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CLIN. CHEM. 39/2, 277–280 (1993)

Selective Removal of β_2-Microglobulin from Plasma Specimens of Long-Term Hemodialysis Patients by High-Performance Immunoaffinity Chromatography

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We examined the suitability of high-performance immunoaffinity chromatography (HPIAC) for removing β_2-microglobulin (β_2-MG) from plasma of patients (n = 26) undergoing long-term hemodialysis. We used immobilized antibody to β_2-MG to prepare an immunoaffinity column with a 0.5-mL bed volume. Plasma samples of 0.5 mL were chromatographed at a flow rate of 0.5 mL/min and pressures <7 kg/cm^2. Although only a small amount of β_2-MG can be removed with a conventional dialyzer, 92.1% ± 0.4% of the globulin could be removed by HPIAC. The column could be reused after acid elution. After 50 cycles the efficiency of β_2-MG trapping was 73% of the original efficiency.

Indexing Terms: *amyloid protein · renal failure*

Amyloidosis appears to be a widespread complication in patients undergoing long-term hemodialysis (*1*) and has been observed in 58% of patients treated for ≥5 years (D. Brancaccio, A. Anelli, P. Padonese, unpublished abstract, 1987). Selective amyloid deposition is usually responsible for carpal tunnel syndrome (*2*), and several reports now indicate that most hemodialysis patients who have been treated for 10 years present one or more clinical or radiological signs of amyloid deposition (*3*).

A relatively new development regarding this complication was the identification of the structure of this type of amyloid: in 1985, determination of the amino acid sequence of the amyloid protein showed β_2-microglobulin (β_2-MG) to be the major fiber constituent (*4*).[4] β_2-MG, a nonglycosylated protein of low molecular mass (M_r 11 800), forms the light chain of the class-I major histocompatibility complex and is present in plasma and on the surface membrane of most nucleated cells (*5*). Because the proximal tubular cells of the kidney are the major site of β_2-MG catabolism, this protein accumulates in the plasma of patients with advanced renal failure and patients on hemodialysis. There is little or no clearance of β_2-MG by cellulose or polyacrylonitrile membrane dialyzers (*6*).

Affinity chromatography with the use of immobilized antibody is a rapid and specific technique for isolating biologically active materials from a variety of different sources (*7–10*). This method has also been used to remove antibody (*11–13*) and antigens (*14*) for medical application.

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Received July 15, 1992; accepted September 1, 1992.

[4] Nonstandard abbreviations: β_2-MG, β_2-microglobulin; HPIAC, high-performance immunoaffinity chromatography; and 2-D, two-dimensional.

This technique is even more useful when the controlled conditions of high-performance immunoaffinity chromatography (HPIAC) are applied. HPIAC is applicable to the rapid isolation and measurement of many different types of biological materials (*15–17*). A packing material that can be used to immobilize antigens and antibodies is the commercially available Affi-Prep 10 (Bio-Rad, Rockville Center, NY).

The HPIAC procedure is an excellent method for removing β_2-MG from plasma samples (*18*). In an attempt to resolve the problem of amyloid deposition in dialysis patients, we used HPIAC to specifically remove β_2-MG from human plasma. We describe studies to evaluate the applicability and effectiveness of HPIAC with a column bearing immobilized anti-β_2-MG IgG as a useful procedure for eliminating plasma β_2-MG in patients on long-term hemodialysis.

Materials and Methods

Reagents. β_2-MG, purified from human urine, was purchased from Calbiochem (La Jolla, CA). Anti-β_2-MG antiserum was obtained from Dakopatts (Glostrup, Denmark) and Protein A–Sepharose from Pharmacia (Uppsala, Sweden). Affi-Prep 10 column for affinity chromatography [30 mm × 4.6 mm (i.d.), 0.5 mL], silver stain kit, and protein assay kit were obtained from Bio-Rad (Rockville Center, NY). Anti-β_2-MG IgG was isolated from the antiserum by use of Protein A–Sepharose. All other chemicals used were analytical grade.

Samples. Samples were obtained from healthy individuals (9 men, 8 women; ages 32–73 years, mean 50 years) and patients on long-term hemodialysis (15 men, 11 women; ages 29–82 years, mean 55 years). The plasma fraction was separated by centrifugation (1500 × *g*, 5 min) of heparinized blood and stored at −20 °C. All patients had received chronic hemodialysis therapy with a Cuprophan membrane dialyzer for 6 months to 7 years. Blood samples were taken from 16 of these patients immediately before and after hemodialysis on two consecutive occasions and from the remaining 10 patients before hemodialysis.

Preparation of immunoaffinity column. Anti-β_2-MG was immobilized on the Affi-Prep 10 by use of the standard Bio-Rad protocol. The total coupling rate was 9.21 mg of IgG per 0.5 mL of Affi-Prep 10 gel. After 10 column elutions and regenerations, no leakage of the immobilized IgG could be detected, as checked by Western blotting (*19*).

Chromatography. The immunoaffinity column (bed volume 0.5 mL) was installed into a Jasco L-800 HPLC system (Japan Spectroscopic, Tokyo, Japan) equipped with a Model 880-PU pump and a Model 875 ultraviolet absorbance detector. The system was also equipped with a Model 880-02 ternary gradient unit with a Model 801-SC system controller, which automatically controlled the elution profile of the system. The column was jacketed and maintained at 4 °C in an ice bath and was isocratically eluted with phosphate-buffered saline (pH 7.2, 0.15 mol/L NaCl) for 10 min at a flow rate of 0.5 mL/min. Samples (500 μL of human plasma) were loaded onto the column by means of the ternary-gradient-unit system controller. During the initial 15 min, immobilized IgG reacted specifically with β_2-MG and the unreacted material ran through the column. The β_2-MG elution phase was then started: a pH gradient was automatically generated from 7.2 to 2.5 by adding 0.5 mol/L glycine-HCl buffer (pH 2.5) to the initial buffer over a 10-min period. The absorbance of the eluate was monitored at 280 nm with a sensitivity setting of 0.16 *A* full scale, and fractions containing the eluted material were collected.

The β_2-MG content in each fraction was determined by a sandwich enzyme immunoassay, which consisted of anti-β_2-MG antibodies immobilized on a solid phase (polystyrene beads) and antibodies labeled with β-D-galactosidase from *Escherichia coli*, as described by Mogi et al. (*20*). The Protein A-purified IgG showed no cross-reactivity with purified IgG, IgM, or albumin from human serum, indicating that this antibody is specific for β_2-MG. Micro two-dimensional (2-D) electrophoresis was performed by use of a modified (*19, 21*) O'Farrell system (*22*).

Western blotting was carried out as described previously (*19*).

Results

We first measured plasma β_2-MG concentrations in 16 patients undergoing hemodialysis. In these patients, plasma β_2-MG concentrations ranged from 35 to 91 mg/L (mean 63.7 ± 3.6 mg/L), which is a 23–61-fold increase over values for healthy individuals (1.3–1.7 mg/L, mean 1.51 ± 0.03 mg/L, n = 17). All samples showed only one band (M_r 12 000), which corresponded to that of the authentic standard, purified β_2-MG (data not shown). Because β_2-MG is not dialyzable by present hemodialysis techniques (*7*), the plasma concentration of β_2-MG would not be expected to be reduced by such treatment. Thus, samples taken after hemodialysis by the conventional procedure (Cuprophan membrane dialyzer) did not have substantially reduced concentrations of β_2-MG: 59.8 ± 5.8 mg/L, a decrease of only 16% ± 5%. This ineffectiveness in eliminating β_2-MG helps to explain why the mean plasma β_2-MG concentration was significantly higher in the patients who had been treated by dialysis for >3 years than in those treated for <1 year (Table 1).

To reduce the β_2-MG concentration, we used HPIAC to specifically remove β_2-MG from plasma and the enzyme immunoassay (*20*) to determine the β_2-MG concentrations. As shown in Table 2, the percentage of

Table 1. Plasma β_2-MG Content According to the Duration of Dialysis Treatment

Treatment length, years	β_2-MG, mg/L
<1 (n = 7)	52.1 ± 3.3
1 to <3 (n = 9)	58.2 ± 6.0[a]
≥3 (n = 7)	69.4 ± 2.1[a]

Mean ± SE.
[a] Significantly different from <1 year, $P < 0.005$ (Student's *t*-test).

Table 2. Pre- and Post-HPIAC Treatment Concentrations of β_2-MG in Plasma

	Pretreatment	Post-treatment	Total β_2-MG trapped, %
	β_2-MG, mg/L		
Control subjects (n = 17)	1.51 ± 0.03	0.010 ± 0.006	99.1 ± 0.04
Hemodialysis patients (n = 26)	57.9 ± 3.0	4.62 ± 0.35	92.1 ± 0.40

Mean ± SE.

total β_2-MG trapped (mean ± SE) was 92.1% ± 0.4%, indicating successful elimination of β_2-MG from the plasma by the anti-β_2-MG IgG immobilized on the Affi-Prep 10 column. The pressure inside the column was <7 kg/cm^2. By analyzing the eluate by micro 2-D electrophoresis, we could effectively determine the specificity of the column. There were no significant changes in 2-D pattern between the original plasma and the flow-through fraction from the HPIAC column (data not shown).

When we subjected samples eluted by acid buffer to 2-D electrophoresis, each sample showed two major spots at pH 5.3–5.7. Both spots corresponded to the same relative molecular mass (11 800), and their mobility was identical with that of standard β_2-MG (data not shown). These findings show that the HPIAC procedure is effective and useful for specific removal of β_2-MG from plasma of patients on long-term hemodialysis.

Next we evaluated the HPIAC column for repeated usage. Repeated loading of plasma and acid elution of β_2-MG from the column led to a gradual decrease in the amount of β_2-MG adsorbed. After 50 cycles of HPIAC, the amount of β_2-MG trapped was calculated to be 73% of that of the original run. However, the protein species adsorbed on the column did not change, as checked by both 2-D electrophoresis and Western blotting, indicating no change in the specificity of the immunoaffinity column (data not shown).

Discussion

Since the identification in uremic amyloid tissue of polymeric fibrillar β_2-MG as the protein marker of a new type of amyloidosis (*4*), there has been intense debate as to whether therapeutic strategies will influence the incidence or natural course of this uremic complication. The renal handling of β_2-MG is supported by the demonstration of proximal tubular uptake and catabolism (*23*) of β_2-MG injected into rats and by the parallel increase in serum β_2-MG. Clearance studies have indicated that the kidney is the major site of catabolism. Thus, it has been postulated that the pathogenesis of this type of amyloidosis in patients undergoing long-term hemodialysis is related to the persistently high β_2-MG concentration in their plasma. Our data support this speculation (Table 1) and confirm that plasma β_2-MG cannot be removed by conventional dialysis.

HPIAC is an easy technique for removing β_2-MG in 1 h; it can be used in any facility by connecting the system to a conventional dialyzer. The immobilized-antibody column remains effective for 100 runs and can be stored refrigerated for as long as 1 year. Because the maximum binding capacity of the column was 1.8 mg of β_2-MG per 0.5 mL of IgG-coupled Affi-Prep 10 under the conditions used, an affinity column with a bed volume of only 100 mL would be of suitable size for adsorption of the β_2-MG from the total plasma of a patient. Thus, this HPIAC procedure should prove useful for medical application and may have broad applicability for the elimination of any plasma components with antigenic or amyloidogenic properties.

Supported by a grant-in-aid from the Japan Health Sciences Foundation, Japan (K.K.).

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CLIN. CHEM. 39/2, 280–283 (1993)

Laboratory Diagnosis of Medium-Chain Acyl-Coenzyme A Dehydrogenase Deficiency by the Amplification Refractory Mutation System

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We used the amplification refractory mutation system (ARMS)—a polymerase-chain-reaction-based method—to detect the 985 A-to-G mutation of the gene coding for the enzyme medium-chain acyl-CoA dehydrogenase (MCAD). The 985 A-to-G allele is thought to account for ~89% of the mutant alleles in this disorder and at least one copy was reported to be present in 98% of affected individuals. ARMS provided a simple and robust method that reliably identified the 985 A-to-G mutant allele in patients either homozygous or heterozygous for this allele. Combined with organic acid analysis, ARMS can provide definitive diagnosis for the great majority of the patients with MCAD deficiency. The method, therefore, should be useful in clinical laboratories involved with the diagnosis of inborn errors of metabolism.

Indexing Terms: *heritable disorders · polymerase chain reaction · gene probes*

Medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency is an autosomal recessive disorder (*1, 2*) that has an incidence of 1:10 000 to 1:30 000 in Caucasian infants (*3, 4*).[3] The disorder is characterized clinically by fasting intolerance, vomiting, lethargy, and hypoketogenic hypoglycemic coma. Patients appear normal before the onset of the first acute episode and during remission between subsequent episodes. The disorder has been associated with sudden infant death syndrome and has a Reye-like clinical presentation (*1–4*). An A-to-G nucleotide transition at position 985 of the MCAD gene was found to be the most common mutation, accounting for ~89% of the mutant alleles (*2*). Previous studies used either *Nco* I digestion of purified polymerase chain reaction (PCR) product or allele-specific oligonucleotide hybridization as the method for the laboratory diagnosis of this mutation (*1, 4–6*). A simple method known as the amplification refractory mutation system (ARMS) has been used in the genotyping of apolipoprotein E alleles (*7*) and known mutations of several disorders (*8, 9*). Here we used ARMS to provide a rapid and reliable method for diagnosing the 985 A-to-G mutation in MCAD deficiency.

Materials and Methods

DNA was isolated from peripheral blood by the method of Miller et al. (*10*). Oligonucleotide primers used for PCR amplification that introduce an *Nco* I restriction site in the mutant allele (*1*) have the following sequences: 5'ATATCATTTATGCTGGCTGAAATGGCCATG3' (sense) and 5'ACCAGAATCAACCTCCCAAG3' (antisense). For ARMS reaction, the sequence of the allele-specific sense primer for the wild type was 5'ATGCTGGCTGAAATGGCAATTA3', and the sequence of the allele-specific sense primer for the 985 mutant was 5'ATGCTGGCTGAAATGGCAATTG3'. To ensure allele specificity, the penultimate base at the 3' end of both primers was deliberately altered to substitute a T for G (*7, 8*). The common (antisense) primer was the same as that used for the *Nco* I digestion method.

PCR followed by *Nco* I digestion was done as described by Yokota et al. (*1*). PCR reactions were performed with the above-described primers. The PCR products were electrophoresed on 2.0% low-melting-point agarose gel. The 87-bp product was then cut out of the gel, extracted with phenol/chloroform, and resuspended in 30 μL of H_2O. A portion was then digested with 2 U of *Nco* I (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37 °C for 2 h, and the products were applied next to the undigested PCR product of the same individual and electrophoresed on 4% agarose gel (NuSieve GTG; FMC Bioproducts, Rockland, ME) containing ethidium bromide.

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[3]Nonstandard abbreviations: MCAD, medium-chain acyl-coA dehydrogenase; PCR, polymerase chain reaction; and ARMS, amplification refractory mutation system.
Received May 11, 1992; accepted September 10, 1992.