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PLASMINOGEN ACTIVATOR-ANTI-HUMAN FIBRINOGEN CONJUGATE

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Summary: A covalent conjugate between the plasminogen activator urokinase and polyclonal rabbit anti-human fibrinogen has been formed using the heterobifunctional coupling reagent N-succinimidyl 3-(2-pyridyldithio) propionate. The resultant urokinase-anti-human fibrinogen conjugate was separated from unreacted material by gel filtration. The conjugate exhibited amidase activity against the small chromogenic substrate pyroglutamyl-glycyl-arginine-p-nitroanilide as well as plasminogen activator activity in an assay employing plasminogen and the plasmin substrate D-valyl-leucyl-lysine-p-nitroanilide. Retention of antibody specificity for fibrinogen was demonstrated using an enzyme linked immunoassay procedure. The conjugate was found to have greater stability in human plasma than unconjugated urokinase.

Human urokinase (UK) is used as a therapeutic fibrinolytic agent in certain disorders such as deep vein thrombosis and pulmonary embolism. UK, which has little if any affinity for fibrin, activates plasminogen not only at the fibrin clot but throughout the circulatory system thereby producing deleterious side effects such as hemorrhaging, tissue damage and depletion of various plasma proteins (1).

Modification of UK to increase its affinity for fibrin should greatly improve its suitability for therapeutic use by enhancing its fibrinolytic properties and concommitantly reducing the unwanted side effects.

This preliminary study demonstrates that an active conjugate can be prepared between UK and an antibody. The results obtained suggest that a similar conjugate between UK and a monoclonal antibody specific for human fibrin, such as those recently described (2,3), can be prepared and used to target this plasminogen activator to fibrin clots in vivo.

Abbreviations used: UK, urokinase; AHF, rabbit anti-human fibrinogen; SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate; S-2444, pyroglutamyl-glycyl-arginine-p-nitroanilide; S-2251, D-valyl-leucyl-lysine-p-nitroanilide.

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MATERIALS AND METHODS

Enzymes and Proteins. Partially purified human urinary UK was kindly supplied by Dr. Genesio Murano (Bureau of Biologics, FDA). Additional UK was prepared by modifications of the methods of White and Barlow (4). UK was further purified by p-aminobenzamidine agarose (Pierce) affinity chromatography as described by Holmberg et al. (5). Plasminogen was prepared by lysine-Sepharose (Pharmacia) affinity chromatography of human plasma (6). Human fibrinogen (Kabi Diagnostics, Sweden) was further purified by lysine-Sepaharose and DEAE-cellulose chromatography (7). Rabbit polyclonal anti-human fibrinogen (Accurate), peroxidase coupled goat anti-rabbit IgG (Sigma) and bovine serum albumin (Sigma) were used without further purification.

Enzyme Assays. UK amidase activity was determined using the chromogenic substrate pyroglutamyl-glycyl-arginine-p-nitoanilide, (S-2444, Kabi)(8). UK plasminogen activator activity was determined using plasminogen and the plasmin specific substrate H-D-valyl-leucyl-lysine-p-nitroanilide (S-2251, Kabi)(9). In both cases, substrate hydrolysis was monitored by observing the increase in absorption at 405 nm.

UK Conjugation to Anti-human Fibrinogen. UK in 0.1 M sodium phosphate buffer, pH 7.5 containing 0.15 M NaCl, was reacted with N-succinimidyl 3-(2-pyridyl-dithio)propionate (SPDP, Pierce) for 30 minutes at R.T. and then dialyzed extensively against the above phosphate saline buffer at 4°C (10). Anti-human fibrinogen (AHF) was partially reduced by one hour R.T. incubation with 0.1 M dithiothreitol at pH 7.5, after which the AHF was separated from the reductant by gel filtration using Sephadex G-25. The AHF and modified UK were combined as the coupling mixture and incubated for 24 hours at R.T.

Modified Enzyme Linked Immunosorbent Assays. Microtiter plate wells (96 well, Linbro) were coated with human fibrinogen by incubating each well with 0.2 ml of 5 ug/ml fibrinogen in phosphate buffered saline (PBS) for 1 hour at 37°C. The plates were thoroughly rinsed with PBS containing 0.05% Tween. Conjugated UK-AHF or unconjugated UK, AHF or mixtures thereof were then incubated in the fibrinogen coated wells for 1 hour at 37°C. After thorough washing of the plates with PBS-Tween, they were examined for: (1) UK amidase activity: the UK substrate S-2444 in 0.05 M TRIS, 0.038 M NaCl, 1% albumin, pH 8.8, was added to the wells; the plates were incubated at 37°C, and the change in absorbance at 405 nm was determined; (2) UK plasminogen activator activity: plasminogen and the plasmin substrate S-2251 in 0.05 M TRIS, 0.1 M NaCl, pH 7.4 were added to the wells, and the absorbance change at 405 nm was determined after incubation at 37°C; (3) AHF binding to the wells: peroxidase-coupled goat anti-rabbit IgG in PBS was incubated in the wells for 1 hour at 37°C . After thorough washing of the plates, phosphate-citrate buffer, pH 5, containing o-phenylenediamine and hydrogen peroxide was added to the wells. After a 5 minute incubation at 37°C, the reaction was stopped with sulfuric acid, and the absorbance change was determined at 490 nm (11). Absorbance changes were monitored using a Biotek microtiter plate reader.

RESULTS AND DISCUSSION

UK and AHF were conjugated using SPDP as the covalent linking agent. When the conjugation reaction was completed, the coupling mixture was subjected to gel filtration on a Sephadex G-200 column (Figure 1). In contrast to uncoupled mixtures of UK and IgG, UK activity was detected in several fractions - the

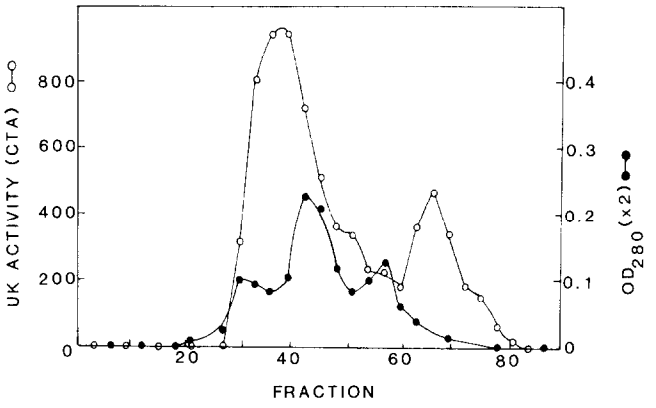

Figure 1. Separation of UK-AHF Conjugate from Unconjugated Reactants by Gel Filtration. Following the UK and AHF coupling reaction described in methods, the reaction mixture was chromatographed on a precalibrated Sephadex G-200 column (2.6 x 43 cm) equilibrated with 0.02 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl, 0.001 M EDTA and 0.02% azide. The closed circles represent absorbance at 280 nm and the open circles the UK amidase activity which was determined as described in methods. The activity of the UK is expressed in CTA (Committee on Thrombolytic Agents) units where 1 CTA unit causes an absorption increase of 0.075 units/ml/hour under the assay conditions. The peak fraction of calibration standards were: ferritin (440,000 daltons) fraction 38; IgG (160,000 daltons) fraction 47; and ovalbumin (47,000 daltons) fraction 65.

first and largest peak emerging just after the void volume of the column and well ahead of the IgG peak. Since the highest reported molecular weight form of UK is approximately 56,000 daltons (12), these results suggest that an active conjugate had been formed. The recovery and distribution of UK activity are shown in Table I.

One of the high molecular weight fractions with UK amidase activity (fraction 34) was examined for the presence of UK-AHF conjugate using enzyme linked

Table I. Recovery of UK-AHF Conjugate

Fraction	Total units (CTA)	Percent recovery This step	Overall
Native UK	69,200	-	100
Product 1	47,808	69	69
Product 2	27,019	56	39
UK-AHF*	12,578	47	18
Unconjugated UK*	8,933	33	13

Product 1: Dialyzed UK after reaction with SPDP.
Product 2: A mixture containing UK-AHF and unconjugated reactants.
* Conjugated and unconjugated UK after separation of the products of the coupling reaction on Sephadex G-200.

Table II. Preliminary Characterization of UK-AHF Conjugate

A. Amidase Activity [1]

Sample	Fibrinogen Coated Wells	Uncoated Wells
UK-AHF Conjugate	0.299 +/- 0.011	0.071 +/- 0.009
AHF	0.053 +/- 0.004	0.055 +/- 0.003
Unconjugated UK and AHF	0.087 +/- 0.003	0.084 +/- 0.004
UK	0.082 +/- 0.002	0.066 +/- 0.004

B. Plasminogen activator and IgG detectable in UK-AHF conjugates bound to fibrin-coated wells

Dilution of UK-AHF Conjugate	Plasmin Activity [2]	AHF Bound [3]
undiluted	>2.0	0.302 +/- 0.007
1:10	0.601 +/- 0.074	0.160 +/- 0.013
1:20	0.421 +/- 0.032	0.116 +/- 0.004
1:40	0.282 +/- 0.015	0.081 +/- 0.003
1:80	0.195 +/- 0.040	0.055 +/- 0.003

1. Absorbance at 405 nm using the substrate S-2444. All assays were run in triplicate.
2. Absorbance at 405 nm (in triplicate, corrected for blanks) after incubation of plasminogen and S-2251 in wells previously washed to remove unbound material.
3. Absorbance at 490 nm (in triplicate, corrected for blanks) due to peroxidase coupled goat anti-rabbit IgG in the presence of o-phenylenediamine and hydrogen peroxide.

immunosorbent assay (ELISA) procedures as described in the methods section. In addition to UK amidase activity, this fraction also demonstrated UK plasminogen activator activity and a strong binding affinity for human fibrinogen (Table II).

The UK-AHF conjugate was stored at 4°C in 0.05 M phosphate buffer, pH 7.5, containing 0.2 M NaCl, 0.001 M EDTA, 0.5 mg/ml albumin, and 0.02% azide. Under these conditions, the conjugate retained over 70% of its initial activity after 3 months. In addition, the amidase activity of the UK-AHF conjugate was much more stable in human plasma than unconjugated UK (Figure 2). This suggests that the conjugate may have a longer $t_{1/2}$ in the circulatory system than native UK.

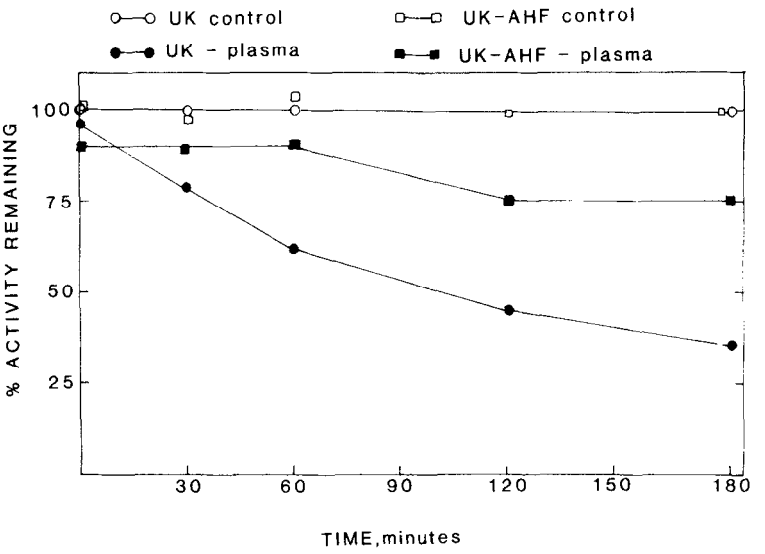

Figure 2. Effect of Human Plasma on the Activity of Native UK and the UK-AHF Conjugate. UK or UK-AHF was incubated with plasma at 37°C. Aliquots, containing comparable units of initial UK activity, were removed after incubation for varying times, and the UK activity remaining was determined using the amidase substrate S-2444 as described in methods.

These experiments demonstrate the feasability of producing functional covalent conjugates between potent plasminogen activators, such as human urokinase, and antibody molecules. The enzymatic activity of the conjugate reported herein also exhibited an increased stability in human plasma relative to unconjugated urokinase. Conjugates of UK or other plasminogen activators with monoclonal antibodies that have high affinity and specificity for human fibrin should therefore prove valuable for therapy of a variety of disorders.

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