Biochemical and Biopharmaceutical Properties of Macromolecular Conjugates of Uricase with Dextran and Polyethylene Glycol

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Uricase (UC) was conjugated with dextran and polyethylene glycol and their biochemical and biopharmaceutical properties were studied. UC–dextran conjugates (UC-D) synthesized by four methods, periodate oxidation, cyanogen bromide, carbodiimide and cyanuric chloride largely retained the UC enzymatic activity depending on the extent to which they modified amino groups. The periodate oxidation method seemed best because it gave a conjugate with high yield and satisfactory activity retention. The conjugate of UC with activated polyethylene glycol (UC-PEG_2) was also obtained with high yield but the remaining activity was somewhat lower than those of dextran conjugates at the same modification extent. UC-D and UC-PEG_2 showed sustained enzymatic activity in plasma after intravenous injection to rats. The advantage of chemical modification of proteins, especially with dextran, by the periodate oxidation method for preparation of a protein-delivery system was thus suggested.

Keywords uricase; dextran; polyethylene glycol; macromolecular conjugate; amino group modification extent; enzymatic activity; intravenous injection; rat; pharmacokinetics

A variety of biologically active proteins and peptides have recently been introduced as candidates for new drugs, but their inherent problems such as being low *in vitro* and *in vivo* stability, rapid *in vivo* clearance due to hepatic uptake and glomerular filtration, and antigenecity have hampered their clinical application. A potential approach to solve these problems seems to be their chemical modification of them by other macromolecules so that they have adequate physicochemical and biological properties for formulation and delivery.[1)]

Uricase (urate: oxygen oxidoreductase, EC 1.7.3.3, UC) is a peroxisomal liver enzyme that catalyzes the oxidation of uric acid to allantoin and has a molecular weight of about 128000 as a tetramer.[2)] This enzyme occurs widely in nature including most animals, but not in humans or birds. In humans and other species lacking this enzyme, treatment with purified UC can dramatically lower the levels of urate in plasma and urine and has potential for therapy of gout and uric acid nephropathy resulting from hyperuricemia. The efficacy of UC treatment, however, is reduced because of the production of an anti-UC antibody with repeated use. To overcome this, modification by covalent attachment was made with monomethoxypolyethylene glycol and its clinical trial was reported.[3)]

In our series of investigations, we have developed various kinds of dextran conjugates of anticancer agents as polymeric prodrugs and demonstrated their usefulness in cancer chemotherapy.[4)] In addition, covalent attachment of dextran was accomplished for soybean trypsin inhibitor (STI) and its prolonged plasma circulation and improved therapeutic effects were demonstrated.[5)] Including these works, the covalent attachment of dextran to proteins is widely known to improve the latter's immunological and pharmacological characteristics, but systematic examinations such as comparative evaluation with other approaches are still insufficient. In the present investigation, we synthesized UC–dextran conjugate (UC-D) by various methods and evaluated its biochemical and biopharmaceutical properties in comparison with those of original UC and UC–polyethylene glycol conjugate (UC-PEG_2).

Experimental

Chemicals UC from *Candida utilis* was kindly supplied by Toyobo, Osaka, Japan. Dextran [T-10 (weight-average molecular weight (Mw), 9900; number-average molecular weight (Mn), 5200), T-70 (Mw, 70800; Mn, 36000)] was purchased from Pharmacia, Uppsala, Sweden. Activated polyethylene glycol [2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine; activated PEG_2 (Mw 10000)] was purchased from Seikagaku Kogyo, Tokyo, Japan. Sodium periodate, cyanogen bromide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and other chemicals were of the finest grade available.

Synthesis of UC-D and UC-PEG_2 The UC-D conjugate was synthesized by the following four methods in which coupling reaction was done at a reaction molar ratio of about 1 : 100 (UC : dextran) and allowed to proceed at 4 °C, unless otherwise stated. The final product was washed with 0.1 M borate buffer (pH 8.5), concentrated by ultrafiltration (UK-200, Toyoroshi, Tokyo, Japan) and lyophilized.[6)]

1) Periodate Oxidation Method[7)]: Dextran (T-10) (1 g) was activated by sodium periodate (107 mg) in 10 ml of water at room temperature for 24 h, washed with distilled water, concentrated by ultrafiltration (UK-10, Toyoroshi) and lyophilized. Then UC (50 mg) was reacted with oxidized dextran (200 mg) in 0.1 M borate buffer (pH 9.5) for 24 h in the dark. The obtained conjugate was reduced by sodium borohydride for 2 h at 4 °C. Conjugate with dextran (T-70) was synthesized in the same way.

2) Cyanogen Bromide Method[8)]: Dextran (T-10) (500 mg) was activated by cyanogen bromide (100 mg) at pH 10.7—10.8. The mixture was subjected to ultrafiltration (UK-10) against distilled water and lyophilized. UC (50 mg) was dissolved in 0.1 M borate buffer (pH 9.5, 10 ml) and added to lyophilized activated dextran (200 mg) by maintaining the pH at 9.0 overnight.

3) Carbodiimide Method[9)]: Carboxyl groups were introduced to dextran (T-10) (1 g) by allowing 6-bromohexanoic acid (1.4 g) to react under an alkaline pH condition. Lyophilized dextran with carboxyl group (200 mg) was dissolved in a solution (10 ml) of UC (50 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (50 mg) was added. The mixture was kept at pH 5.0—5.5 and reaction was allowed to proceed overnight.

4) Cyanulic Chloride Method[10)]: Dextran (T-10) (100 mg) was reacted with cyanulic chloride (40 mg) at 4 °C and pH 7.0. The dextran derivative (20 mg) precipitated and washed by acetone was reacted with UC (5 mg) in 0.1 M borate buffer (pH 10.0) for 2 h.

Synthesis of UC-PEG_2[11)] UC (50 mg) was reacted with activated PEG_2 (200 mg) in 2 ml of 0.1 M borate buffer (pH 9.5) for 2 h at 4 °C. The conjugate was purified by ultrafiltration against 0.1 M borate buffer (pH 8.0) as described above.

Analytical Methods Protein concentration was determined by the method of Lowry *et al.*[12)] using bovine serum albumin fraction V as a standard in the range of 0—200 μg/ml. Dextran concentration was determined by the anthron method.[13)] The degree of modification of amino groups in UC was determined by measuring the amount of free amino groups with trinitrobenzene sulfonic acid (TNBS) using glycine as a standard.[14)]

Examination of Conjugate Formation The formation of the conjugate was confirmed by sodium dodecyl sulfate: polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed using 6.0% stacking and

10.5% resolving gels under a reducing condition with 2-mercaptoethanol.[15] After electrophoresis, the gel was stained with 9% Coomassie brilliant blue and molecular size of each conjugate was estimated from the calibration curve obtained using a calibration kit (Pharmacia, Uppsala, Sweden).

Immunological Test Antisera were prepared against UC and UC-D (T-10) in male Japanese White rabbits. An emulsion of complete Freund's adjuvant containing 3 mg protein of UC or UC-D (T-10) was injected intracutaneously on the animal's back and intramuscularly to the femoral muscle. The rabbits were boosted every 2 weeks for a month with corresponding antigen in incomplete Freund's adjuvant. Blood was obtained from the ear vein 10 d after each injection except for the final one which was obtained from the jugular artery, and the separated sera were stored at −80 °C. The obtained sera were analyzed for immunoglobulin G (IgG) and IgM by the ring test in which a precipitation ring formed between the two-fold serially diluted antisera and the antigen solution (0.2 mg protein/ml of 0.01 M phosphate buffered saline, pH 7.3) in a test tube (diameter, 3 mm) was observed. Ring test titer was expressed by the maximum dilution number of antisera by which a precipitation ring was observed upto 3 h.

***In Vitro* Stability Test** UC, UC-D, and UC-PEG_2 (2.5 unit) was treated with 5 mg of trypsin in 1 ml of 0.05 M phosphate buffer at 37 °C and pH 7.0. Aliquots were taken at various time intervals and the activity of UC measured. Stability tests were also carried out in freshly obtained rat plasma.

***In Vivo* Pharmacokinetic Study** Male Wistar rats (180—210 g) were anesthetized with pentobarbital and injected with UC, UC-D, or UC-PEG_2 dissolved in sterilized saline solution *via* the femoral vein (20 U/kg). At appropriate intervals, blood (200 μl) was sampled from the jugular vein and centrifuged at 3000 rpm for 2 min, and UC activity in the plasma was determined. Plasma concentration profiles were analyzed based on a one-compartment model by the nonlinear least squares regression analysis program MULTI.[16]

Results

Synthesis of UC-D and UC-PEG_2 All four tested methods gave UC-D (T-10) with extents of amino group modification of about 40—60% under standard conditions. On SDS-PAGE, these conjugates showed broad bands with molecular weights apparently larger than 200000, while original UC gave a single major band corresponding to a subunit of UC (Mw, 32000). UC-PEG_2 synthesized under the standard condition had a modification extent of about 30% and was almost immobile on SDS-PAGE.

Figure 1 shows the relationship between the extent of amino group modification and the remaining UC activity in UC-D (T-10) prepared by periodate oxidation method and UC-PEG_2. In this figure, the conjugates synthesized at various reaction molar ratios of UC and modifier are compared. The enzymatic activity of the conjugates decreased in proportion to percents of modified amino groups in both cases. UC-D synthesized by other methods also showed a basically similar relationship to UC-D obtained by periodate oxidation method. The loss of UC activity was somewhat higher in PEG_2 modification than that in dextran modification, as shown in this figure. Throughout these examinations, the periodate oxidation method gave the highest yield with satisfactory activity retention and reproducibility. Since this method is also simple and convenient to apply, the following examination was focused on it.

Immunological Test Tables I and II show representative results of immunogenecity and antigenecity evaluation for UC, UC-D, and UC-PEG_2. Duplicate examinations gave similar results. Both antibody productivity and reactivity to anti-UC antisera of UC were decreased by the conjugation with dextran, especially with high molecular weight dextran (T-70). The conjugation with PEG_2 resulted in a decrease of reactivity to anti-UC antisera of UC to a similar extent as the modification with dextran (T-10).

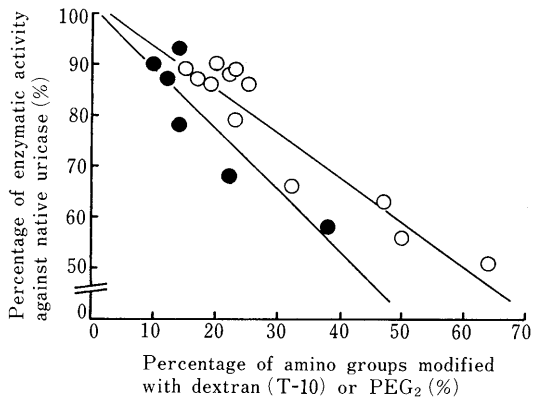

Fig. 1. Relationship between the Percentage of Amino Groups Modified with Dextran (T-10) or PEG_2 and Percentage of Remaining Enzymatic Activity in Comparison with Native Uricase

Dextran (T-10) by periodate oxidation method (○), and PEG_2 (●). Solid regression lines were fitted by the least-squares method; periodate oxidation method, $Y=-0.862X+102.1$ ($r=-0.913$), and PEG_2 method, $Y=-1.119X+100.8$ ($r=-0.907$).

Table I. Effect of Chemical Modification of Uricase with Dextran (T-10) on an Antibody Production in Rabbits

Antiserum	Test antigen	Ring test titers		
		Primary	Secondary	Tertiary
Anti-UC	UC	2	64	128
Anti-UC-D (T-10, 50%)	UC-D (T-10, 50%)	1	16	32

Each pair of rabbits received UC or UC-D immunization with complete Freund's Adjuvant on day 0 and with incomplete Freund's adjuvant on days 14 and 28. The primary, secondary, and tertiary responses were assayed 10 d after each antigen injection. Numbers in parentheses indicate the molar weight of dextran (T-10 = 100000) attached to uricase and the percentage of amino groups modified by dextran.

Table II. Results of Ring Test for UC, UC-D, and UC-PEG_2 against Antisera to UC and UC-D

Antiserum	Test antigen	Ring test titers
Anti-UC	UC	128
	UC-D (T-10, 50%)	64
	UC-D (T-70, 60%)	32
	UC-PEG_2 (10000, 38%)	32
Anti-UC-D (T-10, 50%)	UC	64
	UC-D (T-10, 50%)	32
	UC-D (T-70, 60%)	16

The molecular weight of dextran (T-10 = 10000, T-70 = 70000) and PEG_2 (10000) attached to uricase and the percentage of amino groups modified by dextran and polyethylene glycol are indicated in parentheses.

***In Vitro* Stability** UC showed rapid decrease in its enzymatic activity following a monoexponential equation with a half-life of less than 10 min when incubated with trypsin. UC-D (T-10) also showed relatively rapid activity loss and at 5 min the remaining activity was about 1% of the initial value. On the other hand, UC-D (T-70) gradually lost its activity and more than 30% of the original activity remained after 5 h. A similar result was obtained in UC-PEG_2 and thus UC was stabilized by conjugation with larger dextran or PEG_2. All UC, UC-D (T-10, T-70), and

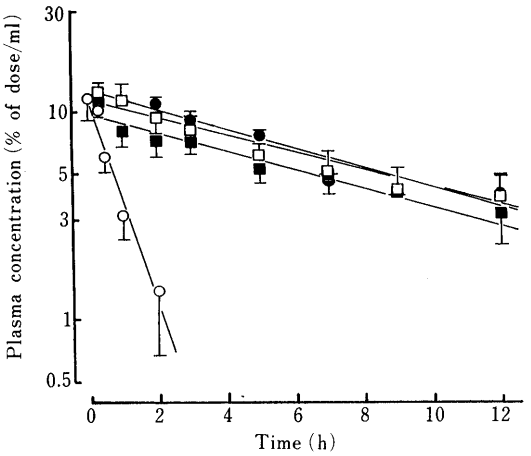

Fig. 2. Plasma UC Activity Time Courses after Intravenous Injection of UC, UC-D, and UC-PEG_2 to Rats

UC (○), UC-D (T-10) with amino group modification extent of 26% (□), UC-D (T-70) with amino group modification extent of 34% (■), and UC-PEG_2 with amino group modification extent of 24% (●). Each point represents the mean±S.D. of 3—5 animals.

PEG_2 were stable in rat plasma and more than 90% of the original activity was kept even after 24 h incubation.

***In Vivo* Pharmacokinetic Study** Figure 2 shows plasma concentrations of UC activity after intravenous injection of UC, UC-D (T-10, T-70), and UC-PEG_2. In all cases, plasma activities were decreased following approximately a monoexponential equation. The plasma concentration of UC rapidly decreased while those of dextran conjugates decreased slowly without any significant differences between them. The distribution volumes and elimination half-lives calculated based on a one-compartment model were 8.5 ml and 0.60 h for UC, 8.7 ml and 6.5 h for UC-D (T-10), 10.7 ml and 7.5 h for UC-D, and 8.0 ml and 6.6 h for UC-PEG_2, respectively. These distribution volumes were considered to correspond to total plasma volume of rats.

Discussion

Dextran has been used as plasma expander in the clinical field since its biocompatibility seems to be better than synthetic polymers. The covalent attachment of dextran to various proteins such as hemoglobin,[17] L-asparaginase,[18] and carboxypeptidase[19] has been reported to increase their thermal and/or proteolytic stability and protect them against immunological reaction. Inhibition of glomerular filtration of smaller protein was also demonstrated in our previous paper.[5] In the present study, a similar approach was applied for UC.

Among four tested methods, periodate oxidation was suggested to be the most preferable as was true in the case of the preparation of STI conjugate.[6] The obtained conjugates showed significant enzymatic activity depending on the extent of amino groups modification in them (Fig. 1). Similar results were observed in PEG_2 conjugation. Although the mechanism of this phenomenon was not clarified, it is useful from a practical viewpoint because we can estimate the activity of the final product from the reaction condition and subsequent substitution extent.

In a stability experiment against trypsin digestion, UC-D (T-10) failed to exhibit sufficient stability, suggesting that considerable exclusion volume of the modifier is necessary to protect protein from enzymatic attack. However, since UC itself was stable in rat plasma, stabilization against proteolytic enzyme does not contribute to the prolongation of biological half-life in rats. The conjugation with dextran and PEG_2 was suggested to inhibit immunological reaction of UC, but the effects were not particularly remarkable because the immunogenecity of UC itself is not high (Tables I and II).

The results of *in vivo* experiments showed that the retention of UC in the systemic circulation was remarkably prolonged by conjugation with dextran and PEG_2. Since stability and immunological reactivity problems do not seem responsible for these phenomena and the difference in distribution volume was relatively small, the increased retention may be correlated with the elimination process of UC from the plasma circulation. In the previous case of STI with a molecular weight of about 20000,[5] inhibition of glomerular filtration due to an increase in molecular size was concluded to be the predominant mechanism of enhanced plasma retention. But this is not the case in the present study, since UC has a large molecular weight and is not excreted through glomerular filtration; this was confirmed by the determination of UC activity in the urine. Consequently, the effect of conjugation must be attributed to an alteration in some irreversible uptake process, probably in the reticuloendothelial system. In our previous paper, we reported that dextran itself was also significantly taken up by the liver as well as cationic macromolecules after intravenous injection into mice.[20] This would suggest an insufficient effect of dextran conjugation. We have found that anionic dextran derivative has very little interaction with the liver in the same system,[20] so we will discuss tissue distribution of UC conjugates with native, anionic, and cationic dextran derivatives in a following paper.

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