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Review

Urokinase - A strong plasminogen activator

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Urokinase (UK) is a serine protease, which specifically cleaves the proenzyme/zymogen plasminogen to form the active enzyme plasmin. It specifically catalyzes the cleavage of the Arg- Val bond in plasminogen. The active plasmin is then able to break down the fibrin polymers of blood clots. Clinically, UK is given to patients suffering from thrombolytic disorders. Among the plasminogen activators, UK provides a superior alternative for the simple reasons of its being more potent as compared to tissue-plasminogen activator and non-antigenic by virtue of its human origin unlike streptokinase. Based on these observations, UK is a strong plasminogen activator. Hence, UK, as one of the most potent plasminogen activators is attracting a great deal of attention. The mechanism of action, physico-chemical properties, *in vitro* production, cloning and expression, and clinical applications of UK are reviewed in this paper.

Key words: Urokinase (UK), plasminogen activators, fibrinolysis, strong plasminogen activator, production, cloning and expression, physico-chemical properties and clinical applications.

INTRODUCTION

Urokinase (UK) is given to patients suffering from thrombolytic disorders like deep vein thrombosis, thrombosis of the eye, pulmonary embolism, and myocardial infarction. This enzyme is a strong plasminogen activator which specifically cleaves the proenzyme/zymogen plasminogen to form the active enzyme plasmin (Lesuk et al., 1967; Kunamneni et al., 2008). It specifically catalyzes the cleavage of the arg–val bond in plasminogen. The active plasmin is then able to break down the fibrin polymers of blood clots (Bennart and Francis, 1976). The initial main source of UK was urine as described by Williams (1951). Later UK was prepared from cultures of human embryonic kidney cells (Bernick and Kwaan, 1967).

In recent years, thrombolytic therapy has revolutionized the treatment of these diverse circulatory disorders such as pulmonary embolism, myocardial infarction, deep vein thrombosis, thrombosis of the eye etc., (Collen et al., 1988). Therapy in thrombosis has been directed towards interference with coagulation mechanism, activation of fibrinolytic system, interference with platelet aggregation or combination of these. In addition, surgical intervention to prevent embolization or to remove thrombi and restore blood flow is of critical importance.

Anticoagulant therapy includes the use of either coumarin drugs, heparin, or a combination of both. The coumarin drugs (warfarin, coumarin and dicoumarol) act by antagonizing vitamin K and result in depression of the concentration of clotting factors [prothrombin (factor II), factors VII, IX and X].

Thrombolytics are the drugs used to lyse thrombi to recanalyze occluded blood vessels. They are curative rather than prophylactic. They work by activating the natural fibrinolytic system.

The plasma fibrinolytic system Fibrinolysis

Fibrinolysis refers to the dissolution of the fibrin blood clot by an enzyme system present in the blood of all mamma-

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lian species (Castillino 1981). The fibrinolytic system consists of the plasma zymogen, plasminogen; its activated product, the proteolytic enzyme, plasmin; plasminogen activators (PAs); inhibitors of both plasmin and PAs 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 PAs (Castellino, 1984). The PAs 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 activity of the fibrinolytic system is modulated by inhibitors that inhibit both PAs 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 (Francis and Marder, 1994).

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

Fibrinolytic system

Extensive studies have been made over the last 25 years to understand the physiology of the fibrin-clot formation (Wu and Thaigarajan, 1996).

The fibrinolytic system and its constituents directly responsible for the dissolution of the fibrin clot are briefly described in Figure 1 (Bick, 1992).

Plasminogen-plasmin system

Plasminogen is a glycoprotein of molecular weight 90-KDa, which is synthesized in the liver. It is converted enzymatically by PAs to the fibrinolytic enzyme, plasmin. cuts away its covalently cross- linked α- chain protuberances. The rather open mesh like structure of a blood clot gives plasmin relatively free access to the polymerrized fibrin molecules thereby facilitating clot lysis.

Plasmin, a plasma serine protease that specifically cleaves fibrins tripple stranded coiled coil segment and Plasmin is formed through the proteolytic cleavage of the 86-KDa zymogen plasminogen, a protein that is homologous to the zymogens of the blood-clotting cascade.
PA is present in the tissue (t-PA), in plasma and in urine (UK). t-PA is localized in the vascular endothelium of veins, capillaries and pulmonary arteries and in the microsomal fraction of cells. t-PA is released into the blood stream in response to a number of stimuli, includeing ischaemia, vasoactive drugs and exercise. Released activator is inactivated rapidly in the blood stream by complexing to tissue plasminogen activator inhibitors (PAIs) and has a half-life of about five minutes.

The major tissue PAIs are synthesized in the liver and in vascular endothelium, but about 30% of the total is probably megakaryocyte-derived and is stored in platelet and granules.

The activator in urine, UK, differs structurally from t-PA and is produced primarily in the kidneys and excreted into the urine where it may help to maintain urinary tract potency. Endothelial UK probably contributes a small proportion of plasma activator activity. Factor XIIa not only intimates coagulation, but also accelerates the conversion of plasminogen to plasmin via a proactivator, kallikrein.

When clotting occurs, a small amount of plasminogen is trapped in the fibrin strands. PA released locally from the vascular endothelium or traumatized tissues, binds to the fibrin of the thrombus and converts plasminogen to plasmin, itself bound to its surface fibrin and in this conformation protected from its otherwise highly effective inhibitor α_2 antiplasmin. Fibrin is then digested. There is little or no plasma fibrinolytic activity because plasmin that is formed in the blood stream from activation of plasma plasminogen is rapidly inactivated by circulating α_2 antiplasmin. The α_2 macroglobulin also acts as a secondary plasmin inhibitor in the presence of excess plasmin.

Fibrinolytics

As in the inflammation reaction, the clotting of blood is an example of a defense mechanism, which can overreact and require therapeutic intervention. It is now known that in normal tissues, there is a constant dynamic equilibrium between blood coagulation (clotting) and fibrinolysis (the process of dissolving the clotted blood). The maintenance of proper balance in this equilibrium is extremely important. If fibrinolysis is increased by a pathological cause, a predisposal to excessive bleeding results. On the other hand, if fibrinolysis is weakened so that clot formation is favoured, conditions occur that are called thromboses (clots formed in and remaining in blood vessels) and embolisms (sudden blockages of blood vessels caused by circulating fragments of clots). These can be life threatening. In chronic cases, cholesterol and other fatty materials may aggregate around clotted deposits in blood vessels. When this pathological condition is well established, it is called atherosclerosis, hardening of the arteries.

Although there are chemical anti-coagulants (such as heparin) available as well as corrective surgical techniques, acute thrombosis and embolisms (which are lumped together as acute thromboembolic vascular diseases) are still the largest single cause of death and disease in the middle-aged and elderly populations of the Western World.

The mechanism used by the body for controlling the equilibrium between clot formation and dissolution is a complex one involving a series of enzymes, proenzymes, activators, and proactivators. A brief outline of the highlights of the process will be helpful in understanding the

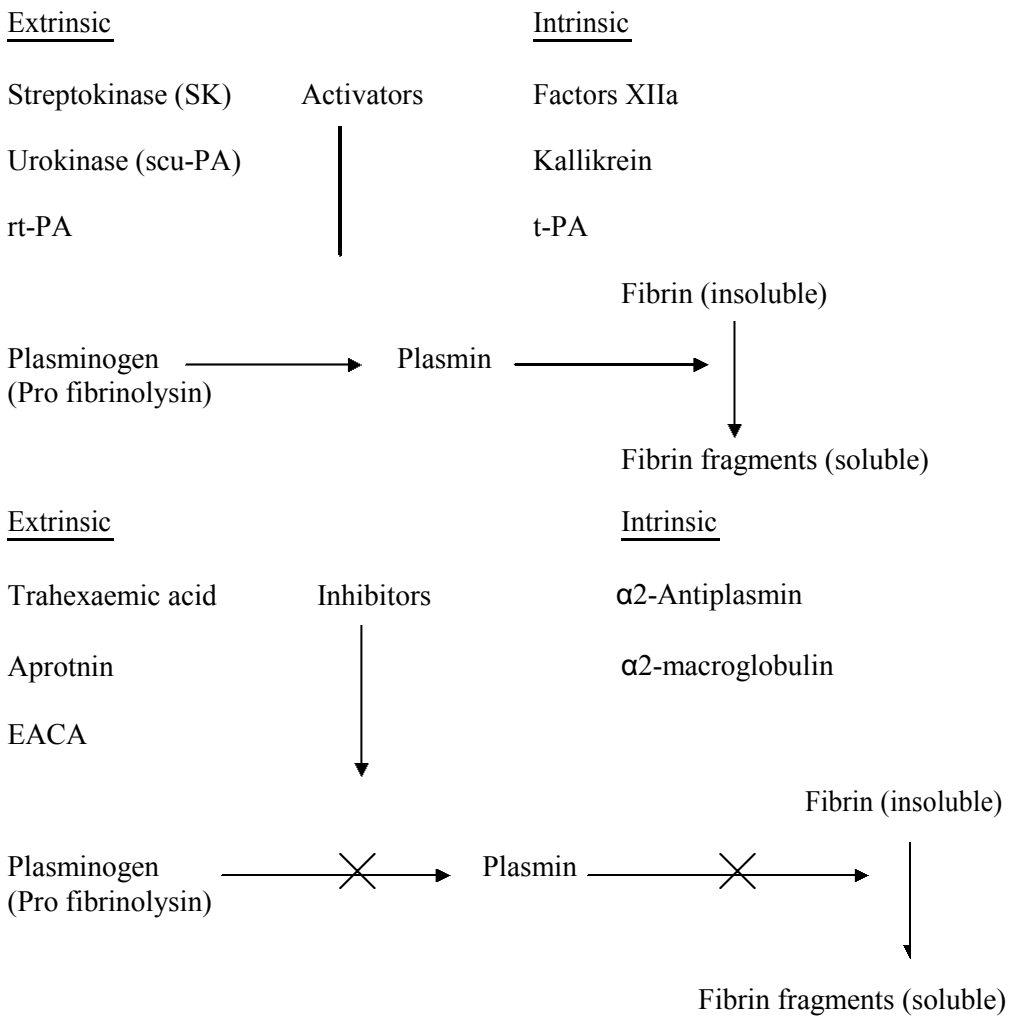

Figure 1. Relationship of components of the fibrinolytic system with activators**.**

therapeutic method. In forming a clot, the plasma protein, fibrinogen, is converted to insoluble fibrin by the enzyme thrombin. Fibrin forms the clot. The enzyme plasmin, which dissolves the clot, exists in the blood as the pro-enzyme plasminogen. Activators convert plasminogen to plasmin for dissolving the clot.

Just as nature uses enzymes in maintaining this crucial balance, so is man learning how to use enzymes to restore the balance once it is lost. Clinical studies have shown that the best approach to therapeutic thrombolysis (dissolution of clots) is an intravenous injection of an enzyme capable of converting plasminogen to plasmin, the enzyme which dissolves the clot. This type of therapy is known as thrombolytic (thrombus or clot-splitting) or fibrinolytic (fibrin-splitting) therapy. The enzymes most frequently used for this are streptokinase (SK) [from bacteria] and UK (from human urine). Three "new" thrombolytic enzymes which have seen some therapeutic use in the last 20 years are Arvin (from a Malayan pit viper), reptilase (from a South American snake), and brinase (from the mould *Aspergillus oryzae*).

Urokinase (UK)

UK is an endogenous substance, which is involved, in many physiological processes. It is a life saving, therapeutically important fibrinolytic enzyme used in the treatment of many disorders requiring dissolution of blood clots.

The main source of UK earlier was its isolation from urine which is a tedious and lengthy procedure and highly expensive also. Further the yield obtained is very less of the order of 6CTA (Committee on Thrombolytic Agents) units/ L urine. This value shows that minimum of 1500 L of urine are required to produce one clinical dose of this enzyme [7500-8000 CTA units].

In contrast UK can be harvested at concentrations of order 800 CTA units/ml from confluent cultures of human kidney cells. As such in recent years human cell cultures are being used for UK production and isolation.

Below is a list of cell lines being used currently for UK production and isolation:

- Human embryo kidney cells

- Human lung edenoma carcinoma cell line.
- Normal human umbilical vein endothelial cells.
- Human fibroblasts.
- Human myeloma cells.
- BHK-21/N cells.

Several forms of UK with different molecular weights were described. A single chain UK-type PA called Saruplase had been prepared. In Great Britain, potency of UK is expressed in arbitrary units known as Plough units. One plough unit is approximately equivalent to 1.5 International Units. In USA, potency is expressed in CTA units. One CTA unit is approximately equivalent to one IU.

UK is secreted from cells as a single chain proenzyme (scu-PA) from which the active two chain enzymatic plasminogen activator (tcu-PA) is derived by proteolysis, the two chains remaining linked by a disulphide bond. The UK that is used clinically is tcu-PA type. UK is enzymic and acts directly as a plasminogen activator and it is not antigenic.

The amino terminal fragment (ATF) of urokinase-type plasminogen activator (u-PA) is 130 amino acid residues long. It consists of 2-α-helicles and two antiparallel β-strands. The ATF contains 2 domains; a growth factor domain and a Kringle domain. It binds fucose (magenta) as a ligand. Fucose is a deoxy-sugar, a monosaccharide with one or more hydroxyl (-OH) groups replaced by a hydrogen (-H). The formula for fucose is $C_6H_{12}O_6$. The ATF also contains 6 disulfide bonds and are as follows: Cys_{11}-Cys_{19}, Cys_{13}–Cys_{31}, Cys_{33}-Cys_{42}, Cys_{50}-Cys_{131}, Cys_{71}-Cys_{113} and Cys_{102} –Cys_{126}.

Physical properties

It had been known UK could be found in multiple molecular sizes. There appeared to be two major forms: low molecular weight (LMW, 33-KDa) and high molecular weight (HMW, 57-KDa). It was found that urinary UK contained predominantly the LMW form. It was suggested that the HMW form is the native structure and that it is converted to the LMW form by cellular proteases. However, under certain conditions, HMW form was found to be more active when assayed with standard enzyme kinetic methods.

The enzyme is moderately stable, showing no appreciable loss in activity over years in lyophilized form or over months in sterile solutions at 1 mg/ml or more at refrigerator temperatures. Stability is decreased at salt concentrations below 0.03 M sodium chloride and precipitation with loss in activity occurs at very low salt concentrations.

In diluting to the levels of activity measured in the fibrinolytic assay, it is advisable to add a protein such as human serum albumin fraction V or gelatin to prevent surface denaturation.

Mechanism of action

u-PA is a strong plasminogen activator which specifically cleaves the proenzyme/zymogen plasminogen to form the active enzyme plasmin. It is a trypsin like enzyme capable of hydrolysing L-lysine and L-arginine esters. It specifically catalyzes the cleavage of the Arg-Val bond in plasminogen by first order reaction. The active plasmin is then able to break down the fibrin polymers of blood clots.

Contraindications

Active internal bleeding, history of cerebrovascular accident, recent trauma of any kind including surgery, intracranial neoplasm, aneurysm, bleeding diathesis, severe uncontrolled hypertension are contra-indicated for UK application.

Interactions

- Platelet inhibitors such as aspirin, indomethacin, etc. can potentiate the action of UK and cause haemorrhage.
- Heparin and oral anticoagulants will increase risk of bleeding.

Adverse reactions

- Superficial bleeding at sites of venous cut down, vascular punctures, etc. may occur.
- More seriously internal bleeding could occur in any internal organ or site.
- Relatively mild allergic reactions such as bronchospasm or rash may occur occasionally.
- Other side effects include chills, blood pressure changes, dyspnoea, palpitation, cyanosis and hypoxemia. Aspirin should not be given for fever.

Assaying UK

Since 1954, when the activity of plasmin and thrombin on synthetic substrates were first demonstrated (Troll et al., 1954; Sherry and Troll, 1954) synthetic substrates have played an important role in the study and characterization of various enzymes involved in blood coagulation and fibrinolysis (Abloundi and Hagan, 1957; Kline and Fishman 1961). Activation of plasminogen to plasmin involves an enzymatic proteolytic step and the activators mediating the action shared the ability of plasmin to split arginine and lysine esters (Alkjaersig et al., 1958). In the past few years, partially purified preparations of UK, the naturally occurring PA have been available and this coupled with a fairly simple assay procedure has stimulated studies with UK relative to its mechanism of action (Alkjaersig et al., 1958), physiological role (Fletcher et al., 1962), signifi-

cance in disease (Sherry and Troll, 1954), and potential for thrombolytic therapy (Fletcher et al., 1962).

Existing assay methods can be divided into three major groups:

(1). The first group consists of indirect assay of UK activity with protein as substrates eg., the fibrin plate method (Haverkate and Bradman, 1975), clot lysis method (Lassen 1958) and caseinolytic methods (Kline, 1971). PAs cannot be assayed directly by these methods but only via their activating action on plasminogen present in or added to the substrate. With the fibrin plate method extremely low levels of enzymatic activity can be determined but long incubation times are required, and the responses found depend on the quality of the fibrin substrate (Haverkate and Bradman, 1975).
(2) A second group of assays, suitable for kinetic studies is based on the determination of hydrolysis of synthetic peptide esters (Bell et al., 1974). The disadvantage of the use of these esters is that esterolytic rather than amidolytic activity of a proteolytic enzyme is measured.
(3) In the third group of synthetic substrates e.g., Acetyl L-lysine p-nitroanilines, amidolysis of the peptide amides is measured (Petkov et al., 1973).

Fibrinolytic assays

The standard system used for measuring PA in cells is an indirect, two-step assay in which plasminogen is incubated with a source of PA and the plasmin activity generated is quantitated by using fibrin, casein or protamine as substrates (Unkeless et al., 1974; Goldberg, 1974; Kessner and Troll, 1976).

Marsh and Gaffney (1977) developed a rapid fibrin plate method for plasminogen activator assay. Their study was carried out to investigate plasminogen-enrichment as a means of shortening the incubation period, which is associated with the fibrin plate method. Fibrin plates were made up to contain 2 casein units of added plasminogen. Each was opaque, firm, did not lyse spontaneously and yielded biometrically valid parallel-line assays for SK and UK.

Jespersen and Astrup (1983) described the reproducebility, precision and required conditions of the firbin plate method for determination of fibrinolytic agents.

Under optimal conditions the assay is sensitive and precise method for the quantitative determination of firbinolytic agents.

Millar and Smith (1983) compared the rapid and highly sensitive solid phase assay with the fibrin plate method for the measurement of UK, SK and the PA in human euglobulin fractions. The solid phase assay was run using Glu- or Lys- plasminogen, and significant differences were observed in the activation of the plasminogen by UK and SK. Very good agreement was obtained between the fibrin plate and solid phase methods in all cases.

Fossum and Hoem (1996) developed a firbin microplate method for the estimation of UK and non-UK fibrinolytic activity in protease-inhibitor-deprived plasma. In this method fibrin clots, with a suitable dye incorporated, were formed in wells of standard high adsorption micro-titer plates.

Roche et al. (1983) presented a rapid and highly sensitive solid-phase radio assay for the measurement of PAs. The method employs a convenient and stable ^{125}I – fibrinogen – latex bead product and can reproducibly detect 0.25 milli PU/ml of UK. This represents a 100-fold increase in sensitivity of UK over radio isotopic solid-phase technique and a 120-fold increase over the sensitivity of the fibrin plate method.

Esterolytic assays

Sherry et al. (1966) investigated the ability of UK to hydrolyze a variety of alpha amino substituted Arg and Lys esters [acetyl -arg methyl ester, benzoyl –arg methyl ester, tosyl –arg methyl ester, lys methyl ester, acetyl –lys methyl ester, benzoyl –lys methyl ester and tosyl –lys methyl ester]. Their observations indicated that UK catalyses a more rapid hydrolysis of lysine esters and its derivatives than the corresponding esters of arginine. Substitution of alpha amino group of lysine methyl esters increases the sensitivity of the ester to hydrolysis. They further reported that acetyl -lys methyl ester is the most sensitive substrate among the various esters tested.

A convenient and highly sensitive colorimetric assay for various proteases such as trypsin, chymotrypsin, plasmin, thrombin and UK was reported (Ninobe et al., 1980). The substrates used were naphthyl ester derivatives of N-tosyl L-lysine, N-acetyl glycyl L- lysine and N -acetyl L-tyrosine. Activity was assayed by colorimetric determination of naphthol released. They reported that this method was more sensitive than the use of corresponding methyl or ethyl ester derivatives.

Barlow and Marder (1980) reported the use of a chromogenic substrate L–pyroglutamyl glycyl L-arginine *p*-nitroanilide [S-2444] for assay of plasma UK levels of patients treated with urinary source or tissue culture source of UK. The *p*-nitroaniline released was measured in a spectrophotometer at 405nm. A linear response relationship between UK concentration and optical activity was obtained, indicating that the method detects UK in quantitative manner.

Kulseth and Helgeland (1993) developed a simple and highly sensitive chromogenic microplate assay for quantification of rat and human plasminogen in plasma samples and subcellular fractions. The assay is based on a conversion of plasminogen to plasmin, using UK as an activator, and a subsequent cleavage of chromogenic plasmin substrate D-alanyl-L-cyclohexylanyl-L-lysine-*p*-nitroanilide-dihydroacetate.

p-Nitroaniline being released by the cleavage is then measured at 410 nm with a microplate reader. The assay includes an acidification step to make plasminogen more

readily activated to plasmin. The method is suitable for analyses of a large number of samples, measuring plasminogen in the nanogram range (0.5 - 50ng/50 l of sample).

Fluorimetric assays

Kessner and Troll (1976) reported a new method for determining plasminogen activator levels. The assay is based on the digestion of N-terminal blocked protamine and subsequent measurement of the exposed amino groups using the flurogenic amine reagent, Fluram.

Nieuwenhuizen et al. (1978) reported fluorigenic substrates for sensitive and differential estimation of UK and t-PA. Two fluorigenic peptide amides have been synthesized, i.e. Boc L-valyl-glycyl-L-arginine-L-naphthylamide and L-valyl- glycyl-L-arginine-2-naphthylamide. The kinetic parameters of plasmin, UK and human uterine tissue plasminogen activator on substrates 1 and 11 have been determined.

Zimmerman et al. (1978) developed a simple, sensitive, direct assay that allowed both rapid measurement and kinetic analysis of PA, independent of plasmin generation. The method employed a synthetic flurogenic peptide substrate 7-(N-CbZ -glycylglycyl argininamido)- 4-methyl coumarin trifluoro acetate. The assay correlated well with the standard ^{125}I-labeled fibrin plate assay using highly purified UK.

***In vitro* production of UK**

The presence in urine of an activator substance capable of effecting transformation of plasminogen to plasmin was first described by Williams (1951) and in the following year by Sobel et al. (1952). The latter group has assigned the name UROKINASE to this activator.

Pepper et al. (1992) demonstrated that fibroblast – conditioned medium induces Madin-Darby canine kidney (MDCK) epithelial cells to form branching tubules when grown in three dimensional collagen or fibrin gels, and that this morphogenetic effect is mediated by hepatocyte growth factor (HGF), also known as scatter factor. In fibrin gels, this effect is inhibited by addition of exogenous serine protease inhibitors, which suggests a role for PAs in the matrix remodeling required for thrombogenesis. They investigated the effect of fibroblast-conditioned medium (CM) and HGF on the production of PAs by MDCK cells. They found that u-PA activity and mRNA were increased 4.9 fold by CM from human Detroit-550 fibroblasts, which lacks thrombogenic activity. The u-PA inductive property of MRC-5 CM was completely inhibited by preincubation with antibodies to recombinant human HGF (rhHGF). Exogenously added rhHGF also increased u-PA activity and mRNA 5.9 fold in MDCK cell, with an optimal effect at approximately 10 mg/ml. MRC-5 CM also increased u-PA receptor mRNA 34.9 fold in MDCK cells, an effect which was inhibited by 71% by preincubating the CM with antibodies to rhHGF, and which was mimicked by exogenously added rhHGF (31.3 fold increase). These results demonstrated that HGF, which induces thrombogenesis by MDCK cells in vitro, also increases u-PA and u-PA receptor expression in these cells. This suggests that the resulting increase in extracellular proteolysis, appropriately localized to the cell surface, is required for epithelial morphogenesis.

By the combined use of zymographies on tissue secretions *in situ* hybridizations, Sappino et al. (1991) explained the cellular distribution of u-PA and t-PA and of their mRNAs in developing adult mouse kidneys. In 17.5-day-old embryos, renal tubules synthesized u-PA while S-shaped bodies produced t-PA. In the adult kidney, u-PA is synthesized and released in urine by the epithelial cells during lining the straight parts of both proximal and distal tubules. In contrast t-PA is produced by glomerular cells and by epithelial cells lining the distal parts of collecting ducts. The precise segmental distribution of PAs suggested that both enzymes might be implicated in the maintenance of tubular potency, by catalyzing extracellular proteolysis to prevent or circumvent protein precipitation.

Valinskey et al. (1981) studied the association of controlled extracellular proteolysis mediated by PA with embryonic tissue remodeling and cell migration in the developing *Bursa fabricius* of quail and chick embryos.

Wojta et al. (1989) stated that vascular origin determines PA expression in human endothelial cells and renal endothelial cells produce large amounts of scu-PA.

Roychoudhury et al. (1999) carried out studies in T-flasks and bioreactor to produce UK using HT 1080 human kidney cell line. While growing the cell line it has been observed that the lag phase is reduced considerably in the bioreactor as compared to T-flask culture. The HT 1080 cell adhesion rate and UK production were observed to be the function of serum concentration in the medium. The maximum UK activity of 3.1×10^{-4} PU/ml was achieved in the bioreactor at around 65 h of batch culture. Since HT 1080 is an anchorage dependent cell line, therefore, the hydrodynamic effects on the cell line were investigated.

Podorolskaia et al. (1999) studied correlative intercommnections between PA activity (fibrin plate method) and level of UK antigen (Ag uAP) and tissue PA antigen (Ag tAP) in urine and blood (ELISA) in 60 patients with chronic glomerulonephritis (CGN) and 38 patients with amyloidosis. High degree of correlation $r = +0.84$ and $P < 0.001$ was found between blood Ag uAP and urine Ag tAP in amyloidosis only.

Iwamoto et al. (1990) studied the effects of thrombin interleukin (IL-1), tumor necrosis factor (TNF) and gamma interferon (gamma-IFN) on the release of PA and inhibitor (PAI) using cultivated human glomerular epithet-lial cells (GEC's) . Their findings indicate that the GEC's participate in the regulation of extracapillary fibrinolysis in the glomerular environment being modulated by thrombin

and cytokines IL-1 and TNF.

Lee et al. (1993) presented a method for determining the plasminogen activation rate by UK via a cascade enzymatic reaction system. A procedure of parameter estimation has been proposed for the determination of the activity of UK and the kinetic constants.

Schnyder et al. (1992) developed a spectrophotometric method to quantify and discriminate UK and t-PAs.

Leprince et al. (1989) developed a colorimetric assay for the simultaneous measurement of PAs and PAIs in serum-free conditioned media from cultured cells.

Ambrus et al. (1979) reported that Plasminogen-rich and plasminogen-poor radiolabeled human fibrin clots were inserted into large veins of baboons and stump-tailed monkeys. The thrombolytic effects of PAs (UK, SK), and plasmin preparations with activator activity (SK-activated human plasmin) and without activator activity (trypsin-activated porcine plasmin, Lysofibrin) were studied. Plasminogen-free and plasminogen-rich clots lysed at equal rates. Preparations with and without activator activity were equally effective as thrombolytic agents. Endogenous activation of plasminogen in the clot thus appears not to be the essential mechanism of thrombolysis. The exogenous pathway of enzyme adsorption to fibrin fibers seems to represent an important thrombolytic mechanism. Clot lysis was achieved with doses of fibrinolytic enzymes, which produced little or no significant hematologic changes including hypofibrinogenemia and decreases of other blood coagulation factor levels.

Jamet et al. (1978) reported that UK and SK transform plasminogen into plasmin by rupture of a Arg- Val bond and the liberation of a peptide with a molecular weight of 6 to 8- KDa. UK is a physiological activator with a direct action. By contrast, SK is an enzyme of bacterial origin and two hypotheses may be advanced to explain its mechanism of action: the formation of a SK-plasminogen complex capable of activating new molecules of plasminogen or the formation of a SK-plasminogen complex within which plasminogen is transformed to plasmin.

Kang et al. *(*1990) reported better pro-UK production at 5% serum as compared to 10% serum supplemented medium from a human kidney CAKI-1 cell line when cultured with cytodex microcarriers in a perfusion bioreactor. The medium can be supplemented with BSA, insulin, transferrin and selenium for preservation of viability in low serum for prolonged culture duration. Therefore the optimal serum concentration for UK production depends on cell type and media additives.

Khaparde and Roychoudhury (2004) reported that a 1% of serum is optimum for UK production as well as for viability of human kidney HT 1080 cells. Similarly, Tao et al. *(*1987) have used 1% of serum as optimum for UK production.

Lacroix and Fritz (1982) reported that the rate of production and release of UK were greatly influenced by a variety of factors including cell density, presence of hormones, incubation temperature and duration of culture.

Suzuki et al. (1989) reported the enhanced UK productivity of 1956 IU/ml when compared to 294 IU/ml in the controls by human normal diploid fibroblasts which were cultured in serum free medium containing phospholipase-A2, phospholipase-C, bradikinin, coenzyme-A and phytohaemagglutinin as inducers.

Chen et al. (1996) have formulated serum-free media by using orthogonal experiments for the growth of genetically engineered Chinese hamster ovary (CHO) cell line 11G and reported an increase of 80% in UK production.

Deutheux et al. (1997) examined the production of UK by culturing human diploid fibroblasts in a serum-free medium supplemented with peptones (protease peptone) and K^+ ions for efficient expression of human gene in *Escherichia coli*. In cultures of 3T3 fibroblasts, the UK activity increased by 20 fold when compared to control cells within 24 h when 100 M of sulphur mustard (SM) was added. Also ryanodine (10 M) amplified the UK upregulation by two fold and dexamethasone (1 M) added directly after SM treatment almost completely prevented the induction of UK at both the protein and mRNA levels.

Jo et al. (1998) developed a serum -free perfusion culture for the production of UK. The cell-growth profile showed a continuous increase in cell density, reaching 5.1×10^7 cells/ml and the production of UK remained stable throughout the culture (1586 ± 247 IU/ml).

Gomes et al. (2000) reported a 10-fold increase in UK activity with simultaneous supplementation of three amino acids, based on their repeated occurrence in the normal pro-UK produced by human kidney cell line HT 1080 culture.

Recently Chen et al. (2004) developed a serum-free medium for the production of UK by adding insulin, a trace element mixture, a lipid mixture, ascorbic acid and pluronic F68 to dulbecco´s modified eagle´s medium (DMEM)/F12 (1:1, v/v).

Usually UK production by mammalian cells depends on the following factors: (i) regulation of UK expression (ii) supply of amino acid building blocks for UK synthesis. Moreover some amino acids like glycine have been known to bring about stabilization of proteins (Chainiotakis, 2004), while arginine is known to induce UK by acting as precursor of nitric oxide, which induces UK production (Ziche et al., 1997).

For UK induction, preferable compounds are saccharides such as glucose, inositol, ribose and deoxyribose, hormones such as adrenaline (Bansal and Roychoudhury, 2006; Bansal et al., 2007).

The enhanced production of PA activity was shown to be a characteristic of many malignant cell types. The intracellular and extracellular levels of PA were demonstrated to be substantially elevated in malignant cells (Christman et al., 1975), cells treated with a tumor promoter (Wigler and Weinstein, 1976), activated macrophages (Unkeless et al., 1974), established cell lines (Mott et al., 1974; Rifkun and Pollack, 1977), granulosa

Table 1. Production of UK using different recombinant cell lines.

Recombinant cell type	UK activity	Reference
Mouse cells LB6	0.8 mg/L/day	Nolli et al. [1989]
Chinese hamster ovary cell line	1000 PU/ml	Avgerinors et al. [1990]
Saccharomyces cerevisiae cells	1863 PU/ml	Turner et al. [1991]
Namalwa KJM-1	3 g/10^6 cells/day	Satoh et al. [1993]
E. coli cells	1500 PU/ml	Tang et al. [1997]
Chinese hamster ovary cell line	860 PU/ml	Jo et al. [1998]

cells during ovulation (Strickland and Beers, 1976), embryonic cells during differentiation (Topp et al., 1976) and hormone treated cells (Katz et al., 1977).

Recombinant studies

Human UK can be used to treat acute thromboembolic events such as venous and arterial thrombosis, pulmonary embolism, intracardiac thrombosis, and systemic embolism. However, the high cost of isolation of UK from either tissue culture cells or urine limits the use of this enzyme as a therapeutic agent. If UK could be obtained from microorganisms by recombinant DNA technology, one might have a more economical method of production.

In order to improve UK production, UK has been expressed in bacteria (Tang et al., 1997; Fahey and Chaudhuri, 2000; Sun et al. 2003; Zhong et al., 2007; Beaton et al., 2005; Gurskii et al., 2005; Ratzkin et al., 1981; Deutheux et al., 1997), fungi (Hiramatsu et al., 1991) , yeast (Wang et al., 2000; Hiramatsu et al., 1989, 1991; Turner et al., 1991), mammalian cells (Nelles et al., 1987; Hu et al., 2006; Innis and Scott, 2001), insect cells (Innis and Scott, 2001) and in plants (Oishi and Zhou, 2000). Different recombinant cell lines that have been used for production of UK are listed in Table 1. Large-scale culture of bacteria or fungi is relatively easy but the main drawback of using prokaryotic system for UK production is the absence of post-translational modification machinery in these organisms. Therefore, the non-glycosylated UK so obtained does not have the same efficacy and pharmacodynamic properties as that of native UK. Mammalian cell lines are hence, preferred for production of UK. A considerable number of mammalian cell lines have been reported to date for UK production.

Tang et al. (1997) developed a system to produce recombinant urokinase-type plasminogen activator (u-PA) in *E. coli*. The u-PA was produced with 6 His-tag at the C-terminus, which was shown to have the same activity, after refolding, as the wild- type protein.

Kohno et al. (1984) reported the establishment of a permanent cell line, TCL- 598, which produces and secretes UK into the medium in smaller quantities.

Hiramatsu et al. (1991) described the production of human pro-UK and its deletion mutants in yeast. They succeeded in producing large amounts of human pro-UK and its mutants as the core-glycosylated forms, which were mainly accumu-lated within yeast endoplasmic reticulum (6667 PU/ml of cul-ture medium). But these accumulated pro-UKs were inactive in their native state and needed to be converted to a biologically active form by a denaturation-refolding proce-dure.

CHO cells are considered ideal hosts for recombinant UK production (Warner, 1999). These cells offer the advantages that they can be easily genetically manipulated, can be adapted for large-scale suspension culture and it can give rise to proteins with glycans which are similar, although not identical and to those found on human glycoproteins.

Interestingly, Kim and Swartz (2004) have illustrated that UK could be efficiently synthesized in *E. coli* based cell- free systems using glutathione redox buffer coupled with the disulphide isomerase to facilitate formation of disulphide bonds. Recently, Roychoudhury et al. (2006) reported that the CHO cell line is known for its unstable karyotype. Loss of recombinant gene copy number and appearance of non- producing populations of cells were predominant causes for instability of production.

Immobilization and bioreactor studies

Wagner et al. (1990) studied the production of pro-UK by a human kidney tumor cell line in long term cultures. Cells were grown on microcarriers, which were retained inside the reactor by sedimentation, or with a spin filter.

Two modes of operation were compared: feed harvest at an average medium exchange rate of 0.3 per day and continuous perfusion at a higher dilution rate of 1.5 per day. In the two systems a stable production of pro-UK could be maintained for more than 400 h. Continuous perfusion yielded a higher cell density than feed harvest resulting in a 2-fold increase in the reactor productivity. But higher final enzyme activities were obtained with harvest recovered medium than in the perfusion medium. The cumulative medium consumption for mass of product was the same in the repeated batch and in the continuous operation mode.

Kang et al. (1990) investigated kinetics of formation of UK from pro-UK in CM and worked for a possibility of lowering the conversion to UK cultivating human cell lines under perfusion operations. A human kidney cell line,

CAKI-1, was cultivated in DMEM with FBS, glutamine and gentamicin without Ca^{2+} to prevent clumping. Cells were then inoculated into a 2L bioreactor with micro-carriers when cell density reached $1 \cdot 10^6$ viable cells/ml. It was observed that better production of pro- UK was obtained with 5% serum containing media than 10% or serum free medium on cytodex II microcarrier under perfusion chemostat operations. Conversion of pro-UK was reduced in the serum containing media.

Senatore and Bernath (1986) had immobilized UK to the inner surfaces of fibrocollagenous tubes (FCT) in an attempt to develop a fibrinolytic biomaterial, which may be suitable for use as a small diameter vascular prosthesis. The enzyme was bound by adsorption followed by glutaraldehyde cross -linking. An *in vitro* kinetic study of immobilized UK was conducted by employing the tubular material as a flow through reactor operated in a batch recycle mode in which the esterolysis of the model substrate, *N*- *α*-acetyl-L-lysine methyl ester (ALME), was monitored as a function of substrate concentration, recycle flow rate, and temperature. Results were compared with data from the soluble enzyme reaction, which was conducted in the presence and absence of 10% swine skin gelatin, in order to identify the specific effects of a collagenous microenvironment. Observed rates for the UK-FCT catalyzed reaction were observed to be dependent on recycle flow rates below 12 ml/min (Re = 107). Apparent Michaelis- Menton rate parameters were determined by nonlinear search technique for two flow rates: one above the critical point for external diffusion effects (Re = 282) and one with in the mass-transfer-limited region (Re = 71). When the later data were corrected for external diffusion by applying the Graetz correlation for laminar flow in tubes to estimate the mass transfer coefficient, the corrected K_m of 6.45 ± 0.38 mM agreed very closely with the diffusion free parameter (that is, 6.13 ± 0.63) . Furthermore, this value was observed to be an order of magnitude higher than that of the soluble enzyme but approximately equal to the K_m of the soluble enzyme in a 10% gelatin environment (8.13 ± 1.53 mM). It is postulated that the difference in kinetic parameters between soluble and collagen immobilized UK is due to an inherent interaction between collagen and enzyme rather than to mass transfer effects. Such an interaction is supported by the effects of collagen on thermal stability and energy of activation.

Human fetal cells (HF) from explants of neonatal fore skins were cultured in DMEM containing calf serum and antibiotics. Microcarrier cultures of these cells were prepared and seeded into microcarrier beads and incubated with medium to grow to confluence (9 - 15 days). Serum free cultures were prepared by rinsing the beads with PBS, and adding medium containing ovalbumin and epidermal growth factor. After 48 h, phorbol myristate acetate was added and the cultures were incubated for an additional 24 h. The culture medium was then collected and fresh medium containing 10% fetal calf serum was added (Eaton et al. 1984).

One of main problems in the cultivation of human and animal cells is the fact that most of these except a few malignant continuous cell lines are anchorage dependent for their growth *in vitro*. This means that they require for growth *in vitro* a suitable solid surface to which they can attach and spread. Hence standard fermentors cannot be applied for cultivation of these cells. Production of a variety of biomolecules required anchorage-dependent cells such as primary cells or diploid cell lines. Van Wezel (1967) discussed the various cultivation systems for these anchorage-dependent cells with special attention to the microcarrier culture system. In these systems, cells are grown on small particles suspended in culture medium by stirring. Cells attach and spread upon carriers and grow out gradually to a confluent monolayer. DEAE sephadex was used as a microcarrier.

Smidsrod and Skjak-Braek (1990) stated that in recent years, entrapment of cells within spheres of Ca^{2+} alginate has become the most widely used technique for immobilizing living cells. This versatile method includes applications ranging from immobilization of living or dead cells in bioreactors, immobilization of plant protoplasts for micropropagation and immobilization of hybridoma cells for production of monoclonal antibodies, to entrapment of animal cells for implantation of artificial organs. This review evaluates the potential of this method on the basis of the current knowledge of structural and functional relationships in alginate gels.

Avgerinors et al. (1990) used a 20 liter stirred tank fermentor, equipped with a 127 mesh ethylene tetraflouro ethylene rotating screen for cell recycle, for the continuous production of recombinant single chain urokinase-type plasminogen activator (rscu-PA) from CHO cells. Viable cell densities between 60 and 74 millions per ml were maintained at medium perfusion rates of 3 to 4 fermentor volumes per day. Cells were retained by the 120-microns nominal opening filter through the formation of "clumped" cell aggregates of 200 to 600 microns in size, which did not foul the filter. The rscu- PA produced over the course of this continuous culture was purified and characterized both *in vitro* and *in vivo* and shown to be comparable to natural scu-PA produced from the transformed human kidney cell line, TCH-598.

Tamponnet et al. (1992) immobilized primary cultivated rabbit articular chondrocytes in calcium alginate beads. Both free and entrapped cells were allowed to grow under normal conditions. After long-term immobilization, the cells still exhibited metabolic activities, patterns of division, synthesis and secretion of extracellular matrix macromolecules such as type II collagen and proteoglycans. After 38 days, immobilized rabbit articular chondrocytes predominantly expressed type II but not type I collagen. Thus, they maintained their cartilage phenotype. After bead lysis, harvested cells showed normal growth patterns when resuspended in culture medium. On the basis of these results, long-duration storage and

large-scale production of extracellular matrix components are being investigated.

Kuo and Bjornsson (1993) developed a simple and sensitive method for the simultaneous determination of free t-PA and u-PA concentration in biological fluids using a solid- phase immuno assay. Microtiter plates were coated with polyclonal goat antibodies and incubated with PA standards or unknown samples. The absorbed PA's were then assayed by incubation with a mixture of plasminogen, poly-L-lysine, and the chromogenic substrate H-D-norleucylherahydrotyrisyllysine-*P*-nitroanilide. Free t-PA and free uPA were detectable in human plasma and urine and in conditioned media from different endothelial cell cultures.

Recently Kumar et al. (2006a) developed a novel type of cell culture device based on supermacroporous polyacrylamide cryogel support with immobilized gelatin for continuous production of UK from human fibro sarcoma HT 1080 and human colon cancer HCT 116 cell lines. The anchorage dependent cells attached to the matrix within 4 - 6 h of inoculation and grew as a tissue sheet inside the cryogel matrix. Continuous UK secretion into the circulating medium was monitored as a parameter of growth and viability of cells inside the bioreactor. A high yield of viable cell count (3.0×10^9 cells/ml) was obtained after continuously running the cell culture reactor for 32 days, during which 152,600 PU of UK was obtained from 500 ml of culture medium. No morphological changes were observed on the cells eluted from the gelatin-cryogel support andre-cultured in normal cell culture flasks. While the cryogel matrix itself is biocompatible with cells, coupling of gelatin makes it particularly suitable for growing anchorage dependent cell lines.

The potential of a cryogel bioreactor as a tool for process development of mammalian cell culture has many advantages. By using cryogel scaffolds as disposables, one can get rid of the problem of contamination and additional expenditures on securing sterile safe guards at considerable extra costs, particularly for scale up processes for continuous runs.

Integrated production and purification of UK from bioreactor

One of the most successful approaches in improving the economy of bioprocesses is to reduce the number of steps involved, as each successive step amounts to considerable loss of the product.

Khaparde and Roychoudhury (2005) successfully developed a two-step integrated process for producing UK from HT 1080 cell line in a hollow fiber reactor to which a sterile 50 ml benzamidine-Sepharose affinity column was coupled for on- line separation of UK. Approximately 4.5×10^4 PU of UK was harvested per day from the integrated set-up continuously for more than 20 days.

Similarly, Kumar et al. (2006b) developed an integrated set-up of gelatin-pAAm cryogel bioreactor was further connected to a pAAm cryogel column carrying Cu(II)-iminodiacetic acid (Cu(II)-IDA-pAAm cryogel), which had been optimized for the capture of UK from the conditioned medium of the cell lines. Thus an automated system was built, which integrated the features of a hollow fibre reactor with a chromatographic protein separation system. The UK was continuously captured by the Cu (II)-IDA-pAAm cryogel column and periodically recovered through elution cycles. The UK activity increased from 280 PU/mg in the culture fluid to 1300 PU/mg after recovery from the capture column which gave about 4.5 fold purification of the enzyme. The integrated bioreactor system operated continuously for 32 days during which no backpressure was observed because of the porous structure of the cryogel matrix. The enzyme eluted from the Cu(II)-IDA-pAAm cryogel capture column was further purified on benzamidine-Sepharose affinity column which gave a preparation of different forms of UK with activity of 13550 PU/mg of protein. This is one of the very few successful UK production strategies using mammalian cell lines.

Clinical applications of UK

UK is used clinically as a thrombolytic agent in the treatment of severe or massive deep venous thrombosis, pumonary embolism, myocardial infarction, and occluded intravenous or dialysis cannulas. Recently, Alteplase has replaced UK as a thrombolytic drug in infarctation.

The clinical use of u-PA is as a thrombolytic agent. However, it is also a prognostic marker for tumors and its structure is the basis for the design of new anti-cancer drugs and is used for the targeting of cytotoxic agents to u-PA receptor expressing cells (Alfano et al., 2005; Blasi and Carmeliet, 2002; Dano et al., 2005; Sidenius and Blasi, 2003).

Conclusions

Thrombolytic diseases are today a major cause of morbidity and mortality world wide. Fibrinolytic enzymes have apparent significance in thrombosis therapy. Therefore great attention has been directed towards a search for thrombolytic agents of various origins with particular reference to agents with more specificity and less toxicity. UK is one such enzyme without immunogenicity and cross reactivity when given to patients. Because of its clinical importance, UK has eluded the interest of researchers as obvious from the lack of reports relating to any kind of technological advancements for its production from *in vitro* cell culture. Developments in cell lines and bioprocess technology have made it possible to produce UK from *in vitro* cell culture. The main source of UK is from cell cultures of lung, heart and kidney tissues. These tissues are normally derived from mammalian source. Cloning of the UK genes followed by heterologous expression provide higher enzyme yields. Therefore, im-

proving the productivity and reducing the production cost are the major goals for the current studies on the UK production.

New bioreactor designs based on supermacroporous cryogel matrices promise major advantages. In the integrated set- up the coupling of purification column with the production bioreactor provides efficient strategy for the production and purification of UK by reducing the process steps and leading to significant improvement in production.

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