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IMMUNOLOGICAL DIFFERENTIATION OF HUMAN TISSUE-NONSPECIFIC TYPE ALKALINE PHOSPHATASES BY A MONOCLONAL ANTIBODY TO THE ENZYME OF HUMAN OSTEOBLAST-LIKE CELLS

BY

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ABSTRACT

Monoclonal antibodies against alkaline phosphatase [ALP; ortho-phosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1.] of cultured human osteoblast-like cells (HBC) were raised in mice. Immuno-reactions of tissue-nonspecific type ALP from human bone, dental pulp, liver and kidney as well as intestinal and placental types to the monoclonal antibodies were compared by a dot immunoassay and ELISA. One clone was able to recognize antigenic differences among tissue-nonspecific type ALPs in addition to intestinal and placental ALPs; it reacted favorably with ALPs from HBC, human bone, kidney and dental pulp, but not with human liver enzyme. Similarly, the antibody immunoreacted with bone-derived ALP but not with liver-derived enzyme present in human serum.

The present monoclonal antibody preparation can be utilized in basic studies as well as in clinical laboratory tests to distinguish minor heterogeneity among human ALPs.

Key words: Alkaline phosphatase, Minor heterogeneity, Monoclonal antibody, Human osteoblast-like cells, Dot immunobinding assay, ELISA.

INTRODUCTION

Human ALPs are classified into four main types: tissue-nonspecific type (kidney/liver/bone type) (Weiss et al. [1]), intestinal type (Henthorn et al. [2]), placental type (Knoll et al. [3]) and placental-like type (Knoll et al. [3]). They are synthesized according to genetically different messages and distinguished by their enzymatic properties. Human kidney/liver/bone and other ALPs that belong to the tissue-nonspecific type (TNSALP), however, have almost identical enzymatic properties such as susceptibility to inhibitors and thermostability.

We have reported that polyclonal antibodies against porcine kidney ALP raised in rabbits cross-reacted with human TNSALP but not with intestinal or placental ALP (Oida et al. [4]). The antibody could be used to distinguish TNSALP clearly from other enzyme types; however, no difference in immunological reactivity was observed among TNSALPs from different sources. Electrophoretic mobility is the only measure routinely used to distinguish that difference in clinical laboratories (Sundblad et al. [5]).

Recently, we found minor immunologic-

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al differences in cross-reactivity among TNSALPs by monoclonal antibodies against swine kidney ALP (Goseki et al. [6]). Human liver ALP scarcely cross-reacted with those monoclonal antibodies. The monoclonal antibody preparations will be useful to distinguish human kidney and bone ALPs from liver ALP. However, it was impossible to distinguish differences in the isoenzymes present in human serum.

In the present study, we have succeeded in raising monoclonal antibodies against the ALP of human osteoblast-like cells (HBC) which were utilized to detect subtle heterogeneities among human TNSALPs of both tissues and serum by their immuno-reactivity.

Materials and Methods

I. Human osteoblast-like cells

A primary culture of HBC was used as the source of ALP antigen. Neonatal human long bone was obtained from a still-born baby and bone chips were explanted in MEM-alpha medium (Gibco Lab.). The cells grown out from the chips were cloned by a limited dilution method according to ALP activity. This cell preparation was established by Drs. Shimokawa and Ibaraki and the details of its cytological characterization will be published elsewhere by them.

HBC cells were homogenized in TBS (10 mM Tris-HCl, pH 7.4 containing 0.9% NaCl), and specific ALP activity was determined to be 0.117 U/mg protein, comparable to that of MC3T3-E1 cells (0.143 U/mg protein) an established mouse osteoblast cell line (Sudo et al. [7]). Bone homogenate had an activity of 0.031 U/mg protein, about a quarter that of HBC.

II. Preparation of human tissue ALPs

Human tissues were prepared as described before (Goseki et al. [6]). Human bone from surgical operations was obtained and human dental pulp was obtained from teeth extracted for orthodontic treatment. Human placenta was from normal deliveries and human blood sera with high ALP activity were selected from among those received in the routine clinical laboratory. The other human samples (kidney, liver and intestine) were autopsy materials.

The tissues were homogenized with distilled water (2 liters/kg of the tissue) and mixed with 1-butanol (1 liter). The aquous phase was separated and the precipitates that appeared at pH 4.9, adjusted with acetic acid, were removed. The supernatant was adjusted quickly to pH 6.5, and the precipitates which appeared by the addition of an equal volume of acetone were collected by centrifugation and dried to acetone powder. HBC, human bone and dental pulp tissues were homogenized in TBS and centrifuged. The supernatants were used in immunological experiments.

III. Enzyme activity assay

ALP activity was determined with 10 mM p-nitro-phenyl-phosphate as a substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer, pH 10.0, containing 5 mM $MgCl_2$ at 37°C (Goseki et al. [6]; Oida et al. [8]). The enzyme activity was also assayed in the presence of various concentrations of inhibitors such as levamisole, L-homoarginine and L-phenylalanine (Goseki et al. [6]). To assay thermostability, the enzyme preparation was treated at 56°C or 60°C for various time intervals (0, 1, 3, 5, 10, 20 and 30 min) and then reacted with the substrate. Minutes required to reduce ALP activity to 50% of the original activity were obtained (Goseki et al. [6]).

IV. Protein determination

Protein concentration was determined according to the method of Lowry et al. [9].

V. Production and screening of mono-

clonal antibodies

A female BALB/c mouse was injected intraperitonealy with 10^7 HBC emulsified with Freund's complete adjuvant (Difco), followed by a second injection of the same amount of cells 4 weeks later. Cell fusion was performed between the spleen cells of the immunized mouse and mouse myeloma cells (Line P3-NS1-Ag4-1, Flow Laboratories, Inc.). The culture media of the hybridoma cells were screened for anti-ALP antibody activity according to the ELISA by Wray and Harris [10].

The cells in positive wells were passaged and cloned three times. Five stable clones were established, and their immunoglobulin classes and subclasses were typed by the Ouchterlony double diffusion test using commerical antisera specific for different classes of murine immunoglobulins (Miles scientific). Two of the clones were IgG_3 and the others were IgM. One of the most stable clones of IgG_3 was designated G3.

The hybridoma cells producing G3 were inoculated intraperitonealy into BALB/c mice pretreated with 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane, Aldrich Chemical Co.). When abdominal swelling was detected after 1–3 weeks, the ascitic fluid was collected, purified by affinity chromatography with Protein A (Affi-Gel Protein A MAPS Kit, BioRad Lab.) and stored at −80°C. The antibody was used at a concentration of 5.4 mg/ml.

VI. Dot immunobinding assay and gradient polyacrylamide gel electrophoresis

Acetone powder of the human tissue was dissolved in TBS. HBC and human bone tissue were homogenized in TBS and centrifuged and the supernatants were used. ALP activity was unified to the level of 0.1 U/ml. A dot immunobinding assay was performed by the method of Hawkes et al. [11]. Samples, 50 μl each, were blotted onto a nitrocellulose membrane (pore size 0.45 μm; BioRad Lab.) with a Minifold (Schleicher & Schuell Inc.). The nitrocelluose sheet was washed with TBS and blocked in 3% skim milk solution in TBS for 40 min. After overnight incubation with an antibody solution diluted with the buffer, the membrane was soaked in horseradish peroxidase-conugated goat anti-mouse IgG (Cappel Laboratories) for 2 h at room temperature. The membrane was washed again with TBS and stained with a peroxidase substrate solution (a mixture of 30 mg of 4-chloro-1-naphthol dissolved in 10 ml of methanol, 50 ml of TBS and 100 μl of 30% H_2O_2).

Human sera with high ALP activity (bone type; 5 samples, liver type; 6 samples) were selected. The sera were first treated with Protein A (Pansorbin, Calbiochem-Behring Corp.) to remove IgG and then electrophoresed with a 4–15% gradient of polyacrylamide gel at 125 V for 2.5 h. The gradient gel was prepared in 0.1M Tris-boric acid buffer (pH 8.3) containing 2.5 mM Na_2EDTA, 0.08% Bis, 0.75% 3-dimethylamino-propionitrile and 0.75% ammonium persulfate. The bridge buffer was 0.1M Tris-boric acid buffer (pH 9.5). ALP activity in the gels was stained by the β-naphthyl phosphate method (Kurahashi and Yoshiki [12]). Migration positions of bone-type and liver-type ALPs were different from each other as reported by Sugita [13].

Each activity band was sliced and homogenized with TBS, and the ALP activity of the homogenate was unified to the level of 30 mU/ml. After overnight incubation, 50 μl or 150 μl of the extract solution was provided for the dot immunobinding assay as described above.

Results

From the results of the inhibition and thermal inactivation experiments shown in Table 1, it was confirmed that HBC ALP

Table 1. Properties of Human Alkaline Phosphatases

Source of ALP	Concentrations or minutes required to reduce ALPs activity to 50% of original activity				
	Lev (mM)	L-HA (mM)	L-Phe (mM)	Heat stability 56°C (min)	60°C (min)
Osteoblast-like cells	0.03	1.00	>20	7.50	1.26
Bone*	0.03	1.17	18	2.65	0.72
Dental pulp	0.03	2.08	19.9	2.56	1.50
Kidney*	0.03	1.18	19.1	5.49	1.51
Liver*	0.03	1.88	>20	3.56	1.85
Intestine*	>1	>10	1.9	21.4	7.22
Placenta*	0.93	>10	2.3	>30	>30

Enzyme activity was assayed by the rate of hydrolysis of p-nitro-phenyl phosphate. The effect of inhibitors was determined in the presence of 5 mM $MgCl_2$ in the assay mixture.
Lev: Levamisole, L-HA: L-homoarginine, L-Phe: L-phenylalanine.
* Data from Ref. [6] (Goseki et al.).

had properties of TNSALPs, clearly different from those of the human intestinal and placental ALPs. However, it was rather difficult to find substantial differences in enzymatic properties among the TNSALPs.

The immuno-cross-reactivity of ALPs from HBC and other human tissues were investigated by a monoclonal antibody G3. The immunological results are summarized in Table 2. Almost the same results as those of the dot immunobinding assay were obtained by the ELISA experiment. This monoclonal antibody reacted with ALPs of HBC or bone, kidney and dental pulp in the order of their reactivity strength. The reactivity of human liver isozyme with the monoclonal antibody was negligible (1/1,000 that of bone type enzyme).

Human serum ALP isoenzymes of liver and bone types were separated by gradient PAGE and tested for their immuno-cross-reactivity. Enzyme activity was stained with β-naphtyl phosphate, and the bands corresponding to the respective ALP types (Sugita [13]) were excised and homogenized with TBS and the extracts were applied to the dot blot immunoassay. The extract of the bone-derived enzyme band reacted clearly with the antibody, but the immuno-cross-reactivity of the liver isoenzyme was as negligible as that of the ALP extracted from human liver (Fig. 1).

Table 2. Immunological Experiments of Alkaline Phosphatases

ALP	Dot-blot	ELISA
HBC	1/10,000	1/10,000
Bone	1/10,000	1/10,000
Kidney	1/10,000	1/1,000
Dental pulp	1/500	1/100
Liver	1/10	1/10
Intestine	—	—
Placenta	—	—

Values are means of maximal dilution of antibody solution with positive immunoreaction.
The initial concentration of the antibody G3 was 5.4 mg/ml.
—: no reaction without dilution.

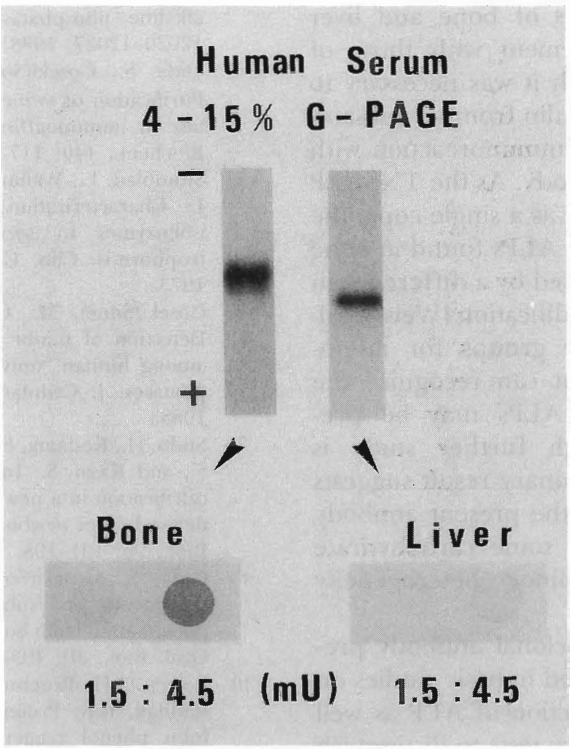

Fig. 1 Immuno-reactivity of human serum ALPs
Enzymatic activity was stained by β-naphthyl phosphate after gradient polyacrylamide gel electrophoresis of human serum ALPs (upper figure). The bands corresponding to bone isozyme (lower left lane) and liver isoenzyme (lower right lane) were sliced and homogenized in TBS and applied to the dot immunobinding assay.

Discussion

Tissue-nonspecific ALPs of kidney, liver and bone have almost identical enzymic properties as shown in inhibition and thermal inactivation tests (Table 1). In this report, we have succeeded in establishing one stable clone (G3) of a monoclonal antibody by selecting five candidates against ALP of human osteoblast-like cells. The immuno-reaction of this monoclonal antibody was intense to ALPs of HBC, kidney, bone and dental pulp, but virtually negative to liver ALP. Human dental pulp ALP showed the same properties as TNSALPs not only in enzymatic study but also in immunological properties (Goseki et al. [14]; Goseki et al. [15]). Human intestinal and placental ALPs did not cross-react at all. These data suggest that there are minor heterogeneities among human TNSALPs, and it is possible to recognize their differences by cross-reactivity with monoclonal antibodies. The present results lend support to the concept that human bone, dental pulp and kidney ALPs have antigenic specificity different from that of human liver enzyme.

The results of immuno-cross-reactivity

of serum isoenzymes of bone and liver types were in agreement with those of tissue ALPs, although it was necessary to remove immunoglobulin from serum samples to evaluate the immunoreaction with the monoclonal antibody. As the TNSALP gene appears to exist as a single copy, the heterogeneity among ALPs found in bone and liver may be caused by a difference in post-translational modification (Weiss et al. [1]). Several epitope groups for monoclonal antibodies that can recognize the difference between ALPs may be presumed. Even though further study is necessary, our preliminary result suggests that the epitope for the present antibody preparation may be some carbohydrate residues causing minor heterogeneity among TNSALPs.

The present monoclonal antibody preparation can be utilized in basic studies on the physiological function of ALP as well as in clinical laboratory tests to distinguish minor heterogeneity among human ALPs.

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