High-Molecular Intestinal Alkaline Phosphatase by Agarose Gel Electrophoresis

Kinue Ooi,¹ Katsuya Shiraki,^{2*} Yoshitaka Morishita,³ and Tsutomu Nobori¹

¹Department of Laboratory Medicine, Mie University School of Medicine, Mie, Japan

²First Department of Internal Medicine, Mie University School of Medicine, Mie, Japan

³Clinical Laboratory Department, Mie University Hospital, Mie, Japan

The presence of high-molecular intestinal ALP (HIALP) overlapping with bone ALP in the $\alpha_2\beta$ region has been demonstrated. In this study we evaluated a method of separating HIALP after its conversion into ALP₅ by the action of protease. Serum samples from patients were mixed with protease at a ratio of 5:1 and left at room temperature for more than 30 min. The protease-treated and nontreated samples were both subjected to agarose gel electrophoresis. Patients who showed a decrease in ALP₃ in the $\alpha_2\beta$ region and an increase in ALP₅ in the β region were regarded as HIALP-positive. HIALP was observed in 26.7–33.1% of patients with liver diseases, collagen diseases, and diabetes mellitus.

Renal disease was ABO blood group-dependent and showed high positive rates for blood groups B and O. The HIALP-positive rate was low (7.1–15.5%) in patients with cardiovascular diseases, malignant tumors, and other disorders. ALP₅ was also observed in 98.4% of HIALP-positive patients with liver diseases. In patients with collagen diseases or diabetes mellitus, the positive rate of ALP₅ was 40.4–66.7%. In conclusion, this method, in which HIALP is converted into ALP₅ by protease pretreatment and is separated from bone ALP, allows HIALP to be identified while other fractions remain unaffected. *J. Clin. Lab. Anal.* 21:140–146, 2007. © 2007 Wiley-Liss, Inc.

Key words: agarose gel electrophoresis; alkaline phosphatase isoenzyme; intestinal alkaline phosphatase; high-molecular intestinal alkaline phosphatase; ABO blood group

INTRODUCTION

Analysis of alkaline phosphatase (ALP) isoenzymes by agarose gel or cellulose acetate membrane electrophoresis can be readily performed and allows the treatment of multiple samples. Therefore, this method is widely performed as a routine examination. However, separation by this method is inadequate, and particularly in the $\alpha_2\beta$ region, a band that overlaps with bone ALP (but is sharper) is frequently observed in patients with disorders such as liver cirrhosis. However, this ALP cannot be clinically regarded as bone ALP, and methods for its separation/confirmation and the identification of its derivation organs are necessary.

In recent years this band has been reported to be high-molecular intestinal ALP (HIALP). In 1988, Van Hoof et al. (1) showed by neuraminidase treatment and the use of anti-intestinal polyclonal/monoclonal antibodies that the band overlapping with bone ALP by agarose gel electrophoresis is intestinal variant ALP. In 1998,

Hoshino et al. (2) showed by polyacrylamide gel electrophoresis (PAGE) that ALP with mobility in the $\alpha_2\beta$ region on cellulose acetate membrane/agarose gel has a high molecular weight. Matsushita et al. (3) were able to separate this ALP variant from bone ALP by converting it into ALP₅ by subtilisin treatment.

We had also analyzed ALP isoenzymes by electrophoresis with cellulose acetate membranes as supporting medium and therefore had the same problem in that the

Abbreviations: ALP, alkaline phosphatase; IALP, intestinal alkaline phosphatase; HIALP, high-molecular intestinal alkaline phosphatase; NIALP, normal-size intestinal alkaline phosphatase; DM, diabetes mellitus; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; PAGE, polyacrylamide gel electrophoresis.

*Correspondence to: Katsuya Shiraki, First Department of Internal Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. E-mail: katsuyas@clin.medic.mie-u.ac.jp
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separation between HIALP and bone ALP was impossible. In this study, patients' serum samples were analyzed by a new method in which agarose gel electrophoresis is performed after conversion of HIALP into ALP₅ by protease treatment. Since this method allows electrophoresis of protease-treated and untreated serum samples placed side by side, HIALP can be detected and quantified by comparing these samples. This method may be clinically useful. We compared HIALP among various diseases using this method.

MATERIALS AND METHODS

Subjects

The subjects of this study were 630 inpatients and outpatients of our hospital. They consisted of 216 patients with chronic liver diseases, 121 with collagen diseases, 35 with cardiovascular diseases, 58 with malignant tumors, 30 with diabetes mellitus, 13 with renal disease, and 22 with other disorders. In addition, 99 healthy subjects served as controls.

The chronic liver diseases included chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, primary biliary cirrhosis (PBC), alcoholic liver injury, and autoimmune hepatitis. The collagen diseases included systemic lupus erythematosus (SLE), Sjögren syndrome, dermatomyositis, polymyositis, scleroderma, and mixed connective tissue disease (MCTD). The cardiovascular diseases included ischemic heart disease, cardiac failure, and valvular heart disease. The malignant tumors included pulmonary cancer, gastric cancer, leukemia, malignant lymphoma, prostatic cancer, and bladder cancer, etc. The diabetes mellitus was non-insulin-dependent diabetes mellitus (type II).

Methods

ALP values were measured with a Vitros 250 Chemistry System (Ortho-Clinical Diagnosis, Raritan, NJ, USA) using a Test Methodology ALKP (Ortho-Clinical Diagnosis). The measurement principle was the measurement (at 400 nm) of p-nitrophenol formed in an alkaline state by p-phenylphosphoric acid. Normal reference values were 100–350 U/L.

ALP isoenzymes were manually analyzed with Quick Gel ALP (K.K. Helena Kenkyujo, Saitama, Japan) as agarose gel and Quick ALP as an ALP reagent (K.K. Helena Kenkyujo), which were developed for the Electrophoresis Processing Analyzer Epalyzer 2 (K.K. Helena Kenkyujo). The measurement principle of Quick ALP was densitometry at a wavelength of 570 nm to obtain the fraction of formazan formed using 2-indoxyl phosphate disodium salt as a substrate and nitroterazolium blue (NTB) as a color developer.

For the detection of HIALP, patients' serum samples were mixed with protease (*Streptomyces griseus*, K.K. Helena Kenkyujo) at a ratio of 5:1 and left at room temperature for more than 30 min. The pretreated samples and untreated samples were subjected to electrophoresis, being placed side by side. HIALP was converted into normal-size intestinal ALP (NIALP, ALP₅) by the action of protease, and patients showing a decrease in ALP₃ and an increase in ALP₅ were regarded as HIALP-positive. In some of the patients, IALP was confirmed based on no changes in mobility after pretreatment with neuraminidase (*Clostridium perfringens*; Sigma-Aldrich, St. Louis, MO).

Measurement was manually performed by the method in which HIALP is converted into ALP₅ by protease and, agarose gel (Quick Gel ALP) developed for the Epalyzer 2 is used as supporting medium, and conditions were determined. The measurement conditions were protease treatment for 30 min, electrophoresis at 250 V for about 20 min, staining time for 25 min, decoloring/fixation time with 70% of methanol for 15 min, and washing with water for 10 min followed by washing with replaced water for 20 min (total washing time = 30 min). The samples were measured for each Quick Gel. Protease treatment resulted in the completion of the reaction after about 30 min. Placenta ALP was also observed in the $\alpha 2\beta$ region but could be distinguished because of its heat resistance.

RESULTS

Detection of HIALP by Agarose Gel Electrophoresis

Figure 1 shows zymograms of ALP isozymes obtained after electrophoresis of serum samples from protease-treated (sample: protease = 5:1) and -untreated patients. After visual observation of the zymograms, patients who showed a decrease in the sharp band overlapping ALP₃ and an increase in ALP₅ were regarded as HIALP-positive. Patients 1–6 had chronic liver diseases and showed ALP₅. After protease treatment, HIALP indicated by the arrow decreased while ALP₅ increased, showing HIALP-positive results. In patient 1, since the reaction between the sample and protease was slow, neuraminidase treatment was performed for confirmation. Lane 2 shows a pattern after neuraminidase treatment. Though liver ALP (ALP₂) and bone ALP shifted toward the cathode with respect to the origin, HIALP as well ALP₅ showed no changes in mobility. In patient 6, high-molecular hepatic ALP (ALP₁) did not change after protease treatment.

Figure 2 shows ALP isozyme patterns after densitometry (wavelength = 570 nm) of samples from patients 2–4 in Fig. 1. The upper row indicates nontreated serum

Fig. 1. Detection of HIALP using protease and neuraminidase: ALP isoenzyme in six patients with HIALP separated by agarose gel electrophoresis. Protease treatment: serum incubated over 30 min at room temperature. Neuraminidase treatment: serum incubated overnight at room temperature.

samples, and the lower rows indicate protease-treated samples. The left side was the anode, and from the anode side, ALP₁, ALP₂, ALP₃, and ALP₅ were separated. ALP₁, ALP₂, ALP₃, and ALP₅ indicate high molecular hepatic ALP, hepatic ALP, bone ALP overlapping with HIALP (only bone ALP in the lower row), and IALP, respectively. We confirmed that the %CV was under 5% (data not shown).

Case 2 was a 65-year-old male with liver cirrhosis showing an ALP of 722 U/L and blood group B. Patient

3 was a 59-year-old female with chronic hepatitis showing an ALP of 467 U/L and blood group O. Patient 4 was a 65-year-old female with PBC showing an ALP of 629 U/L and blood group O. Changes in ALP₁ and ALP₂ were negligible, but ALP₃ decreased while ALP₅ increased. In patient 2, ALP₃ changed from 25.6% to 15.2% and ALP₅ from 48.3% to 63.9%. In patient 3, ALP₃ changed from 50.8% to 32.9% and ALP₅ from 16.2% to 30.6%. In patient 4, ALP₃ changed from 39.8% to 17.7% and ALP₅ from 8.6% to 26.8%.

Fig. 2. Changes in ALP isoenzyme patterns in three patients with HIALP before and after protease treatment. Upper: before protease treatment; lower: after protease treatment.

Fig. 3. Appearance of HIALP in various diseases.

Comparison of the HIALP-Positive Rate Among Various Diseases

As shown in Fig. 3, the HIALP-positive rate was high in liver diseases, collagen diseases, renal diseases and diabetes mellitus (26.7%–33.1%) but low in cardiovascular diseases and malignant tumors and other disorders (8.6–15.5%).

Relationship Between HIALP and NIALP (ALP₅)

Though the HIALP-positive rate was similar among liver diseases, collagen diseases, and diabetes mellitus (34.2%–37.5%), the positive rate of ALP₅ was 53.9% in liver diseases, 31.3% in diabetes mellitus, and 24.2% in collagen diseases (Fig. 4, left).

Fig. 4. Relationship between HIALP and NIALP (ALP₅) in various diseases.

When the appearance of HIALP was compared with that of ALP₅ (Fig. 4 right), ALP₅ was observed in 65 (98.5%) of 66 HIALP-positive patients with liver diseases and about 40–67% of HIALP-positive/negative patients with collagen diseases/diabetes mellitus. Evaluation of HIALP-negative patients showed ALP₅ in 126 patients with liver diseases (58.9%), 17 with collagen diseases (16.3%), and 4 with diabetes mellitus (20.0%). There were marked differences among diseases.

Relationship Between HIALP and ABO Blood Groups

The possible relationship between the appearance of HIALP and ABO blood groups was evaluated in liver diseases, collagen diseases, cardiovascular diseases, and malignant tumors (Fig. 5). Considering that the ABO blood group distribution shows A:O:B:AB = about 4:3:2:1, there was almost no bias in blood groups in the diseases. For blood group A, HIALP was observed in five patients (5.9%) with liver diseases and collagen disease in one patient (1.9%) but in none with other diseases. For blood group AB, HIALP was observed in one patient with liver disease (7.7%). For blood groups B and O, there were many HIALP-positive patients with liver diseases or collagen diseases. The HIALP-positive rate in liver diseases was 16/37 (43.2%) for blood group B and 38/63 (60.3%) for blood group O, and that in collagen diseases was 16/22 (72.7%) for blood group B and 23/35 (65.7%) for blood group O. In cardiovascular diseases, HIALP was present in ONE patient with blood group B and one patient with blood group O. In malignant tumors, HIALP was positive in one patient

with blood group B and two with blood group O. The positive rates in cardiovascular diseases and malignant tumors were lower than those in liver diseases and collagen diseases but suggested high positive rates in patients with blood groups B and O.

DISCUSSION

IALP (ALP₅) migrates in the β region by cellulose acetate membrane electrophoresis, and its high incidence in liver cirrhosis has long been known since the report in 1965 by Fishman et al. (4), who used an amino acid inhibition method. The incidence of ALP₅ has been reported to be high in liver cirrhosis (4–6), diabetes mellitus (7), and renal failure (8). Langman et al. (9) observed ALP₅ in about 38% of normal secretors with B/O blood groups, but not in subjects with A/AB blood groups or nonsecretors with any blood group, suggesting a blood-group dependency. All of these studies dealt with NIALP (ALP₅).

In 1981 Komoda et al. (10) showed the presence of two principal components of IALP by chromatography. Since 1988, studies have shown that one of the principle components is HIALP, which migrates in the $\alpha_2\beta$ region by cellulose acetate membrane or agarose gel electrophoresis and is ABO blood-group dependent (1,11–13). Investigators observed the presence of the variant type of IALP by performing electrophoresis using agarose gel and cellulose acetate membranes as the supporting media after treatment with neuraminidase or anti-intestinal antibody (1,11). Subsequently, Van Hoof (12) and Matsushita et al. (3) reported methods of converting HIALP into ALP₅ by phospholipase C and

Fig. 5. Relationship between HIALP and ABO blood groups.

subtilisin treatment, respectively. The results of PAGE performed by Hoshino et al. (2) and Matsushita et al. (3) suggested that the variant has a high molecular weight. In addition, no changes in mobility after neuraminidase treatment showed the absence of sugar chains, and the results of treatment with anti-intestinal antibody indicated the intestinal type (1,11). Based on these findings, the band that is detected in the $\alpha 2\beta$ region and overlaps with ALP_3 was confirmed to be HIALP.

However, the previously reported methods are used for HIALP measurement in research and are too complicated to be directly used as routine examination methods. Chromatography requires a special apparatus. PAGE is difficult to perform because it requires the constant production of gels with the same composition, and operator skill to read the fractionized bands. Pretreatment with an anti-intestinal antibody is accurate but expensive. Neuraminidase, which requires a reaction time for 1 day at room temperature, is useful for the confirmation of some samples but inappropriate for the treatment of all samples.

Due to recent advances in the automation of routine examinations in all departments of sample examination, automated mass treatment in a short time is indispensable. Electrophoresis has also been automated to minimize differences between examiners. Recently, the Epalyzer 2 (K.K. Helena Kenkyujo), which allows the addition of new items and improved measurement methods, has come into general use. This analyzer is operated according to the input program and is optimal for mass treatment. However, since changes in the program are necessary to change conditions, it is difficult to achieve consistent analyses at each institution until the optimum conditions can be determined.

In this study we evaluated the method of converting HIALP to ALP_5 using agarose gel (Quick Gel ALP), which was developed as a supporting medium for the Epalyzer 2, and protease for pretreatment. This method has the following advantages: 1) The procedure is easy, multiple samples can be treated, and good results can be obtained in a relatively short time. 2) Since protease degrades only HIALP, changing its mobility, and

negligibly affects the other fraction values, one can perform quantification by calculating the ALP₃ and ALP₅ fraction values before and after protease treatment. Protease appears to specifically degrade HIALP and does not degrade high-molecular hepatic ALP (ALP₁), though both types of ALP have high molecular weights.

In this study the HIALP-positive rate differed among diseases and was associated with ABO blood groups in both patients and normal adults. The positive rate was high in patients with liver diseases and collagen diseases showing blood group B/O, and low in patients with cardiovascular diseases, malignant tumors, or other disorders.

The presence of HIALP was clarified only recently. The mechanism of its appearance in the blood or its composition remains unclear. HIALP appears to be produced by in vivo binding of ALP₅ to various substances. The majority of previous studies on IALP dealt with ALP₅. There have been only a few studies on HIALP (1,11), and no study to date has focused on the association between collagen diseases and HIALP. It is possible that the half-life of HIALP is prolonged in renal failure compared to other diseases due to delayed renal excretion of ALP, especially HIALP. In diabetes mellitus, since diabetic nephropathy is a representative complication, the above in vivo mechanism in renal failure may also be involved.

Though both the mechanism of the appearance of HIALP in the blood and its composition are still unclear, this method is of value because HIALP, which cannot be differentiated from bone ALP by conventional methods, is converted to ALP₅, and its derivation organs can be identified. The Kasahara isoenzyme (14,15) is a variant of IALP that has long been known and occasionally appears in hepatocellular carcinoma. Therefore, the presence of IALP in liver diseases is clinically important. In liver and collagen diseases, detailed analyses of IALP by this method, as well as accurate analyses of the pathological conditions, are necessary to evaluate their association.

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