Quantitative Measurement of p21 Protein in Human Serum by Time-Resolved Fluoroimmunoassay

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The p21 protein (a non-*ras* 21 kDa protein) binds to cyclin-dependent kinases and is an inhibitor of the kinases in mammalian cells.¹⁻⁴ It can be transcriptionally activated by wild-type p53, but not by mutant p53,² and functions to block cell-cycle progression in many human neoplasms.⁵ It has been reported that human p21 protein is related to many human tumors, such as urogenital cancer⁶⁻⁸ and lung cancer.⁹ Although an enzyme-linked immunosorbent assay (ELISA) method has been established for the measurement of p21 protein in human sera,^{7,8} the method is only semi-quantitative. Serum samples were first coated on the wells of a microtiter plate, and then the rabbit anti-p21 antibody, biotinylated goat anti-rabbit IgG antibody, and peroxidase-labeled avidin were added.⁸

Time-resolved fluoroimmunoassay (TR-FIA) using an europium chelate as the label is an established immunoassay method, and has been widely used for bioassays.¹⁰⁻¹⁷ Recently we synthesized a new tetradentate β -diketonate-Eu³⁺ fluorescence chelate, 4,4'-bis(1'',1'',1'',2'',2'',3'',3''-heptafluoro-4'',6''-hexanedion-6''-yl)-chlorosulfo-*o*-terphenyl (BHHCT)-Eu³⁺, and characterized its application for highly sensitive TR-FIA.¹⁸ The BHHCT-Eu³⁺-labeled antibody, streptavidin (SA), SA-BSA conjugate (BSA is bovine serum albumin), and drug-BSA conjugate have been used for the highly sensitive measurement of tumor markers,^{18,19} immunoglobulin,²⁰ hormone²¹ and drugs.^{22,23}

In the present study, we developed a novel quantitative measurement method of p21 protein in human sera. The method is competitive-type immunoassay, in which biotinylated p21-BSA conjugate and BHHCT-Eu³⁺-labeled SA-BSA are used. The assay was carried out on a 96-well microtiter plate coated with rabbit anti-goat IgG antibody. After a goat anti-human p21 antibody was reacted in the wells, the competitive reaction of biotinylated p21-BSA and p21 standard solutions (or serum samples) with goat anti-human p21 antibody was performed. Finally BHHCT-Eu³⁺-labeled SA-BSA was added to each well to react with the biotinylated p21-BSA, and solid-phase time-resolved fluorescence measurements were carried out. The method gives the detection limit of 1.07 ng/ml and the

large dynamic range up to *ca.* 2000 ng/ml for human p21 protein. The intra-assay coefficient of variation (RSD) and the recovery are approximately in the range of 2–8% and 85–110%, respectively, for the human serum samples.

Experimental

Apparatus and materials

The 96-well microtiter plates (Fluoro Nunc Modules) were used. Time-resolved fluorometric measurements were carried out on a DELFIA 1234 fluorometer (Wallac) under these measurement conditions: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 0.2 ms; window time, 0.4 ms; and cycling time, 1.0 ms. A human p21 protein (control peptide) and a goat anti-human p21 (N-20) polyclonal antibody (affinity-purified IgG, reacts with p21 of human origin by Western blotting and immunohistochemistry, and not cross-reactive with p27 or other mitotic inhibitors) were purchased from Santa Cruz Biotechnology, Inc. The p21 standard solutions were prepared by diluting p21 control peptide with 0.05 M Tris-HCl buffer of pH 7.8 containing 5% BSA, 0.1% NaN₃, and 0.9% NaCl. A rabbit anti-goat IgG antibody was purchased from Bethyl Laboratories, Inc. Human serum samples were collected from healthy people and were stored at –20°C before use.

Preparation of the biotinylated p21-BSA conjugate

The preparation basically followed the biotinylation procedure for the methamphetamine-BSA conjugate.²² After repeated dialysis of 1.0 ml of p21 protein (100 µg/ml) at 4°C against 3 l of saline, 1.0 ml of 0.1 M phosphate buffer of pH 7.0 containing 0.5 mg of BSA was added to the solution. While stirring, 0.1 ml of 1.0% glutaraldehyde was added. The solution was stirred at room temperature for 1 h, and further incubated at 4°C for 24 h. To the solution was added 2 mg of NaBH₄, and the solution was stirred at room temperature for 2 h. The solution was dialyzed twice each for 24 h against 3 l of saline at 4°C and then diluted to 3.0 ml with water, to which 25 mg of NaHCO₃ and 4 mg of sulfosuccinimidyl-6-(biotin-amido)hexanoate (NHS-LC-biotin, Pierce Chemical Co.) were added. After the solution

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Fig. 1 Schematic representation of the immunoassay for p21 protein in human sera.

was stirred at room temperature for 1 h and further incubated at 4°C for 24 h, it was dialyzed twice at 4°C against 3 l of 0.1 M NaHCO₃ containing 0.25 g of NaN₃, each time for 24 h. The biotinylated p21-BSA conjugate solution was stored at -20°C. When it was used in immunoassay, the solution was diluted 400-fold with 0.05 M Tris-HCl buffer of pH 7.8 containing 0.2% BSA, 0.1% NaN₃, and 0.9% NaCl.

Preparation and labeling of the SA-BSA conjugate with BHHCT

Preparation and labeling of the SA-BSA conjugate, SA(BSA)_{0.9}(BHHCT)₄₆, were described in the previous report.²⁰ It was diluted 700-fold with 0.05 M Tris-HCl buffer of pH 7.8 containing 1.0 × 10⁻⁷ M EuCl₃, 0.2% BSA, 0.1% NaN₃, and 0.9% NaCl and was heated at 56°C for 2 h before use in immunoassay.

Immunoassay of human p21

One hundred microliters of 0.1 M carbonate buffer of pH 9.6 containing 15 µg/ml of rabbit anti-goat IgG antibody were incubated in a 96-well microtiter plate for 24 h at 4°C. The wells were rinsed twice with 0.05 M Tris-HCl buffer of pH 7.8 containing 0.05% Tween 20 (buffer 1) and once with 0.05 M Tris-HCl buffer of pH 7.8 (buffer 2). To each well was added 50 µl of 0.05 M Tris-HCl buffer of pH 7.8 containing 6 µg/ml of goat anti-human p21 antibody, 0.2% BSA, 0.1% NaN₃, and 0.9% NaCl. The plate was incubated for 24 h at 4°C, and rinsed twice with buffer 1 and once with buffer 2. After 50 µl of 1:1 mixed solutions of biotinylated p21-BSA and p21 standard solutions (or human serum samples) were incubated at 37°C for 2 h in the wells, the wells were rinsed twice with buffer 1 and once with buffer 2. To each well was added 50 µl of the SA(BSA)_{0.9}(BHHCT)₄₆-Eu³⁺ solution. After incubation at 37°C for 1 h, the wells were rinsed 4 times with 0.05 M Tris-HCl buffer of pH 9.1 containing 0.05% Tween 20, and subjected to solid-phase time-resolved fluorometric measurement.

Results and Discussion

Evaluation of the immunoassay method

The immunoassay procedure of the present method is shown

Fig. 2 The influence of biotinylated p21-BSA concentration on the fluorescence intensity in the measurement of p21 protein. The concentrations are expressed on the dilution factors in the figure.

in Fig. 1. In the method, a secondary antibody (rabbit anti-goat IgG antibody) was used for the well coating, and the biotinylated p21-BSA conjugate was used for the competitive reaction. The present method is complicated compared with the method in which goat anti-human p21 antibody is coated on the wells directly; it was found that the goat anti-human p21 antibody used in this assay can not be effectively coated on the wells by the general physical absorption method. By using the present method, the goat anti-human p21 antibody was immobilized on the solid-phase successfully. We also tested the use of a biotinylated p21 protein for the competitive reaction; however, a significant signal could not be obtained. This fact indicates that the direct biotinylation of p21 with NHS-LC-biotin was not effective, or that the binding activity of p21 with anti-p21 antibody was lost after the biotinylation. We have reported that a methamphetamine-BSA conjugate can be easily biotinylated by using the same reaction,²² since a BSA molecule

Fig. 3 The calibration curve for human p21 protein.

has 59 amino groups,²⁴ some of which are used for binding to a drug molecule but others still remain unbound, and can be used for binding to biotin. By using this method, the biotinylated p21-BSA was prepared and the immunoassay using it gave successful results. The effect of biotinylated p21-BSA concentration (1/100, 1/200, 1/400 and 1/800 dilution) on the competitive reaction was examined. The results, as shown in Fig. 2, indicate that the concentration of 1/400 dilution is optimal, since the slope of the calibration curve still continues going down only with the dilution factor to 0.2 ng/ml of p21.

Quantitative measurement of p21 in human serum samples

The calibration curve of human p21 protein is shown in Fig. 3. The detection limit, defined as the concentration corresponding to twice the standard deviation of the background signal, is 1.07 ng/ml, which is low enough for the assay of p21 protein in human sera.⁸ The method also gives a large dynamic range of 1 to 2000 ng/ml.

The precision and recovery of p21 protein added to human serum samples are shown in Table 1. The results of 2–8% RSD for $n = 6$ (the standard deviation was calculated with $n - 1$) and 85–110% recovery show that the present method can be employed for the general clinical analysis of p21 protein in human sera with good accuracy and precision. In addition, the present method can also be used for the analysis of p21 protein normal human tissues and various tumor tissues.

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Table 1 Analytical precision and recovery of p21 protein added to serum samples (serum A and serum B)

Added/ng ml ⁻¹	Found/ng ml ⁻¹	RSD, % ($n = 6$)	Recovery, %
(Serum A)	5.10	4.25	—
50.0	48.3	7.20	86.4
20.0	24.5	4.13	97.0
5.00	9.67	5.25	91.4
(Serum B)	4.12	6.31	—
50.0	49.4	2.10	90.6
20.0	24.7	7.87	102.9
5.00	9.57	3.65	109.0

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