Plasma Alkaline Phosphatase (ALP) in IS Rats and its Possible Origin

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ABSTRACT. The activities of alkaline phosphatase (ALP) from plasma at several ages were determined, and the origin of plasma ALP was investigated on the basis of the electrophoretic migration and the optimum pH in IS strain of rats (IS rats) and SD strain of rats (SD rats). Both in IS and SD rats, the activity was highest at the youngest group (8 weeks), and it decreased rapidly with age. The activities in IS rats at 8 and 10 weeks of age were 63% and 66% of those in SD rats. At 20 and 30 weeks of age, the difference was not clear. The zymogram gave two bands for SD rat plasma. One of the bands was regarded as the intestinal isozyme and the other was the osseous and/or the hepatic isozymes. The zymograms of IS rat plasma gave only one band which fairly consistent with that of the osseous or hepatic isozyme, and no intestinal isozyme was detected. The mobility of the intestinal isozyme was not completely identical between IS and SD rats. Thus, the molecular difference in the intestinal isozyme was thought to be a cause of the difference in the ALP activity and isozyme composition of the plasma between these two strains. The zymograms for other tissues did not show any difference between IS and SD rats. The effect of the change in pH on ALP activity also suggested that the plasma ALP in IS rats did not contain any intestinal isozyme.—*Key words:* alkaline phosphatase, Ishibashi rat.

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INTRODUCTION

The IS strain of rats (IS rats) was established from a crossbred between Wistar and wild rats and are characterized with congenital vertebral malformation [5]. Several investigators [10, 14, 18] reported that the plasma alkaline phosphatase (ALP) in IS rats was lower than in other strains. Takahashi *et al.* showed that the ALP isozyme of the intestine was virtually undetectable in the plasma of IS rats [18]. The anomaly in IS rats were considered to be similar to the hypophosphatasia in human, because of a low level of plasma ALP in addition to the vertebral abnormalities. However, the genes affecting plasma ALP activity are reported to be separable from the genic factors affecting the vertebral anomalies [10]. A definite cause of the low level of plasma ALP in IS rats remains uncertain.

Plasma ALP in rats were considered to be originated from the intestine, bone and liver [1, 3, 9, 11, 15, 17]. The ALP activity and the composition of isozymes in plasma were affected by some factors such as age and feeding condition. In this study, the plasma ALP activity in IS and SD (control) rats at several ages was determined and the tissue origin was examined by the electrophoretic mobility and the optimum pH of the isozymes.

MATERIALS AND METHODS

The IS rats used in this study were maintained in our laboratory and F24 progeny of full-sib mating. The SD rats were obtained from Charles River Japan, Inc. Blood samples were obtained from the vena cava caudalis by a heparinized syringe at 8, 10, 20 or 30 weeks of age. Samples of the upper intestinal mucosa, thigh bone, kidney and

liver were obtained at 8 or 10 weeks of age. The intestinal mucosa was scraped by a slide glass, and the bone was crushed in an iron mortar and pestle. The kidney and the liver were minced with scissors. These samples were homogenized by Ultra Tax (Ika-Werk) in 5 volumes of 0.25 M sucrose, and the crude ALP preparations were prepared according to the method of Morton [12]. The homogenate was stirred in an ice-water bath, and n-butanol was added gradually until a concentration of 25% v/v was reached. Then the mixture was stirred for more 1 hr and centrifuged at 20,000 $\times g$ for 20 min at 4°C. The aqueous phase was collected and dialyzed for 40 hr at 4°C against three changes of 0.01 M Tris-HCl buffer (pH 7.5).

ALP activity was determined by measuring the liberation of phenol from phenylphosphate basically according to the method of Kind and King [7]. Two ml of the assay mixture consisted of 50 mM bicarbonate buffer (pH 9.2–pH 10.6), 5 mM disodium phenylphosphate, 2.2 mM 4-aminoantipyrine, 2 mM $MgCl_2$ and an extracted sample at appropriate dilution. After incubation for 30 min at 30°C, the reaction was stopped and red color was developed by the addition of 2 ml of 210 mM H_3BO_4 –5.8 mM $K_3Fe(CN)_6$. The absorbance was measured at a wavelength of 500 nm. The activity was described in terms of n mol/ml or mg/min. Protein concentration was determined by the method of Lowry *et al.* [8].

The isozymes of ALP were separated by the electrophoresis, using the cellogel and the discontinuous buffer system [13]. A 0.26 M Tris-borate buffer (pH 9.1) was used for the anode buffer, and a 0.03 M barbital buffer (pH 8.0) was used for the cathod buffer. Sialic acid in the samples was catabolised by incubation with neuraminidase [16] in a 0.5 M acetic acid-sodium acetate buffer (pH 5.0) for 24 hr at 37°C. The current, 0.5 mA/cm cellogel membrane, was supplied for 40 min.

After electrophoresis, a cellogel was soaked for 30 min at 37°C in 10 ml of a 0.1 M 2-amino-2-methyl-1, 3-propanediol, containing Naphthol AS-BI phosphate and 20 mM $MgCl_2$, and the substrate was made to react with ALP. Then the cellogel was stained with 5 mg Fast blue RR salt in 10 ml of a 0.1 M 2-amino-2-methyl-1, 3-propanediol.

RESULTS

Plasma ALP activities at several ages were shown in Fig. 1. The activity at 8 weeks was highest both in IS and SD rats and it decreased rapidly with aging. The activities in IS rats at 8 and 10 weeks were significantly lower (63% and 66%, respectively) than those in SD rats. The activities in IS rats at 20 and 30 weeks of age were not significantly different from that of SD rats.

The effect of pH on plasma ALP activity in IS and SD rats at 8 and 20 weeks was shown in Fig. 2. Both at 8 and 20 weeks of age, the maximal ALP activity in IS rats was observed at pH 10.2. The maximal ALP activities in SD rats were slightly shifted to the lower pH range. The relative activities at pH 9.4 and 9.6 in IS rats at 8 weeks were significantly lower than those in SD rats, but between pH 10.4 and 10.6 IS rats had significantly higher activity than SD rats. The similar tendency was observed at 20 weeks of age, but the difference was much attenuated.

Changes in ALP activity under different pH were shown in Fig. 3 using the intestinal mucosa, bone, kidney and liver preparations from IS and SD rats. The effect of pH on ALP activity from respective tissues were almost identical between the two strains. The optimum pH of ALP activity from the intestine was pH 9.6, and the optimum pH ranged from 10.0 to 10.2 in other tissues. The changing pattern of plasma ALP activity under different pH in IS rats was similar to those of the bone, kidney and liver, but the pattern in SD rats was intermediate between those of the intestinal mucosa and the bone.

Fig. 1. Changes in plasma ALP activity in IS and SD rats with aging. ●, IS rats; ○, SD rats. The means $\pm$ S.D. for 6 animals are given by points and vertical bars. The statistically significant difference between the two strains with the same age was obtained from Student's t-test. **, $p < 0.01$; *, $p < 0.05$.

Fig. 2. Effect of pH on plasma ALP activity in IS and SD rats at 8 (A) and 20 (B) weeks. For other symbols, see Fig. 1 legend.

Fig. 3. Effect of pH on ALP activity of the intestine, bone, kidney and liver preparations in IS and SD rats. ●, IS rats; ○, SD rats; I, intestine; B, bone; K, kidney; L, liver. Each point shows the means of 3 determinations.

Fig. 4. Effect of pH on ALP activity of the tissues and the plasma preparations in IS and SD rats. The values are expressed as the ratio of ALP activity at pH 9.6 to that at pH 10.15. ■, IS rats; □, SD rats; I, intestine; K, kidney; B, bone; L, liver; P, plasma; 8W, 8 weeks; 10W, 10 weeks; 20W, 20 weeks; 30W, 30 weeks. For animals used and the statistical analysis, see Fig. 1 legend.

Fig. 5. Electrophoretic migration and densitometric scans of plasma ALP before and after the digestion with neuraminidase. A, before digestion; B, after digestion; IS, IS rats; SD, SD rats; 8W, 8 weeks; 20W, 20 weeks; O, origin.

The ratios of the ALP activities of the plasma and the tissues at pH 9.6 to 10.15 (activity at pH 9.6/activity at pH 10.15) were shown in Fig. 4. The ratios of the same tissues were almost equal between IS and SD rats. The ratios of the intestinal mucosa were higher than others. The ratios obtained by the bone, kidney and liver preparations were almost equal, ranging around 80%.

The ratio of the plasma in IS rat were significantly lower than that in SD rats at every age examined. The ratios of the plasma increased slightly with aging, but there were no significant differences. The ratios obtained in IS rats plasma were similar to those in the kidney, bone and liver preparations. In SD rats, the ratios at various ages differed each other. The difference at 10 and 30 weeks of age was statistically significant. The values obtained in SD rat plasma were slightly higher than those in the bone, liver and kidney, and definitely lower than that in the intestinal mucosa.

The zymograms of the plasma isozyme from IS and SD rats were shown in Fig. 5. The ALP from IS rats gave a single band, whereas that from SD rats gave two bands. The band from IS rats stayed slightly behind the fast band from SD rats, but clearly before

Fig. 6. Plasma ALP activity in SD rats at 8 and 20 weeks. Two bands electrophoretically separated were determined. , fast band; , slow band; 8W, 8 weeks; 20W, 20 weeks. Each column and vertical bar indicates the mean $\pm$ S.D. of 6 animals.

the slow band from SD rats. After the neuraminidase digestion, the ALP from IS rat plasma migrated to the cathodal side as a broad band. The fast band from SD rat plasma migrated to the same position as in IS rats, and the slow band stayed at the same position as that before the digestion. Between 8 and 20 weeks of age in SD rats, the ratios of the ALP activity of the fast to slow band before the digestion were reversed as shown in Fig. 6. The slow band was more potent at 8 weeks of age, whereas the fast one became more potent at 20 weeks of age.

The zymograms of the tissues from IS and SD rats before and after the neuraminidase digestion were shown in Figs. 7 and 8. ALP of the intestinal mucosa from IS and SD rats gave a single band. The migration of these two bands did not completely coincide; and the band of IS rats went slightly ahead. The migrations of the band from the intestinal mucosa and the slow band from the plasma were identical in SD rats. The isozyme band of the intestinal mucosa could not be detected in the plasma of IS rats. The neuraminidase digestion failed to effect the migration of the

Fig. 7. Electrophoretic migration of ALP from plasma and various tissues before digestion with neuraminidase. I, intestine; B, bone; L, liver; P, plasma; IS, IS rats; SD, SD rats.

intestinal isozyme, and this remaining band was situated at the identical position to the band of the plasma in SD rats which was also not affected by neuraminidase.

The migrations of ALP isozymes from the bone and the liver were the same between IS and SD rats. Before the neuraminidase digestion, plasma ALP in IS rats migrated almost similarly to the fast band of either the bone or the liver. The two bands of SD rat plasma migrated identically with those of the bone or the liver. After the neuraminidase digestion, the plasma ALP in IS rats and the slow band of SD rat plasma migrated cathodically as the osseous isozymes or the slow band of the liver. The fast band of SD rat plasma was situated at the intestinal band of SD rats. The zymograms of the kidney in both strains were fairly complex and yielded a broad or multiple fractions before the neuraminidase digestion (data were not shown). After neuraminidase digestion, however,

Fig. 8. Electrophoretic migration of ALP from plasma and various tissues after the digestion with neuraminidase. I, intestine; B, bone; L, liver; K, kidney; P, plasma; IS, IS rats; SD, SD rats; O, origin.

only a single band was distinct. The migrations of the band from the kidney ALP and that of plasma ALP in either strains did not coincide as shown in Fig. 8.

DISCUSSION

ALP activity of plasma was higher in young animals than in adults, and it decreased with aging. Low plasma ALP in IS rats was reported at 8 to 10 weeks [10] and 20 weeks [18] of age. The present results basically confirmed previous reports. Because of the rapid decrease in plasma ALP activity both in IS and SD rats with aging, the difference in ALP activity between 2 strains will become more unclear at 20 and 30 weeks. The variation on growth process between these two

strains should also be considered for the comparison of ALP activity.

ALP has been found in many tissues, though precise physiological role is still not clear. The tissues with high ALP activities are those concerned much with the active transport process such as the intestinal mucosa, tubule renales, bile canaliculi, osteoblast, and placenta. Thus, the function of the enzyme in these tissues might be related to the active transport mechanism. The changes in plasma ALP activity and absorption rate of lipid by the intestine were well correlated [17, 18], and moreover, high osseous isozyme activity was found in human infant with a high ossification activity [2].

The tissues containing high ALP activity were to be the source of plasma ALP. Previous investigators showed by electrophoresis that intestinal ALP isozyme was detected in rat plasma especially when the rat was fully fed [15, 17] or affected with some disease such as diabetes [9]. The high osseous ALP isozyme activity was found in fasted rat plasma by electrophoresis or a kinetic method [15]. It was reported that some ALP isozyme found in plasma was immunologically identical with the hepatic isozyme [11]. Other experiments using electrophoresis [6] showed that the hepatic isozyme was increased by ligating the bile duct, whereas this isozyme was not found in the plasma of control rats. The ages and other conditions in these experiments were not the same, so the results could not be generalized. Nevertheless, from these observations it is surmised that the plasma ALP in rats may be a mixture of the isozymes from the intestine, liver and bone. The contents and proportion of each isozyme may vary according to the differences of feeding, age, disease and some other conditions.

Changing pattern in plasma ALP activity under different pH were different between IS and SD rats (Fig. 2). The plasma ALP of IS rats had a pattern similar to that of the bone, kidney and liver, so the origin of plasma ALP

is presumably some of these tissues. The plasma ALP of SD rats had an intermediate pattern between the intestinal and the osseous-renal-hepatic isozymes. Hence, the plasma ALP would be a mixture of isozymes from those multiple tissues. The intestinal ALP had the optimum pH shifted slightly to a neutral range (Fig. 3), which was in agreement with a previous report [15]. The ratio of the ALP activities obtained at pH 9.6 and 10.15 was calculated for a simple parameter to express the effect of pH on enzyme activity. The comparison of these figures among ALP preparations from plasma and tissues indicated the same conclusion as obtained on the basis of the optimum pH.

The ALP zymograms from plasma and the tissues also showed the same indication about the origins of plasma isozymes: The fast band from plasma ALP in SD rats migrated identically with the fast fraction from the liver or the bone before and after the digestion with neuraminidase. Thus the origin of the band from plasma ALP in SD rats was thought to be one or both of these tissues. The slow band observed before the digestion in the liver and the bone preparations from both strains was proved identical with each other: the band did not coincide with that of the plasma in the either strains after the neuraminidase digestion. The plasma isozyme of IS rats migrated with slight different mobility compared with the corresponding bands from SD rat plasma or other tissues. After the neuraminidase digestion, however, the band migration was identical with that of the fast one of the plasma isozymes in SD rats and fast one of the osseous and hepatic isozymes. Thus, the plasma isozyme in IS rats would be essentially the same as the isozyme developed as these bands.

In IS rat plasma, no intestinal isozyme was found, whereas the zymogram of the plasma in SD rats revealed that intestinal isozyme was a major component in the plasma of young animals. The low ALP activity in the

plasma of IS rats may be due to this absence of the intestinal isozyme as reported previously [18]. This seems to contradict the result that the ALP activity was also found in the intestinal mucosa in IS as well as SD rats. The intestinal isozyme in IS rats may be somewhat dissimilar to that of other strains; its liberation from the intestinal mucosa into the blood may be blocked, or its disappearance rate from blood may be higher than in other strains. Disappearance rates of exogenous ALP isozymes from the plasma are reported to be different each other [14], and the intestinal ALP isozyme injected intravenously reported to disappear rapidly from the circulation in rats [17]. Moreover, only a fractional increase of plasma ALP activity after feeding was detected in IS rats in contrast with the obvious increase in the other strains [18]. A slight disagreement in the electrophoretic migration of the intestinal ALP between IS and SD rats in this experiment might suggest the mutation of the intestinal ALP isozyme in IS rats.

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要 約

IS ラットの血漿アルカリホスファターゼ (ALP) 活性および由来について: 藤瀬 浩・西川祥子・横山英子・小林好作・石橋正彦¹⁾ (麻布大学獣医学部臨床病理学教室, ¹⁾育種学教室)——IS ラットと対照 SD ラットの、加齢にともなう血漿アルカリホスファターゼ (ALP) 活性変化を測定するとともに、血漿 ALP の由来臓器を電気泳動と至適 pH を用い検討した。両ラットとも血漿 ALP 活性は8週齢で最も高く、加齢にしたがい急激に低下した。IS ラットの8週および10週齢の活性は、それぞれSD ラットの63%および66%であった。20週および30週齢でも同様の傾向はみられたがその差は明瞭ではなかった。SD ラットの血漿電気泳動像には2本の泳動帯が観察され、1本は小腸アイソザイム、他は骨あるいは肝アイソザイムもしくはその両者に一致していた。IS ラットの血漿電気泳動像には1本の泳動帯が観察され、それは骨あるいは肝アイソザイムに類似しており、小腸アイソザイムは検出されなかった。両ラットの血漿 ALP の泳動帯は一致せず、この差が両ラットにおける血漿 ALP 活性および含有アイソザイムの差をもたらすと考えられた。他の臓器 ALP では、両ラット間に差がなかった。ALP 活性に対する pH の影響でも、IS ラットの血漿 ALP には小腸アイソザイムが含有されないことが支持された。