Development of Immunoassays for Human Urokinase

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ABSTRACT

The purpose of this work, is to develop radioimmune assays (RIA) and enzyme-linked immune assays for measurement of pro-urokinase and the two active forms of the enzyme. Polyclonal and monoclonal antibodies, with desired specificities against preselected synthetic regions of urokinase (UK), were obtained by immunization with the respective synthetic peptides and used to develop RIA for zymogen and the two activated forms of UK.

INTRODUCTION

Continuous flow electrophoresis in microgravity has been employed in the separation of kidney cells into subgroups (1). It is necessary, however, to determine whether the separated cells retain function. Kidney-cell subgroups produce various amounts of UK. Presently, the assay for UK depends on the measurement of enzymic activity. This method is not very sensitive and it is an unreliable indicator of the amount of secreted UK because of the production of enzyme inhibitors by the cells. Furthermore, the method is unable to detect the zymogen, which is the initial synthetic product. The zymogen is synthesized as a single chain protein composed of 411 amino acid residues (2-4). This single chain zymogen has no enzymatic activity. Activation of the zymogen involves cleavage of the peptide bond Lys.158-Ile.159 and the loss of Lys-158, to produce a two-chain high molecular weight active UK (3,4). In this active enzyme, the A chain (residues 1-157) is linked to the B chain (residues 159-411) by a disulfide bond between Cys-148 and Cys-279 (3,4). Cleavage of the bond between Lys-135 and Lys-136 removes the segment 1-135 and gives a low molecular weight active enzyme (5,6) in which peptide 136-157 (A1 chain) is linked to the B chain by the above disulfide bond. Production by kidney-cell subgroups of normal, or even high, levels of UK cannot be measured when little or none of the zymogen undergoes activation.

In order to better monitor kidney cell separation and obtain a sound correlation with viability and activity of the various cell groups, a very precise method for measurement of all of the forms of urokinase (i.e. zymogen and the two active forms) is needed. The purpose of this work is to develop radioimmune assays (RIA) and enzyme-linked immune assays for measurement of the three forms of the enzyme. These assays depend on the preparation of polyclonal and monoclonal antibodies with specificities directed against preselected regions of UK. These preselected regions are made synthetically and the synthetic peptides are used as immunogens to obtain antibodies of the desired specificities (7-9). These antibodies will be used to develop RIA and ELISA assays for zymogen and activated UK.

It should be noted that development of these assays for UK will have a useful clinical spin-off. The enzyme is secreted by human carcinomas, especially in the lung, prostate, breast and colon cancers (10-13). By developing a sensitive assay for UK, it will be possible to detect these cancers early, even before a solid tumor becomes visible. Early detection will be extremely useful for design of therapy.

Abbreviations: UK, urokinase; RIA, radioimmune assay; BSA, bovine serum albumin; PBS, 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2

METHODS

Preparation of peptides and peptide conjugates

Peptides were used as immunogens for the preparation of antibodies that possess pre-determined specificities. The locations of these regions within the UK polypeptide chain are shown in Table 1. These peptides were (or are being) synthesized, purified and analyzed by previously published procedures (7-9). Synthetic peptides were coupled to exhaustively succinylated (Su) derivatives of BSA, or hen lysozyme or to the native proteins after activation of the carrier carboxyl groups by conversion to the p-nitropheny-lester groups (14). The extent of coupling was determined by amino acid analysis of acid hydrolysates of the peptide-protein conjugates.

Antisera against peptides

Antibodies against the synthetic peptides were raised in mice by two methods (a) Immunization by peptide-protein conjugates: Mice were injected and boosted with the peptide-SuBSA conjugate (50 µg per mouse) as an emulsion in complete Freund's adjuvant in the footpads and subcutaneously in the neck. Serial bleedings from 10 days prior, up to 230 days after, the first injection will be collected and studied separately. (b) Immunization with free peptide: In view of the finding (7) that small synthetic peptides (6 residues or larger), when immunized in their free form in complete Freund's adjuvant, will stimulate an in vivo antibody response, peptides were injected into mice in their free form (i.e. without coupling to carrier). Each animal received peptide (25-50 µg per mouse) as an emulsion in complete Freund's adjuvant distributed into three sites as above. The animals were boosted with similar doses 3 weeks after the first injection and thereafter monthly. Serial bleedings from 10 days before, up to 200 days after, the initial injection were studied separately.

Preparation of monoclonal antibodies of preselected specificities

The recent discovery (7) that synthetic peptides will evoke antibody formation when used as immunogens in their free form (i.e. without coupling to a carrier) has been exploited in this laboratory to prepare monoclonal antibodies with preselected submolecular binding specificities to desired protein regions (15). Peptides representing antigenic sites as well as synthetic peptides representing surface regions that are not antigenic when the whole molecule is used as an immunogen have been shown to produce antisera and subsequently monoclonal antibodies of preselected specificities (16,17).

Mice were immunized as above with a given synthetic peptide (25-50 µg) in complete Freund's adjuvant and boosted and test-bled at 3 week intervals until high antibody titer was obtained in the test sera. Somatic cell fusions, hybridoma selection, limiting dilution cloning and subcloning, and hybrid cell expansion were performed as described by Schmitz et al. (15). Expanded subclones were also injected into BALB/cByJ mice (2×10^6 cells/1.0 ml fresh tissue culture media) that had been primed with pristane (Sigma Chemical Co., St. Louis, MO). Ascites fluid were collected, clarified and stored frozen at -20°C until screened for the presence of hybridoma antibodies.

Radiolabelling of proteins

Immune IgG preparations or protein A were radiolabelled with ^{125}I (Amersham Corp., Arlington Heights, IL) using the chloramine-T method (18). Unbound ^{125}I was separated from the radiolabelled sample by gel filtration on Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). Protein-associated ^{125}I was assayed by precipitation with 10% (v/v) trichloracetic acid.

Table 1. Synthetic peptides representing different regions of human urokinase*

A. Peptides from the region 1-135 (to make antibodies specific for this region)

	Peptide	Structure
(1)	1-12	S.N.E.L.H.Q.V.P.S.N.C.D
(2)	59-73	R.G.K.A.S.T.D.T.M.G.R.P.C.L.P
(3)	102-111	C.R.N.P.D.N.R.R.R.P
(4)	103-113	R.N.P.D.N.R.R.R.P.W.C
(5)	126-135 (G at 131)	C.M.V.H.D.G.A.D.G.K
(6)	127-135	M.V.H.D.G.A.D.G.K
		59 73
(7)	(59-73)-(103-113)	R.G.K.A.S.T.D.T.M.G.R.P.C.L.P 103 113 R.N.P.D.N.R.R.R.P.W.C
		102 112
(8)	(102-112)-(126-135) G at 131)	C.R.N.P.D.N.R.R.R.P 126 135 C.M.V.H.D.G.A.D.G.K

B. Peptides specific for the zymogen (to make antibodies specific for the single chain zymogen).

(9)	155-162	P.R.F.K.I.I.G
(10)	154-163	R.P.R.F.K.I.I.G.G.E

C. Peptides from the B chain (to make antibodies that will react with all forms of the enzyme).

(11)	177-189	Y.R.R.H.R.G.G.S.V.T.Y.V.C
(12)	205-215	C.F.I.D.Y.P.K.K.E.D.Y
(13)	222-233	S.R.L.N.S.N.T.Q.G.E.M.K
(14)	319-341	L.I.S.H.R.E.C.Q.Q.P.H.Y.Y.G S C.L.M.K.T.T.V.E
(15)	282-293	S.M.Y.N.D.P.Q.F.G.T.S.C
(16)	401-411	S.H.T.K.E.E.N.G.L.A.L
(17)	(177-189) (205-215)	Y.R.R.H.R.G.G.S.V.T.Y.V.C C.F.I.D.Y.P.K.K.E.D.Y

* The single letter notations of the amino acids are: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. From ref. 52.

Screening for antibody binding by plate assay

Polyvinylchloride protein assay plates (Costar, Cambridge, MD) were incubated for 3 hours at 37°C with excess (1.5 µg in 50 µg of PBS/well) test and control antigens, washed extensively with PBS, and blocked with 1% BSA in PBS (100µl/well) for 1 hr at 37°C to prevent non-specific binding of subsequent reagents. After washing, the plates were used for binding antibody.

Sera, culture supernatants and clarified ascites fluids were screened for anti-UK antibodies using a solid phase RIA described by Sakata and Atassi (19) as modified by Schmitz et al. (15). This assay was also used to determine antibody binding specificities to peptides. Briefly, RIA plates that had been coated with the appropriate test antigens (various forms of UK or peptide conjugates) were incubated for 3 hours at 37°C with an antibody preparation (50 µl/well) appropriately prediluted in PBS-BSA so as to maximize specific binding. The plates were subsequently washed with PBS and amplified with excess (1:1000 dilution of the stock reagent in PBS-BSA) rabbit anti-mouse IgG + IgM antisera (Litton Bionetics, Kensington, MS) for 2 hrs at 37°C. After washing, the plates were developed with excess (2.0×10^5 cpm in 50 µl PBS-BSA/well) ^{125}I-labelled protein A for 2 hrs at room temperature, washed, and then separated into individual wells that are counted in a gamma counter (Beckman Instruments, Inc., Irvine, CA). Results are corrected for nonspecific (0.1-2%) binding detected in control wells not coated with test antigen but blocked with BSA.

When peptide-SuBSA conjugates are the immunizing antigens then antibody responses were analyzed by peptide-lysozyme (not succinyl lysozyme) conjugates since lysozyme and SuBSA do not cross-react immunochemically. Correction for non-specific binding is derived from binding to lysozyme control. When the free peptides were the antigens then antibodies were analyzed on peptide-SuBSA conjugates and employing lysozyme and SuBSA as controls.

RESULTS AND DISCUSSION

Antibodies that react specifically with the zymogen and the active form that has an A chain (residues 1-157) and a B chain (residues 159-411)

These will be antibodies that react specifically with the region 1-135 of the A chain. Certain regions of the molecule were selected for synthesis on the basis of the prediction that they have a high probability to be on the surface. In order for an antibody to bind to a protein, the regions it recognizes must be on the surface of the protein (20-22). However, since these predictive methods are not entirely reliable, several areas (peptides 1-8, Table 1) were selected for synthesis in order to improve our chances of success (i.e. obtaining anti-peptide antibodies that will react with the whole molecule). These peptides have all been synthesized, purified and characterized and are now being immunized into mice in order to prepare polyclonal and monoclonal antibodies that possess the desired specficities.

Antibodies that distinguish the zymogen from the active forms

The conversion of the zymogen to the active enzyme, involves scission at Lysine-158. We have synthesized a peptide centered around Lysine-158, fanning out three residues in each direction (peptide 9, Table 1). Also another peptide was made, elongating four and five residues on the two sides of Lys-158 (peptide 10, Table 1). These peptides were used as immunogens. Antibodies against the intact peptide will be expected to react only with the zymogen (in which the bond 158-159 is intact) and not with the active forms (in which this bond cleaved and Lys-158 is lost).

After injection of the peptides into outbred mice, and fusion of their spleen cells, 17 monoclonal antibodies were selected for their specificity to one of the peptides (154-163). Table 2 shows the results of this screening. These monoclonals were tested for binding to the different forms of UK by the western blot method. The nitrocellulose paper was incubated with cell culture supernatants followed by ^{125}I-labelled rabbit anti-mouse IgG. Three of the monoclonals showed specific binding to single chain UK. The clones involved are now being expanded and the monoclonal antibodies purified for further characterization and testing.

Table 2. Titer of monoclonal antibodies prepared by immunization with peptides 153-162 and 154-163.

Clone No.	Antibodies bound (cpm) by BSA-Urokinase Peptide	BSA-Nonsense peptide	BSA Only Blank
A3-E5-A7	71,813	803	901
A3-E5-B8	47,816	1,321	876
A3-E5-G10	58,503	1,454	1,311
A3-E5-H12	85,240	1,138	1,158
A3-E5-B3	59,674	871	821
A3-E5-D1	36,729	934	877
C2-A4-H4	7,633	1,855	928
C2-A4-F10	40,659	3,053	1,448
C2-A4-F11	91,196	6,987	1,726
D4-A5-E7	4,962	1,393	1,430
D4-C1-A5	25,631	1,579	1,355
D4-C1-G4	90,645	3,419	1,497
D4-C6-A4	72,303	1,101	1,045
D4-C6-AB	43,309	946	2,171
D4-C6-E12	82,320	1,405	1,055
D4-C6-F7	68,513	1,280	868
D4-C6-G12	43,013	1,109	781

note: group A; mice were immunized with free peptide 153-162
group C; mice were immunized with free peptide 154-163
group D; mice were immunized with BSA-conjugated peptide 154-163

Antibodies specific for the B chain

These will constitute general purpose antibodies that react with all forms of the enzyme. Several areas (peptides 11-17, Table 1) were selected from the B chain on the basis that they would have a high probability of being on the surface. These peptides have been (or are being) synthesized, purified and characterized. Polyclonal and monoclonal antibodies against these peptides should react with the immunizing peptides and with all forms of urokinase.

As part of this work, we have also carried out studies designed to determine optimum culture conditions, for kidney cells in bioreactor and also to find the nutrient requirements after electrophoretic separation of these cells. It is also necessary to determine whether the cells require any specific nutrients after flight experiments relative to earth-based duplicate cultures of the same cells. Depletion of certain nutrients would indicate the need for appropriate modifications in culture media composition for better support of cell growth and proliferation in future experiments.

OTHER STUDIES

Using high pressure liquid chromatography (HPLC) and amino acid analysis, we have analyzed 164 samples of culture media that were supplied to us by JSC. The results have been delivered to NASA (JSC) and a computer analysis of the data is now being carried out by NASA scientists.

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