[Regular Paper]

Correlation of electrophoretically separated alkaline phosphatase isozyme 3 with osteocalcin in healthy adults

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SUMMARY

Activities of alkaline phosphatase isozyme 3 (ALP₃) in healthy adults (27 males and 36 females), ranging in age from 24 to 67 years old with a mean and standard deviation of 45.3 ± 9.4 , were estimated from the total activities of ALP and the fractions of ALP₃ among ALP isozymes separated by electrophoresis on cellulose acetate membranes. When mean values among different blood groups were compared, the ALP₃ activity and age in blood group B were higher than those in blood group AB, and the osteocalcin level in blood group B was highest. ALP₃ activities in male and female groups were not different. In females, ALP₃ activity correlated positively with the level of osteocalcin, but not with that of parathyroid hormone (PTH), although the total ALP activity correlated with both osteocalcin and PTH. Age correlated with both ALP₃ and PTH, but not with osteocalcin. The wide distribution of ALP₃ activity observed in the healthy adults, ranging from 44 to 168 IU/l with a mean and standard deviation of 89.3 ± 28.4 , could be explained by the modulation of osteoblastic activity and indirectly by the age-dependent alteration of parathyroid status in female subjects.

Key words: alkaline phosphatase, isozyme, electrophoresis, healthy adults, osteocalcin.

INTRODUCTION

Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1.; ALP) exists in multiple molecular forms, which are coded for by at least three distinct gene loci, namely the gene for the placental ALP, the gene for the adult intestinal

ALP and the tissue-unspecific gene for the ALP from liver, bone and kidney and probably other sources.^{1,2)} Post-translational modifications further complicate the multiple forms of ALP as may be seen in circulating blood plasma. These isozymes of ALP could be differentiated by a combination of electrophoresis and inactivation (or inhibition) by heat or chemicals as reviewed

健康成人における電気泳動で分離されたアルカリフォスファターゼアイソザイム3とオステオカルシンの相関

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in a recent article.³⁾

Among the techniques available for separation of serum ALP isozymes,³⁾ agarose gel,⁴⁾ or cellulose acetate⁵⁾ electrophoresis is widely used because of its simplicity and separation of a maximum number of clinically important multiple forms of ALP in one step; namely, ALP₁, high-molecular weight ALP; ALP₂, liver ALP; ALP₃, bone ALP; ALP₄, placental ALP; ALP₅, adult intestinal ALP; and ALP₆, ALP-immunoglobulin complexes.⁶⁾ Only ALP₁ and the variant ALP^{7,8)} overlap in the agarose, but not polyacrylamide, gel electrophoresis.⁵⁾ A drawback of this method is a poor resolution of ALP₂ and ALP₃. In fact, the activities of ALP₃ in sera of healthy adults varied widely when ALP₃ was separated by cellulose acetate electrophoresis and quantitated by densitometry. In an attempt to see whether the variation bears physiological significance or not, the activities of ALP₃ thus determined were evaluated by comparing with several parameters of bone metabolism, including serum levels of Ca, inorganic phosphate (P_i), parathyroid hormone (PTH) and osteocalcin (OC). The results indicated that the wide distribution of ALP₃ reflects varying levels of osteoblastic activity.

MATERIALS AND METHODS

Serum samples were collected from staffs of Kagawa University and of Yura Hospital at the time of periodical check for hepatobiliary disease by blood analysis, which includes alanine aminotransferase, γ -glutamyltransferase and hepatitis B surface antigen (Hepanostika HBsAg Micro-elisa system; Organon Teknika B.V., Boxtel, Holland). Serum levels of ALP were determined by a modified method of Bessey and Lowry⁹⁾ at 37°C using an Autosera ALP kit (Daiichi Pure Chemicals Co., Ltd., Tokyo) on subjects who had negative results for the above blood tests. Cases having increased serum levels of ALP above 280 IU/l (37°C) (cf. Ref. 9) were further evaluated, and the cases having physical or other laboratory

abnormalities were excluded. ALP isozymes were separated by electrophoresis on cellulose acetate membranes (Titan III Iso-Vis, Helena Laboratories, Beaumont, Tex.) and stained with naphthol AS-BI phosphate (sodium salt) and Fast Blue RR salt according to Saga.⁵⁾ Stained ALP bands were identified by the nomenclature system of Suzuki et al.⁶⁾ Band intensities were quantitated by densitometry with an Auto Scanner FLUR-VIS, Helena Laboratories, and expressed as per cent of the total intensity. ALP isozyme activities were tentatively calculated from the percentages of isozymes and the total ALP activity (IU/l at 37°C) determined as mentioned above. Serum levels of intact PTH, osteocalcin, Ca and inorganic phosphate (P_i) were determined on frozen sera. Intact (1-84) PTH was estimated by a two-site immunoradiometric assay using an Allégro intact PTH kit (assay range, 5-1500 pg/ml), Nichols Institute (San Juan Capistrano, CA) provided through Nippon Medipysics Co., Ltd. Osteocalcin was measured by radio-immunoassay with a Human Osteocalcin RIA kit (assay range, 0.25-60 ng/ml), Compagnie Oris Industrie S.A. (Gif-sur-Yvette, France) supplied through the Green Cross Co. (Osaka, Japan).

Results were expressed as means $\pm$ standard deviations (SD) for two groups of male and female and for four groups of different blood groups (A, 8 males and 8 females; B, 6 males and 11 females; AB, 3 males and 6 females; and O, 10 males and 11 females). Significant levels of differences between mean values were analyzed by the Student's *t* test.

RESULTS

Sera from healthy subjects showed two to four bands of ALP isozyme. ALP₂ and ALP₃ were present in all the samples analyzed, although their proportion varied widely from one case to another. ALP₁ and ALP₅ were present independently in a limited number of cases. ALP₄ was not detected in any of the sera tested. Representative zymograms and their densitometric tracings

Fig. 1. Representative zymograms of ALP isozyme and their densitometric tracings.
 Case 1: ALP₁, 4%; ALP₂, 54%; ALP₃, 30% and ALP₅, 12%. Case 2: ALP₁, 0%;
 ALP₂, 35%; ALP₃, 57% and ALP₅, 8%.

are shown in Fig. 1. The resolution of ALP₂ and ALP₃ was not necessarily satisfactory in all the cases studied. However, the mobilities of ALP₂ and ALP₃ in poorly resolved cases did not appear to differ from those of the corresponding well-separated cases. Since ALP₅ is detected at higher rates among subjects of blood groups B and O (secretor type),¹⁰⁾ the proportions of other ALP isozymes would be altered by the presence of ALP₅.

ALP₁ may have a similar but much less effect. In order to see whether this effect is limited to the proportions of ALP isozymes or affects the isozyme activities which were calculated from the percentages of individual band intensities and the total ALP activity, the mean isozyme activities for different blood groups are compared in Table 1. Sex differences are also shown in this table. Mean ALP₅ activities of blood groups B and O were higher than that of blood group A as generally accepted.¹⁰⁾ The mean activity of ALP₃ in blood group B was higher than those of blood groups A and AB, and conversely the

mean activity of ALP₂ in blood group B was lower than those of blood groups A and AB.

Some of the differences were statistically significant as indicated in the table. The mean age also showed a tendency similar to that of ALP₃; namely, the mean age in blood group B was higher than in blood group A or AB, the latter being a statistically significant difference. The mean osteocalcin level was also higher in blood group B as compared with that in blood group A, AB or O. The difference in serum Ca level between blood groups A and O was minor, even though it was statistically significant. In males the fraction of ALP₃ was lower and the activity of ALP₂ was higher than in females, although the activity of ALP₃ showed no difference between them. It was apparent that those differences in ALP activities were unrelated to the presence of ALP₅. In order to evaluate the differences in ALP isozyme activities among the above studied groups, correlation coefficients among the enzyme activities and related parameters were calculated, and the correlation coefficients with *p*

Table 1. ALP activities, ALP isozyme fractions and activities, and related compound levels in serum for different blood groups and sex in healthy adults.

No. of Cases	Blood groups				Sex		Total (range) 63
	A 16	B 17	AB 9	O 21	Male 27	Female 36	
Age (years)	44.8 ± 12.6	47.2 ± 8.0	39.7 ± 7.4	46.5 ± 7.8	46.7 ± 9.3	44.2 ± 9.4	45.3 ± 9.4 (26.5-64.1)
ALP (IU/l)	185.8 ± 38.8	191.4 ± 38.5	169.7 ± 28.9	187.9 ± 40.4	195.1 ± 40.1	178.6 ± 34.9	185.7 ± 37.8 (110.0-261.3)
ALP ₁ (%)	0.38 ± 1.03	0.82 ± 1.74	0.44 ± 0.88	0.10 ± 0.44	0.48 ± 1.34	0.36 ± 0.96	0.41 ± 1.13 (0-2.67)
ALP ₁ (IU/l)	0.74 ± 2.02	1.46 ± 3.19	0.79 ± 1.58	0.85 ± 2.43	0.81 ± 2.28	0.69 ± 1.95	0.74 ± 2.08 (0-4.90)
ALP ₂ (%)	51.4 ± 9.7	41.4 ± 8.1	50.2 ± 10.8	44.9 ± 6.9	49.0 ± 8.0	44.4 ± 9.8	46.4 ± 9.3 (27.8-64.9)
ALP ₂ (IU/l)	95.2 ± 25.4	78.6 ± 18.5	86.2 ± 27.6	84.3 ± 22.6	94.9 ± 22.9	79.0 ± 21.5	85.8 ± 23.3 (39.1-132.5)
ALP ₃ (%)	45.0 ± 10.3	51.9 ± 8.6	44.6 ± 12.2	48.3 ± 8.4	45.1 ± 8.4	50.0 ± 10.3	47.9 ± 9.7 (28.4-67.4)
ALP ₃ (IU/l)	84.3 ± 28.6	100.6 ± 31.3	74.0 ± 18.8	90.7 ± 26.8	88.8 ± 29.0	89.8 ± 28.3	89.3 ± 28.4 (32.6-146.1)
ALP ₅ (%)	3.2 ± 2.9	6.4 ± 5.1	4.8 ± 2.6	6.8 ± 3.5	5.4 ± 3.6	5.6 ± 4.3	5.5 ± 4.0 (0-13.5)
ALP ₅ (IU/l)	5.5 ± 4.8	12.0 ± 9.3	8.6 ± 5.5	12.6 ± 5.9	10.4 ± 6.8	9.8 ± 7.5	10.1 ± 7.2 (0-24.5)
PTH (pg/ml)	20.8 ± 5.9	20.6 ± 4.7	20.4 ± 6.5	18.5 ± 5.6	18.9 ± 4.9	20.7 ± 6.0	19.9 ± 5.6 (8.8-31.0)
OC (ng/ml)	2.94 ± 1.65	4.16 ± 1.69	2.91 ± 1.54	3.03 ± 1.52	3.45 ± 1.63	3.18 ± 1.68	3.30 ± 1.65 (0-6.60)
Ca (mEq/l)	4.80 ± 0.22	5.00 ± 0.24	4.88 ± 0.20	4.95 ± 0.20	4.90 ± 0.19	4.90 ± 0.25	4.9 ± 0.22 (4.46-5.34)
P _i (mg/dl)	3.40 ± 0.47	3.46 ± 0.51	3.18 ± 0.46	3.19 ± 0.55	3.11 ± 0.45	3.47 ± 0.50	3.31 ± 0.51 (2.30-4.33)

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (range), mean ± 2 SD. OC, osteocalcin.

values below 0.05 are listed in Table 2.

Generally, higher positive correlation coefficients were obtained in the female group. The highest and meaningful correlation coefficient was present between ALP₃ activity and osteocalcin level. Slightly less but similar correlations were found between the fraction of ALP₃ or total ALP activity and osteocalcin level. PTH correlated with total ALP and tended to correlate with ALP₃ activities ($r = 0.256$). In the male group, a negative correlation was found between PTH and serum Ca levels and a positive correlation between osteocalcin and serum P_i levels. The correlation coefficients between total ALP activity and ALP₃ activity were generally higher than those between total ALP activity and ALP₂

activity, indicating that the change in total ALP activity reflects the change in ALP₃ activity to a greater extent than that in ALP₂ activity, even though the proportions of ALP₂ and ALP₃ to the total ALP activity were similar.

DISCUSSION

The wide distribution of ALP₃ activity in healthy adults was first analyzed by comparing the mean values of ALP₃ and related parameters among different blood groups and between male and female groups. The higher activity of ALP₃ in blood group B than in blood group AB could be explained by the higher mean age in blood group B as well as the predominance of female in this blood group since a significant positive

Table 2. Significant correlation coefficients obtained among ALP activities, ALP isozyme fractions and activities, and related compound levels in serum of male, female and total subjects.

			Age	PTH	OC	Ca	P _i	ALP
Age (years)	M		—					
	F		—					
	M + F		—					
PTH (pg/ml)	M			—				
	F		0.360	—				
	M + F		0.276	—				
OC (ng/ml)	M				—			
	F				—			
	M + F				—			
Ca (mEq/l)	M			—0.401		—		
	F					—		
	M + F					—		
P _i (mg/dl)	M				0.411		—	
	F						—	
	M + F						—	
ALP (IU/l)	M							—
	F		0.355	0.366	0.483			—
	M + F				0.260			—
ALP ₂ (%)	M							
	F							
	M + F				—0.288			
ALP ₂ (IU/l)	M							0.696
	F							0.567
	M + F						—0.284	0.650
ALP ₃ (%)	M							
	F				0.351			
	M + F				0.304			
ALP ₃ (IU/l)	M							0.812
	F		0.464		0.558			0.763
	M + F		0.297		0.401			0.762

M, male (27 cases); F, female (36 Cases); and M+F, total (63 cases). Values with $p < 0.05$ are presented. OC, osteocalcin.

correlation was found between the age and ALP₃ in the female group. The higher value of osteocalcin in blood group B may be similarly explained since there was a high positive correlation between the ALP₃ and osteocalcin, although there was no direct correlation between the age and the osteocalcin level. Such a discrepant result as this is reported under varying pathological conditions and considered as reflecting different aspects of osteoblast function.^{11,12)}

The positive correlation existing between the age and the total ALP activity may be interpreted as indicating that the total ALP activity is

reflecting the age-dependent increase of ALP₃ activity in female. This is well documented¹¹⁾ and also supported by the findings in this study that the correlation between the total ALP and ALP₃ activities was higher than that between the total ALP and ALP₂ activities. The age also correlated with PTH, although the PTH level and the ALP₃ activity showed no significant correlation. A negative correlation between PTH and Ca levels as well as a positive correlation between osteocalcin and P_i levels were found only in males. Thus, the serum ALP₃ level in female subjects is directly related to the osteoblastic activity and

indirectly related to the age-dependent alteration of parathyroid status. Although no statistically significant positive correlation was found between the age and the serum level of osteocalcin, the age-dependent increase in serum osteocalcin level in females has been well documented.¹¹⁾ The higher activity of ALP₂ in male has not been reported elsewhere, and its physiological significance can not be assessed.

It must be remembered that the increases in the fraction of ALP₂ cause decreases in the fraction of ALP₃. Accordingly, the determination of relative activities of ALP₂ and ALP₃ has little significance in clinical evaluation of the results. The presence or absence of ALP₁ and ALP₅ had no marked effect on the proportions of other ALP isozymes, because they were minor components even if they were present in healthy subjects. The ALP₃ activity calculated from the total ALP activity and the fraction of ALP₃ was found in this study to be a useful parameter of bone metabolism independent of ALP₂ activity. Since the substrates used for the determination of total ALP activity (*p*-nitrophenyl phosphate) and for visualization of ALP isozyme (naphthol AS-BI phosphate) were different, the ALP₃ activity calculated from the total activity and the proportion of ALP₃ does not necessarily represent the activity of existing ALP₃. It should be considered rather an index related to ALP₃. Nevertheless, it had a high correlation with osteocalcin, and hence it could be regarded as a bone-type ALP for a practical purpose.

Currently used immunosassay of ALP₃ is less satisfactory than the present electrophoretic assay since ALP₃ activity was estimated indirectly with a monoclonal antibody having a 5-fold greater affinity for ALP₂ as compared with that for ALP₃ and by assuming only ALP₃ and ALP₂ were present in test samples.¹¹⁾ Accordingly, the present electrophoretic assay of ALP₃ is acceptable as the currently available, clinically useful, though not the best, method of ALP₃ analysis.

Rosalki and Foo¹³⁾ employed wheat-germ lectin in cellulose acetate electrophoresis to improve the separation of ALP₂ and ALP₃ by reducing the mobility of ALP₃. Although wheat-germ lectin in cellulose acetate electrophoresis improved the separation of ALP₂ and ALP₃ by reducing the mobility of ALP₃, the separation of ALP₃ and ALP₅ became consequently less marked, leaving a possibility of the overlap of these ALP bands. When the lectin was included in agarose gels, a precipitating bone ALP appeared,³⁾ making the separation of ALP₂ and ALP₃ further complicated. It may be worth-while, however, to carry out a similar study on the bone-type ALP separated by the method of Rosalki and Foo in an attempt to obtain additional evidence of the clinical importance of the determination of ALP₃ activity.

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要 旨

健康成人 (男 27 例, 女 36 例) のアルカリフォスファターゼ・アイソザイム 3 (ALP₃) の活性を, ALP の総活性およびセルロース・アセテート膜上で電気泳動によって分離した ALP アイソザイム中の ALP₃ の割合から求めた. 平均値を血液型各群で比較すると, B 型例の ALP₃ 活性および年齢は AB 型群のそれらよりも高く, B 型例のオステオカルシン値は他の血液型例より

も高かった. ALP₃ 活性は男女間で差がなかったが, ALP₃ の総活性に対する割合は男性のほうが低かった. 女性の ALP₃ 活性はオステオカルシン値と正の相関を示したが, 副甲状腺ホルモン (PTH) 値とは相関がみられなかった. しかし ALP の総活性はオステオカルシンおよび PTH と相関した. 年齢は ALP₃ および PTH と相関したがオステオカルシンとは相関しなかった. 健康成人における ALP₃ 活性の変動 (89.3 ± 28.4 IU/l) は, 女性における年齢依存性の副甲状腺機能の状態および骨代謝に, より直接的な関連因子による骨芽細胞機能の変調によって説明することができた.