Expression of intestinal-type alkaline phosphatase mRNA in liver of Akp3 knockout mice

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SUMMARY

Previously, we discovered that the rat liver showed enhanced expression of intestinal-type alkaline phosphatase (ALP) on high fat-feeding. Here we tested the hypothesis that intestinal-type ALP (Akp3 or Akp6) might also be regulated in the liver of Akp3–/– mice upon high fat-feeding. C57Bl/6J male Akp3–/–, Akp3+/–, or wild-type mice were fed a normal control or high-fat diet and the effects on intestinal-type ALP mRNA in liver were investigated. We found that Akp3 mRNA is only expressed in the intestine and not in the liver, while Akp6 mRNA is expressed in both the upper intestine and liver of wild-type mice. The intensity of Akp6 mRNA expression in the liver was not enhanced in the Akp3–/– mice compared with the wild-type mice fed on a high-fat diet. The nucleotide sequence of the PCR product of Akp6 from the liver was identified as being the same as that of Akp6 in the intestine. This is the first report concerning IAP mRNA expression in the mouse liver, but further studies will be needed to determine if this ectopic expression of intestinal-type ALP is associated with any unique function.

Key words: alkaline phosphatase, liver, knockout mice, mRNA expression

INTRODUCTION

Alkaline phosphatase (ALP: orthophosphoric monoester phospho-hydrolase, alkaline optimum, EC 3.1.3.1.) hydrolyzes a variety of monophosphate esters into inorganic acid and alcohol at a high optimum pH (pH 8–10). Studies on the genes encoding this enzyme family revealed that there are four ALP isozymes in the human body: tissue-nonspecific (liver/bone/kidney: TNSALP)1, intestinal (IAP)2, 3, placental (PLAP)4, 5, and placental-like or germ cell (GCAP)6. In humans, TNSALP shows 52 and 57% homology with PLAP and IAP, respectively4, and the sequences predicted from cloned cDNAs encoding ALPs show that IAP and PLAP are 87–90% identical at the amino acid level5, 7. As a result of studies on cDNAs encoding ALP isozymes, it is known that the primary structure in the catalytic region is well-conserved in ALPs of humans, animals, and E. coli. Based on studies of hypophosphatasia, a systemic skeletal disorder resulting from TNSALP deficiency, TNSALP has been shown to be indispensable for bone mineralization by controlling the concentration of inorganic pyrophosphate, a potent calcification inhibitor8–13. Recently, we identified a significantly stronger association between single nucleotide polymorphisms (SNPs) in the TNSALP gene (787T>C) (rs3200254) associated with the low or high bone mineral density (BMD) among 501 postmenopausal women14, 15.

In rats, ALP is classified into two types: TNSALP and IAP. IAP is present in the membrane surrounding neutral fat droplets in the microvilli of the intestinal mucosa during fat absorption, and is thought to transport dietary lipids from the intestinal tract into the circulation as a component of unilamellar membranes called surfactant-like particles (SLPs)16, 17. A single gene for human IAP has been isolated, and the multiple forms of mRNAs encoding human IAP are due to differences in polyadenylation20. Although most species express a single IAP, several kinds of IAP have been identified in three species, the mouse18, rat19, 20...
and cow(21). Two different cDNA clones, IAP-I and IAP-II, for rat intestinal ALP were isolated by Lowe et al.(19) and Strom et al.(20), respectively. Strom et al. found that the expression of IAP-II mRNA was specifically enhanced by 1α,25-(OH)2D3 administration. The two isoforms are products of two distinct genes and their cDNA sequences show 79% identity at the amino acid level. Functional differences between IAP-I and IAP-II were suggested by different regulation of the expression of the two mRNAs(22), as well as by structural and catalytic differences(23).

In mice, five different ALP loci have been identified: TNSALP, IAP, embryonic (EAP), Akp6, and Akp-ps1. These ALP genes correspond to different proteins: Akp2 encodes TNSALP, Akp3 encodes IAP, Akp5 encodes EAP, Akp6 encodes a novel IAP-like isozyme expressed globally in the gut (thus called gIAP), and Akp-ps1 encodes the inactive pseudo-type ALP. The sequence of Akp3 is similar to rat IAP-II. Recently, Narisawa et al.(18) demonstrated that gIAP was upregulated in the jejunum and ileum of Akp3 knockout mice, and in the same distribution following chow or high fat-feeding in both of wild type and Akp3–/– animals. This Akp6 gene has a sequence homologous to rat IAP-I gene. The Km (Michaelis constant) value for the protein translated from the Akp6 gene was 30% lower than that from the Akp3 gene using p-nitrophenylphosphate (p-NPP) as a substrate at pH 9.8.(18)

Previously, we reported the expression of mRNA encoding IAP-II in the rat liver and its increase on high fat-feeding(24). We expected that the expression of mRNA encoding Akp6 corresponding to rat IAP-II in the mouse liver and its increase on high fat-feeding.

In the present study, we examined the effect of fat-feeding on the expression of mRNA encoding mouse IAPs (Akp3 or Akp6), and we revealed the effects of high fat-feeding on the expression of IAP in liver tissue compared to wild type and Akp3–/– animals.

**MATERIALS AND METHODS**

**Animals and tissue preparation**

An Akp3+/− mouse line(25), with a theoretical 75% 129/Sv and 25% C57Bl/6j gene background, was maintained by sibling mating. The genotypes of the offspring were identified by PCR using genomic DNA extracted from the tail. We used the littermate wild-type (WT) mice as controls. The mice were kept at 23°C under a 12:12-h light-dark cycle with free access to chow and water. Eight- to ten-week-old mice were fed a 4% fat standard chow (MEQ; Oriental Yeast, Tokyo, Japan) a synthetic 25% lard-added MEQ high-fat chow (30% total fat)(17). All experiments were approved by the Animal Care Committee of Saitama Medical University. Under anesthesia, the small intestine (upper jejunum) was removed from the mice, opened longitudinally, and washed in cold 0.9% saline.

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA from the intestine or liver was isolated by RNA later (Ambion Inc., Austin, TX, USA). Poly(A)+RNA was purified by oligo(dT)-cellulose column chromatography (QuickPrep micro mRNA purification kit, Amersham Biosciences, Piscataway, NJ, USA). As a template for PCR, single-stranded cDNA was prepared from 200 ng of poly(A)+RNA using Ready-to-go You-Prime First-Strand Beads (Amersham Pharmacia Biotech). For the Akp3 nucleotide sequence, the PCR primers Akp3-up (nucleotide position 625–642) and Akp3-down (nucleotide position 941–958) were used (GenBank: NCBI sequence data: NM007432)(20). In order to detect Akp6, a sense-primer (Akp6-up) and an anti-sense primer (Akp6-down) were designed on the basis of the Akp6 nucleotide sequence (GenBank: NCBI sequence data AK008000). Akp6-up spans nucleotide positions 882–902 and Akp6-down spans 1,333–1,354. PCR primers for Akp2(26) and Akp5(27) were used. The PCR conditions were as follows: 5 cycles at 94°C (1 min), 50°C (1 min), and 72°C (1 min), and 25 cycles at 94°C (30 sec), 55°C (30 sec), and 72°C (30 sec), followed by 10 min at 72°C. The amplified samples were analyzed using 5.25% polyacrylamide gel electrophoresis (PAGE). The stained gels were observed with UV light. The density of the photograph was determined by densitometry (AE6920M, ATTO, Tokyo, Japan). The PCR products were directly sequenced using a Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) with a Gene Rapid sequencer (Amersham Pharmacia Biotech).

**Enzyme activity and protein assays**

ALP activity was determined with 10 mM p-NPP as a substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer containing 5 mM MgCl2, pH 10.0, at 37°C. Protein concentrations were determined using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA).

**Statistics**

Values are shown as the mean ± standard error (S.E.). Comparisons between groups (WT vs. Akp3+/−) were performed using the unpaired Student’s t-test. Differences were considered significant at p<0.05. Analysis was conducted using SPSS 13.0J (SPSS, Inc., Chicago, IL, USA).

**RESULTS AND DISCUSSION**

Body weights (g), liver weights (g), and ALP activities of liver tissue among the WT, Akp3+/−, and Akp3−/− mouse group which were all fed a high-fat diet are summarized in Table 1. There were no significant differences in the liver weights and ALP activities among these groups.

Total RNA was extracted from the small intestine or the
liver in the WT, Akp3\(^{+/-}\), or Akp3\(^{-/-}\) mouse groups, and single-stranded cDNA were prepared from 1 \(\mu\)g of total RNA as a template for PCR.

Expression of Akp3 (encoding IAP) and Akp6 (encoding gIAP) mRNAs in the liver under normal feeding are shown in Fig. 1. Akp3 was expressed strongly in the intestine of the WT mouse, but not expressed in the intestine of Akp3\(^{-/-}\) (data not shown). In the liver, Akp2 (encoding TNSALP) was expressed mainly in WT and Akp3\(^{-/-}\) mice, while Akp6 mRNA was detected at very low levels in both of WT and Akp3\(^{-/-}\) mice.

Fig. 2 shows the expression of Akp3 and Akp6 in the liver under high-fat feeding. The expression of Akp3 mRNA was not detected in the livers of WT, Akp3\(^{+/-}\), and Akp3\(^{-/-}\) mice fed the high-fat diet. Akp6 was expressed in the liver of WT, Akp3\(^{+/-}\) and Akp3\(^{-/-}\) groups, but the intensity of Akp6 expression was low and similar among the WT, Akp3\(^{+/-}\) and Akp3\(^{-/-}\) groups.

To confirm the identity of Akp6 in the intestine or liver under high-fat feeding, and we confirmed that this sequence was identical to the mouse intestinal Akp6 nucleotide sequence (Fig. 3). Akp6 cDNA was cloned and sequenced from Akp3\(^{-/-}\) mouse intestinal RNA, and a faster body weight gain in Akp3\(^{-/-}\) mice was observed when these animals were fed a high-fat diet\(^{18}\). In addition to the physiological role of IAP in dietary lipid transport, there are several valuable studies suggesting other functions of IAP. It was demonstrated that heat shock induced IAP in rat IEC-18 cells and IAP may play an important role as a dephosphorylating enzyme under stress conditions\(^{28}\). Koyama et al. reported that ALP reduced lipopolysaccharide (LPS) toxicity through

<table>
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<td>3.2±0.3</td>
<td>0.64±0.09</td>
</tr>
<tr>
<td>Akp3(^{+/-})</td>
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<td>3.2±0.5</td>
<td>0.64±0.06</td>
</tr>
<tr>
<td>Akp3(^{-/-})</td>
<td>4</td>
<td>57.7±3.9</td>
<td>3.8±0.4</td>
<td>0.76±0.08</td>
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Each value represents the mean±S.E.
Male Akp3\(^{-/-}\), Akp3\(^{+/-}\) and WT mice were fed a high-fat diet.

![Fig. 1. Expression of ALP genes in the liver of mice fed on the control diet.](image)

A: Detection of Akp3, Akp6, and GAPDH RNA sequences by PCR and 5.25% polyacrylamide gel electrophoresis. Total RNA was extracted from the liver of WT or Akp3\(^{-/-}\) mice fed the control diet. Lanes 1–4: WT mouse; lanes 5–8: Akp3\(^{-/-}\) mouse. B: Diagrams show the relative density of the PCR products. Results are the mean±S.E. of 4 animals.

Table 1. Body weights, liver weights, and ALP activities of liver tissue.

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Each value represents the mean±S.E.
Male Akp3\(^{-/-}\), Akp3\(^{+/-}\) and WT mice were fed a high-fat diet.
Most recently, Goldberg et al. demonstrated that IAP has the ability to detoxify LPS and prevent bacterial invasion across the gut mucosal barrier using WT and Akp3<sup>−/−</sup> mice and in vitro assays with human and rat intestinal cell lines<sup>30</sup>. They concluded that IAP might be a gut mucosal defense factor maintained by enteral nutrition<sup>30</sup>.

In the present study, we showed that the mouse liver weakly expressed one of the IAP’s (Akp6) mRNA but not Akp3 mRNA, and this expression was not enhanced in the liver of Akp3<sup>−/−</sup> mice compared with that of WT mice on a high-fat feeding. Previously, we discovered that rat IAP-I mRNA was expressed only in the intestine and not in the liver, while IAP-II mRNA was expressed both in the intestine and liver<sup>24</sup>. On high-fat-feeding, the expression of rat IAP-I mRNA increased only in the intestine while that of IAP-II mRNA increased both in the intestine and liver<sup>24</sup>. However, the reason why the expression of rat IAP mRNA in the liver is enhanced by high fat-feeding is unclear. Mice and humans have a gallbladder, but rats do not. In rat bile, the presence of a heat-stable intestinal type ALP in addition to TNSALP is well-known<sup>31</sup>. The intake of food, especially fat, was followed by a higher level of IAP in the serum of rats than in any other species<sup>32</sup>. Bile duct ligation also induced a marked elevation of not only hepatic but also intestinal ALP<sup>33</sup>, and the uptake of IAP by the rat liver from the circulation was reported<sup>34</sup>. These data show that IAP induction in the liver following high fat-feeding is not similar in rats and mice.

In summary, this study demonstrates the presence and identity IAP mRNAs expression in the murine liver and the effects of high fat-feeding. Further analysis of the promoter regions will help to elucidate if this ectopic expression of intestinal-type ALP is related with any other function.

**ABBREVIATIONS**

ALP, alkaline phosphatase; TNSALP, tissue-nonspecific alkaline phosphatase; IAP, intestinal alkaline phosphatase; RT-PCR, reverse transcription polymerase chain reaction.
REFERENCES


Fig. 3. Nucleotide sequence of the PCR product from liver poly(A)+RNA under control-diet feeding using Akp6 primers is compared to the mouse intestinal ALP (Akp6) sequence (GenBank: NCBI sequence data AK008000) (top sequence).

Matches between the two sequences are marked by asterisks.

22) Xie Q, Alpers DH. The two isozymes of rat intestinal alkaline phosphatase are products of two distinct genes. Physiol Genomics. 2000;3:1–8.


