin also catalyzes activation of factor XIII (fibrin-stabilizing factor), which then catalyzes formation of ε-(γ-glutamyl) lysine bonds between the monomers of fibrin [4].

PN degrades fibrinogen or fibrin initially from the carboxy terminal end of the A-α chain to form fragment X. Subsequent asymmetric degradation at the amino terminal of the molecule generates fragments Y and D. Further digestion by PN of fragment Y results in the formation of an additional D fragment and a fragment E [4, 9].

Fibrinolytic system inhibitors

Natural inhibition of the fibrinolytic system occurs both at the level of plasminogen activation and also at the level of plasmin action [3, 20, 21]. The inhibitor which acts at the level of plasminogen activator is called plasminogen activator inhibitor-1 (PAI-1). The inhibitors which act at the level of plasmin are many, the most important being alpha-2-antiplasmin and alpha-2-macroglobulin [22].

Plasminogen activator inhibitor-1

Plasminogen activator inhibitor-1 is a fast acting inhibitor of TPA and UK [23], found at low concentrations in the blood. It is a serpin consisting of 379 amino acids with Arg_{346}–Met_{347} as the active sites [20].

Alpha-2-antiplasmin

It is a single chain glycoprotein of the serine protease inhibitor (serpin) superfamily, with molecular weight of about 70 kDa [5, 24]. It is composed of 452 amino acids with Arg_{364}–Met_{365} as the reactive sites [25]. It inhibits PN activity by forming a rapid, enzymatically inactive stoichiometric 1:1 irreversible complex with PN [24, 26]. Alpha-2-antiplasmin reacts very rapidly with plasmin in two steps, first to form a reversible but inactive complex, which then slowly converted into an irreversible complex. The first step of the reaction depends on the presence of free lysine-binding sites and a free active centre in the plasmin molecule [24]. Although α-2-antiplasmin is presumed to be the primary inhibitor of PN activity [24]. The complex of PN with SK is less reactive towards α-2-antiplasmin than PN [27].

Alpha-2-macroglobulin

The native molecule of α-2-macroglobulin, molecular weight 726 kDa is a tetramer, comprised of four identical subunits of 185 kDa each [28]. Under certain conditions PN may also react with α-2-macroglobulin [29]. Alpha-2-macroglobulin is an effective, although slow inhibitor of PN activity [22]. It has been demonstrated through in vivo studies that the complex of PN with PG activators such as SK when injected intravenously into mice, reacted rapidly with α-2-macroglobulin and was cleared from the circulation within 10 min [30].

Plasminogen activators

The fibrinolytic system can be activated essentially by two physiological activation pathways viz., PG activation by pharmacological activators and the activation of PG proactivator by active Hageman factor (a serine protease), which further activates PG to PN [2]. The most commonly and widely used (clinically approved) PG activators are SK, UK and TPA.

These are also called thrombolytic agents and extensively used in the treatment of various thrombolytic disorders. Direct plasminogen activators are serine protease with a high substrate-specificity for plasminogen. They hydrolyse the Arg_{560}–Val_{561} peptide bond in HPG to yield the active enzyme, plasmin, that carries out fibrinolysis.

Plasminogen activators can be divided into two broad categories, one related to eucaryotic origin while second from the bacterial origin. Plasminogen activators from bacterial origin includes SK from *Streptococcus* sp. and staphylokinase (SAK) from *Staphylococcus* sp. [31–34] which activates plasminogen by an indirect mechanism.

Another activator acylated plasminogen streptokinase activator complex (APSAC) which is clinically approved, represents a complex of SK with HPG in which the active site of HPG has been acylated. The deacylation of the active site in vivo is believed to provide longer plasma half-life to the molecule [35].

Eucaryotic PG activators involve mainly TPA and UK from human and they activate PG directly. Prourokinase (Pro-UK) [25] is an another example. Another eucaryotic PG activator, bat plasminogen activator (bat-PA) was identified in salivary glands of South American vampire bats [36].

The pro-UK is obtained from urine and is a precursor to UK which gets activated once introduced into blood stream.

The SAK is produced from certain strains of *Staphylococcus auerus*. It has a mechanism of action similar to that of SK, in that it forms a binary complex with plasmin(ogen), which then converts other PG to PN and a mechanism for fibrin specificity was proposed [32, 37, 38]. In an effort to understand the mechanism of plasminogen activator, Collen et al. [32] have resolved the crystal structure of recombinant SAK and recognized the three non-over lapping immunodominant epitopes. Recently the gene coding for SAK has been cloned and expressed in *E. coli* [32, 39] and *Bacillus subtilis* [40]. Recombinant SAK was shown to be able to induce fibrin specific clot lysis on a human plasma milieu [37, 38].

Because of the recognized shortcomings of the available plasminogen activators, attempts are underway to develop improved recombinant variants of these compounds [7, 38, 41–44]. The major advantage of SK is its low cost of approximately $200 per dose. It remains the most commonly used fibrinolytic in Australia.

Second-generation plasminogen activators

Although several plasminogen activators are currently being used for thrombolytic therapy it has been realized that none of them has all the desired properties in the same molecule. To improve the functional characteristics of various plasminogen activators, genetically engineered mutants of those were generated. One of such mutants, consisting of only Kringle 2 (K_2) and protease (P) domain of TPA showed prolonged half-life and was relatively fibrin-selective compound [41]. Deletion of the amino acid sequence Arg_{179}–Ser_{184} results in an urokinase mutant which is resistant to inhibition by PAI-1 [42].

Recombinant chimeric plasminogen activators have been constructed in the recent past primarily using different regions of TPA and single-chain urokinase type plasminogen activators (SCU-PA) [38]. The chimeric proteins might thus combine the mechanisms of fibrin-selectivity of both molecules.

Chimeric molecules of TPA and U-PA were produced. The functional properties of these recombinant TPA/U-PA chimeric plasminogen activators were similar to those of U-PA, but their affinity for fibrin was lower than that of recombinant TPA (r-TPA) [38]. The pharmacokinetic and thrombolytic properties of such chimeras were not superior to those of intact SCU-PA in a rabbit jugular vein thrombosis model [43].

The thrombolytic potency and the fibrin specificity of the new generation thrombolytic agents are not as pronounced in human as was anticipated from several animal models. Therefore, the quest for better thrombolytic agents still continues.

Streptokinase (SK)

Streptokinase (SK) (EC 3.4.99.22) is an extracellular non-enzymatic protein produced by various strains of β-haemolytic Streptococci. SK is a single-chain protein of molecular weight 47 kDa containing 414 amino acids, having isoelectric pH 4.7. The enzyme has its maximum activity between pH 7.3 and 7.6. The capacity of SK to cause lysis of blood clots was first described by Tillet and Carner in 1933 and the effect was thought to be a direct enzymatic action on fibrin. Milstone in 1941 demonstrated that SK achieved its effect through activation of plasma protein. At first SK was called fibrinolysin, until it was found that it induced fibrinolysis indirectly through activation of a plasma protein in the fibrinolysis system in man. The term streptokinase was then coined by Christensen [45] to describe the bacterial extract.

Unlike UK and TPA, SK has no proteolytic activity of its own and thus activates PG to PN indirectly by first forming a high affinity equimolar complex with PG (SK–PG activator complex).

Mechanism of SK action

The particularly fascinating feature of this plasminogen activator is that it has no proteolytic activity by itself and thus activates HPG to PN indirectly by first forming a high affinity equimolar complex with PG (SK–HPG) which is not affected by α-2-antiplasmin. The ability of SK to form an equimolar complex with HPG has been demonstrated [46, 47].

There are two important steps in the SK-catalyzed activation of plasminogen [48–50]. The first step involves formation of the proteolytic species which serves as the agent which catalyzes the cleavage of the Arg_{560}–Val_{561} peptide bond necessary for the activation of plasminogen. The events which occur in the formation of this activator species are summarized below [4].

$$SK + HPN \rightleftarrows SK \cdot HPG \rightarrow SK \cdot HPG' \rightarrow SK^{*} \cdot HPN$$

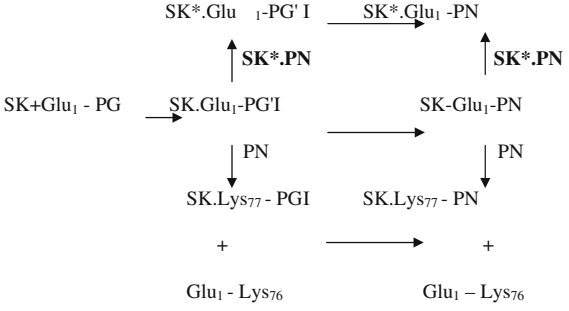
$$SK + HPN \rightarrow SK^{*} \cdot HPN$$

[SK*: proteolytically modified form of SK]

Here, SK and HPG bind in an equimolar complex, yielding SK.HPG. As a result of this interaction a conformational alteration occurs in the complex, yielding SK·HPG′, possessing active site. This active site resides in the plasminogen moiety of the complex [50].

This complex is not stable and is rapidly altered to a complex (SK*·HPN) of streptokinase and human plasmin (HPN). This is accomplished by an intramolecular cleavage of the Arg_{560}–Val_{561} peptide bond in the SK.HPG′ complex, catalyzed by the active site in the plasminogen moiety of the complex [51, 52]. Following the formation of HPN, in the complex, SK is proteolytically modified to an altered form SK* [49, 50, 53]. This altered form of SK possesses a molecular weight of 36 kDa [53], suggesting that peptides of total molecular weight of approximately 9 kDa has been removed. SK* appears to possess the same functional properties as does native SK.

The molecular events which occur during the formation of plasminogen activator and later the formation of SK*·HPN are shown below [52] .



Apart from its role in the activation of the PG molecule of the SK–PG complex to SK–PN, SK plays a characteristic role in modulating the substrate specificity of this 'partner' plasmin(ogen), from a general protease to a protease highly specific for substrate molecule of plasminogen [54]. They investigated the change in substrate specificity by studying the proteolytic, esterolytic and bovine PG activation activity of PN as a function of SK concentration. They found that with increase in the amount of SK there was a fall in general proteolytic activity, but a concomitant increase in bovine PG activation activity. Both these changes saturated at a stoichometric 1:1 concentration of SK and PN. Also SK* enhances the catalytic efficiency of plasmin, at least towards the peptide substrate studies [4].

SK can catalyze activation of plasminogen in a species-specific fashion. No synthetic substrate has yet been found for SK. The only protein substrates known are plasminogens from human [55], monkey [55, 56],

baboon [56], chimpanzee [56], cat [55], dog [55] and rabbit [55, 57]. Sheep PG is refractive to SK activation. However, all species of plasminogen are activated equally well by the SK*·human Lys_{77}–PN complex [54, 55].

Recent evidence has shown that a protein, which potentiates the activity of SK towards human plasminogen activation, exists in plasma. In both cases, it was found that the same effect could be observed when fibrinogen [58, 59], fibrin [58] or fibrinogen degradation products [60] were added to the system of interest.

Kim et al. [61] reported that the Asp_{41}–His_{48} region in a SK–plasminogen binary complex plays an important role in binding to a substrate plasminogen. The C-terminal domain of SK is involved in plasminogen substrate recognition and activation [62–64]. The role of an adjacent region, i.e., residues 48–59, on plasminogen activation has been discussed [65]. Wu et al. [66] concluded that the coiled coil region of SK gamma-domain, SK (Leu_{314}–Ala_{342}), plays very important role in HPG activation by participating in virgin enzyme induction and stabilizing the activator complex. Sazonova et al. [67] studied the mechanism of a bacterial plasminogen activator using *S. uberis* plasminogen activator. They concluded that gamma-domain is not required for a SK activation of bovine plasminogen.

SK binds preferentially to the extended conformation of plasminogen through the lysine binding site to trigger conformational activation of plasminogen [68, 69]. SK–plasminogen activator complex interacts with plasminogen through long range protein–protein interactions to maximize catalytic turnover [70]. The first 59 amino acid residues seem to have multiple functional roles in SK [71, 72]. Without these N-terminal residues, SK has an unstable secondary structure. Loss of residues 1–59 greatly reduces the activity of the remaining SK fragment (i.e., residues 60–414) [72]. Species-specific plasminogen activation has been demonstrated using 56 isolates of the pathogenic group C Streptococci [73].

Physico-chemical properties of SK

The physical and chemical properties of SK were reported by De Renzo et al. [74] and Talyor and Botts [75]. The SK is a monomeric protein, of molecular weight 45–50 kDa as determined by equilibrium sedimentation. It migrates on gel electrophoresis as an α-globulin and has an extinction coefficient of 9.49, with an isoelectric point of 4.7 [53, 74, 75]. It is free from cystine, cysteine, phosphorous, carbohydrates and lipids. The nitrogen content is 14.5%. Optical rotatory dispersion studies suggest that the helix content is 10–12%. Its specific viscosity is 0.1.

SK structure

Jackson and Tang [76] first determined the complete amino acid sequence of SK by automated Edman degradation of its cynogen bromide and protease-derived fragments. The entire nucleotide sequence of SK gene from *S. equisimilis* H46A was determined by Malke et al. [77]. The sequencing was performed after cloning a 2568-bp Pstl fragment containing the SK gene from the genome of *S. equisimilis* H46A into *E. coli*. The investigators also identified the nucleotide sequences of the adjacent regions involved in the control of transcription and translation of the SK gene. Homology studies have also been performed among the SK genes [78] as well as amino acid sequences [79] from different groups of Streptococci to compare and contrast the regions of variability in the SK sequence. Both studies suggested a considerable degree of heterogeneity among the various streptokinases produced by different groups of Streptococci.

Structural domains (conformational properties) of SK

Most of the structural information available on SK has been gathered from biophysical techniques such as CD, NMR, FT-IR and different scanning calorimetry [80–87], or through other relatively indirect studies such as by determining the physico-chemical properties of different fragments of SK [53, 62, 71, 72, 82, 88–91].

The presence of multiple domains in SK with different structural–functional properties have been shown by several investigators. Scanning calorimetric analysis of SK suggested that the protein is composed of two distinct domains [83].

In order to understand the role of the amino-terminal region of SK i.e. residues 1–59 of SK, synthetic approach was adopted [92]. It was found that the N-terminal domain complements the low plasminogen activation ability of the SK 60–414 fragment by enhancing it upto several hundred fold. Further, the N-terminal region reveled it to be composed of two smaller PG-specific. Recently, the crystal structure of SK complex with HPN light chain has been studied [93]. Similarly, the crystal structure of SK beta domain has been discussed [93, 94]. Thermal denaturation of SK has been discussed [95] and heat stability of specific domains of the enzyme has been studied [85]. How SK activates plasminogen has been the focus of extensive research [4, 52, 61–66, 70, 96–100]; nevertheless,

mechanisms of activation of plasminogen by SK are still being elucidated [68, 69, 101].

Immunogenecity of SK

The immunogenecity of SK was noted by Tillet and Garner [102] shortly after discovering its fibrinolytic effect. They found that SK was inactivated in blood samples from patients with recent Streptococcal infection as a result of the presence of neutralization of antibodies.

A measurable level of antibodies to SK is nearly omnipresent in the population as a consequence of the high frequency of Streptococcal infection [103, 104]. Many different anti-SK platelet-activating antibodies are known to exist and occur widely [105]. Extensive work has been reported on the identification of antigenic regions of SKs [106–109]. New antigenic domains were identified [109] as recently as 2001. The domains (sites) responsible for the antigenecity of SK were studied by Reed et al. [110]. They suggested that some segments are more immunogenic than the others.

Chemical modification of SK

To overcome the side effects of SK, attempts have been made at the chemical modifications of SK to enhance its stability characteristics by preparing polyethylene glycol (PEG)–SK adducts [30, 111, 112]. The main aim of this modification was to prepare conjugated derivatives of SK, which would be less immunogenic than the native form of SK and also improves its in vivo half-life.

Wu et al. [66] prepared mutants of SK in which one or more amino acid residues in the region of Pro_{58}–Lys_{59}–Ser_{60}–Lys_{61} were replaced by other amino acids become better fibrinolytic agents. The mutants are resistant to the hydrolytic inactivation of plasma clots. He showed that, the preferred mutant comprises replacing the Lys_{59} residue with glutamic acid. The alteration of the functional properties of SK α-domain deletion and point mutations in which individual domain interactions are affected may also result in rearrangement of the mutant-SK·plasminogen/plasmin interactions and contribution from lysine binding site-independent and -dependent binding to complex stability [113]. This suggests that loss of function accompanying such mutations may not be easily interpreted as loss of a specific function of a particular domain without consideration of the interdependency of the domain interactions with plasminogen/plasmin.

Galler [114] disclosed SK derivatives having platelet glycoprotein-binding domains. These derivatives produce higher local concentrations of plasmin in vivo as compared to unmodified SK. These derivatives are useful in treating thromboembolic disorders. They can be made by recombinant or by chemical synthesis in conjugation.

Production of SK

SK occurrence in Streptococci strains

In 1874, Billroth identified globular microorganisms growing in chains in purulent exudates from erysipelas lesions and infected wounds. Similar organisms, eventually named Streptococci, were isolated from the blood in scarlet fever. In 1903, Schotmuller [115] proposed that the different varieties of *Streptococcus* sp. could be classified on the basis of their capacities to haemolyze erythrocytes. Further in 1919, Brown introduced the term alpha, beta and gamma to describe the three types of haemolytic reactions observed on blood agar plates. The elaborate work of Lancefield in the early 1930's on β-haemolytic Streptococci, differentiated them into a number of immunological groups designated by the letters A to O [116].

The main sources of SK are β-haemolytic Streptococci of the Lancefield groups A, C and G. The group C is preferred for SK production, as they do not produce erythrogenic toxin.

Christensen [45] isolated a group C strain of *Streptococcus equisimilis* (H46A) (ATCC 12449) from human source. It has been widely used as the source for the production of SK. This strain was selected from over a hundred fibrinolytic isolates, because it produced the most active SK. Also, it does not produce erythrogenic toxin and is not so fastidious in its growth requirements as the majority of group A strains, so that a semi-defined medium could be easily formulated for its optimal growth and SK secretion [45, 117]. This strain has also served as the principal source for the SK gene, which has been used to express the protein in *E. coli*, *Streptococci and yeast* [77, 118, 119].

SK occurrence in recombinant strains

The gene coding for SK has been cloned from *S. equismilis* H46A into *E. coli* by Malke and Ferretti [120]. Malke [79] studied the SK gene (*skc*) from various sources and suggested its polymorphic nature. Kapur et al. [121] found that alleles of SK in 47 isolates of *S. pyogenes* have a mosaic structure.

The gene coding for streptokinase *skc*, from *S. equisimilis* H46A was expressed in several heterologous gram positive and gram negative bacteria [77, 120, 122]. The SK gene has been expressed in *E. coli* [123, 124]. Muller and Malke [125] cloned *skc* in erythromycin resistant plasmid and introduced into *Streptococcus equisimilis* H46A, to enhance SK production. They got erythromycin resistant clones producing SK at high level.

Ko et al. [126] have constructed pSK100, a high-level secretory expression plasmid for SK. It produced about 5,000 u/ml SK in LB–ampicillin medium. The SK gene was also expressed in Eucaryotic cells such as *Pichia pastoris*, [118, 127].

Fermentative production of SK

A very complex, rich medium supplemented with various growth factors is generally required for the optimal growth of several strains of Group A haemolytic Streptococcus [128]. Christensen [45] used a modified medium of that of Bernheimer et al. [128] for the production of SK by *Streptococcus equisimilis* H46A. This medium contained peptone, phosphate salts, glucose, additional mixture of vitamins (biotin and riboflavin), amino acids (tryptophan and glutamine) and nucleic acids (thiamine, adenine and uracil). The principal modification was a 75% reduction in the amount of glutamine added. Also as only certain lots of casein hydrolysate supported massive growth of the strain H46A, it was substituted with Difco neopeptone, so that massive growth could be obtained uniformly under these conditions. The low glucose concentration first used permits growth to become well established overnight without excessive production of acid. Then the remaining glucose was added in the following morning and incubation continued to obtain high cell density. The pH during the fermentation was maintained at neutral by the addition of 5 N NaOH at regular intervals. If the pH was not controlled, growth as well as SK production was also greatly diminished.

Rosenberger and Elsden [129], investigated the effect of both glucose and tryptophan limitation on the growth in continuous culture of *Streptococcus faecalis*. They concluded that to obtain maximal cell yield per unit energy source, the energy source should be the limiting factor. On the other hand, for product formation, the energy source should be in excess and some other metabolite should comprise limiting factor. Von Polnitz et al. [130] reported a process for the preparation of SK. This process especially relates to measures for reaching high yields in the biological production of SK. Also they described a method for the biological preparation of SK comprising adding, prior to inoculation, polyoxyethylene sorbitan monoolate to the culture medium at a concentration from 0.01 to 0.1%. Davies et al. [131] investigated the effect of amino acids on the steady state growth in continuous culture of group A Streptococci with dilution rate of 0.5 h and constant pH 7.5. They determined the cell yield and found that all the 14 amino acids tested, could function as a limiting factor except alanine.

Holmström [132] studied the production of cells and SK at various dilution rates, pH, and temperature in complex medium supplied with excess glucose in continuous culture of β-haemolytic Streptococci group C strain H46. He used a modification of Christensens medium for the growth. He found that at pH. 7.0, productivity of cells and SK as well as the yield was constant with respect to glucose, all increased with increasing dilution rate in the range of 0.1–0.5 h. The production of SK was found to be a function of both growth rate and cell concentration. Also the production of SK in continuous culture was found to be 2.3 times higher than batch culture.

Feldman [117] used a medium containing corn steep liquor, cerelose, KH_2PO_4 and $KHCO_3$ with pH 7.0. He could achieve an increase of the SK yield by using corn steep liquor instead of casein hydrolyzate with strain H46A after 12 h fermentation at 34°C. Baewald et al. [133] employed a simple and cheap culture medium for the production of a high yield SK from *S. equisimilis*. The medium containing yeast autolyzate or corn steep liquor as nitrogen source, glucose, K_2HPO_4, KH_2PO_4, $NaHCO_3$, $CH_3COONa \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $FeSO_4$ and $MnCl_2 \cdot 4H_2O$, with pH 7.2–7.4. at 28°C, 20–24 h with agitation. They could achieve a high yield of SK. An excellent yield of SK was obtained employing a mutant strain of Streptococcus using the following medium and conditions: Casein or Serratio peptidase hydrolyzated casein, glutamine, cysteine and yeast extract as nitrogen source, pH 6.8–7.2, 35–38°C, 50–500 rpm agitation and 0.1–1.0 vvm aeration. They could get over 8500 u/ml SK yield [44].

Van De Rijn and Kessler [134] described a chemically defined medium (CDM) for the growth of group A Streptococci. It contains glucose, adenine, uracil, guanine, buffer solution, salt solution, vitamins and amino acids. The advantages of this medium over all of the earlier CDM media are: small inocula may be used without any prior adaptation regimen; the Streptococci grow with doubling times comparable to those in complex media with higher culture densities. The group A Streptococci grown in this medium retained production of SK and other extracellular proteins. McCoy et al. [73] used the CDM of

Van De Rijn and Kessler [134]. Ozegowski et al. [135] studied the influence of the cultivation temperature on growth and production of SK from group C Streptococci *Streptococcus equisimilis* H46A, the range being 28–43°C. They found that the strain was able to grow in the entire temperature range with maximum growth at 28°C as optimal. The amount of SK depends on both cultivation temperature and biomass production.

Another commonly used enriched medium for the production of SK is brain heart infusion (BHI) medium. It is obtained from bovine brain and heart tissues. Malke and Malke and Ferretti [120] used this medium and got good growth of *Streptococcus equisimils* H46A. Suh et al. [136] used the same medium with 6.5% BHI at pH 7.4 for the production of SK by a strain of group C Streptococci, isolated from human patient. The maximum activity of SK was detected in the exponential growth phase. Nemirovich—Danchenko et al. [137] produced SK from group C Streptococci, *Streptococcus equisimilis*. They cultivated the strain in a medium composed of glucose, dipotassium hydrogen phsophate, magnesium sulphate, potassium hydrogen carbonate and casein hydrolyzate containing phenol red and supplemented with a low-molecular weight ultrafiltrate of Todd Hewitt broth for the production of SK to study the pathogenic group C Streptococci. They demonstrated species-specific plasminogen activation using 56 Streptococcal isolates.

Estrada et al. [124] expressed the SK gene from *S. equisimilis* in *E. coli.* They could produce a recombinant SK at levels more than 10-fold greater than what they obtained by using a group C Streptococci. Ko et al. [126] used LB–ampicillin medium for the production of SK by the high-level secretory expression plasmid pSK100, which produced about 5,000 u/ml of SK. The medium contains Bacto-tryptone, yeast extract, sodium chloride and 50 μg/ml ampicillin at pH 7.3. Narciandi et al. [138] examined the influence of culture conditions on the production of the recombinant fused protein Kringle (SK) expressed in *E. coli* under the control of the Ipp–Iac promoter. They used two different media: complex SOC medium and synthetic medium. The SOC medium contains Bacto-tryptone, yeast extract, sodium chloride, potassium chloride, magnesium sulphate, magnesium chloride, glucose supplemented with 20 μg/ml of thiamine–HCl and 50 μg/ml of ampicillin [139]. The synthetic medium contains KH_2PO_4, $C_6H_8O_7$, $(NH_4)_2 \cdot HPO_4$, $MgSO_4 \cdot H_2O$, glucose, supplemented with 1 ml of trace metal solution, 20 μg/ml of thiamine HCl and 50 μg/ml of ampicillin [138]. The pH of the medium was controlled at 6.8 with aqueous ammonia (25%). One mM of isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added as inducer of the expression system. The production of SK was induced by adding 1 mM IPTG to the medium. The plasmid encoded a SK that lacked the 13 N-terminal amino acid residues of the normal protein. This enhanced productivity of the recombinant protein and enabled secretion into the extracellular medium [140]. At least a part of the N-terminal domain is known to be functionally relevant in SK [141], but this may not include the first 13 residues [140]. Other reports have also described IPTG-induced production of recombinant SK [142, 143].

Lee et al. [140] showed the enhanced production and secretion of SK into extracellular medium in *E. coli* by removal of 13 N-terminal amino acids. They used LB–ampicillin medium as production medium at 37°C for 12 h. The culture was induced by adding 1 m M IPTG. Yazdani and Mukherjee [143] clone the SK gene under the T_7 protomer containing the ompA secretory signal sequence. The T_7 RNA polymerase was induced with IPTG. The biomass concentration was reduced while there was abnormal increase in activity.

Patnaik [144] applied the principal component analysis (PCA) for fed-batch fermentation for the production of SK to identify the variables, which are the essential to formulate an adequate model. He observed that the suitability of PCA to formulate minimal model for industrial scale fermentations. Malke et al. [145] studied the expression of the SK gene, skn using southern hybridization analysis. This led to the localization of the core promoter region of *skc* and identification of the active upstream region required for full promoter activity.

Potential global health care implications of new production technologies

Commercial production of SK requires special attention to biosafety considerations because the protein is potentially immunogenic to process workers [44]. In addition, care is necessary if SK is being produced using natural strains of Streptococci because all SK producing Streptococci are potentially pathogenic. The various safety considerations relevant to production of biopharmaceutical proteins have been discussed by [44, 146].

The increasing incidence of thromboembolic diseases has sustained the search for new agents able to stimulate the natural fibrinolytic system [147]. The first generation of antithrombotic agents includes bacterial SK and human urine UK. Because these molecules lack specificity for the fibrin clot, important efforts

have been made to produce, using recombinant DNA technology, agents presenting higher fibrin clot selectivity such as TPA and SCU-PA. In parallel, several laboratories are presently attempting to create mutants and hybrids plasminogen activators displaying improved thrombolytic properties with respect to the natural molecules.

Diwedi et al. [148] reported that reperfusion which was the primary end point was assessed using a combination of enzyme (Creatine Kinase) peaking and ST segment resolution was observed in 68.2% (58) in the recombinant SK and in 69.4% (34) patients in natural SK group whereas, rates reported in literature by the present criteria vary from 59 to 82% [149, 150]. A randomized comparative study was carried out in over 200 patients where a reperfusion rate of 67.1 and 70.7 was observed with recombinant and natural SK respectively [151]. A similar rate of 70.6% was reported from previous Indian studies with natural SK [152]. Safety profile assessment, which was the secondary end point in this study, was similar in both the recombinant SK and natural SK group. These events seen were similar to those reported by other studies. Incidence of bleeding was 4.2% (4) in the recombinant SK group while it was 5.4% (3) in the natural SK group, which are similar to the rates reported in literature vary between 0.5 and 15.2% [149, 150]. No anaphylactic reactions were seen in the recombinant SK group whereas one was reported from the natural SK group. Adverse effects of thrombolytic therapy commonly include hypotension 4.5–15%, fever 5%, rigors, nausea and vomiting 46% and bleeding (0.5–15.2%). The mortality rates were low for both the groups {3.1% (3) and 5.5% (3)}, however this small study did not have the statistical power to show the difference in the two comparable treatment groups. Postthrombolysis, cardiogenic shock was observed in 1 (1.2%) and 2 (3.6%) in the recombinant and natural SK groups respectively whereas reinfarction was seen in 2 (2.4% and 3.6%) patients respectively in both the groups which is much less than in other studies. This study has its limitations as the sample size is small and the markers used for assessing reperfusion were non-invasive instead of the gold standard that is angiography, although the non-invasive methods have been validated against angiography and are proven surrogate markers of reperfusion. Since this trial was a randomised controlled trial the instigators were of the opinion that the conditions to see the efficacy and safety need some factors to be controlled including patients not more that 65 years of age and without any co-orbid conditions that might influence the outcome. A larger study with potency assessed by angiography and without influencing factors of age or others would give a precise assessment of reperfusion.

SK is the most widely used thrombolytic agent particularly in India while others like tPA are expensive. Reviews comparing other thrombolytic agents have shown that streptokinase to be as good as that of TPA or recombinant PA in terms of both efficacy and safety [153, 154]. Recombinant SK has the advantage of not containing streptolysin and streptodornase unlike Streptococci-derived natural SK, which might make it safer, and will be cheap which is very relevant to our country's population. This technology can be further used to make more modifications in these agents to make them more safe and efficacious.

Conclusions

Comparative clinical trails and cost-effectiveness considerations suggest that SK is the drug of choice for thrombolytic therapy in clinical practice, but its use is not risk free. Cloning of the SK gene in non-pathogenic microorganisms has enabled production of recombinant SK that eliminates any risk of inadvertent inoculation of patients and production personnel with potentially pathogenic Streptococci. SK can be produced inexpensively in bulk via bacterial fermentation. Various chemical modifications were used to prepare conjugated derivatives of SK, which would be less immunogenic than the native form of SK, improving plasminogen activation, and also improves its in vivo half-life in circulation. These goals have been attained to various degrees also by producing mutated and engineered SKs. The SK domains responsible for antigenicity, stability and plasminogen activation appear to overlap to some degree. These derivatives are useful in treating thromboembolic disorders. They can be made by recombinant or by chemical synthesis in conjugation.

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