Kinetics of mRNA expression of alkaline phosphatase isoenzymes in hepatic tissues from glucocorticoid-treated dogs

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Objective—To clone segments of the canine liver alkaline phosphatase (LALP) and corticosteroid-induced alkaline phosphatase (CIALP) genes and use these clones to determine the tissue source of CIALP, the kinetics of LALP and CIALP mRNA expression for glucocorticoid-treated dogs, and the correlation between LALP and CIALP transcript concentrations and isoenzyme activities.

Sample Population—Tissues obtained from 7 dogs treated with prednisone (1 mg/kg, SC, q 24 h) for up to 32 days and 1 untreated (control) dog.

Procedure—Gene segments of LALP and CIALP were obtained by reverse transcription-polymerase chain reaction (RT-PCR) assay. The tissue source of CIALP and IALP mRNA was determined by northern blot analysis of tissues from 1 of the glucocorticoid-treated dogs. Hepatic tissues and serum samples were obtained from the 6 remaining glucocorticoid-treated dogs on days 0, 2, 5, 10, and 32 of prednisone treatment, and relative expression of LALP and CIALP mRNA was correlated with LALP and CIALP activity.

Results—A 2,246-bp pair (bp) segment of canine LALP and a 1,338-bp segment of CIALP were cloned. The Northern blot analysis revealed CIALP mRNA expression in hepatic tissues only after glucocorticoid treatment. Kinetics of serum and hepatic LALP and CIALP were paralleled and were obtained by reverse transcription-polymerase chain reaction (RT-PCR) assay. The tissue source of CIALP, IALP, and CIALP mRNA was determined by northern blot analysis of tissues from 1 of the glucocorticoid-treated dogs.

Conclusions and Clinical Relevance—The liver is the most likely source for CIALP in dogs. Analysis of kinetics of serum and hepatic LALP and CIALP mRNA suggests that glucocorticoid treatment, both are regulated by modification of mRNA transcript concentrations, possibly through differing mechanisms.

In dogs, increases in serum and tissue alkaline phosphatase (ALP) isoenzyme activities have been associated with excess circulating glucocorticoids. For these conditions, 2 isoenzymes comprise the greatest activity in serum (ie, liver ALP [LALP] isoenzyme and corticosteroid-induced ALP [CIALP] isoenzyme). Liver ALP is a product of the tissue nonspecific (TNS) ALP gene and has activity in the liver and serum of clinically normal dogs. The TNS ALP gene also yields bone and kidney ALP isoforms.

Corticosteroid-induced ALP is unique to dogs, and there is little or no activity in serum of clinically normal dogs. Corticosteroid-induced ALP is believed to be a product of the liver; however, the exact tissue source of synthesis has not been confirmed and is somewhat controversial. Immunohistologic examination and staining for ALP activity of hepatic tissue from dogs experimentally treated with glucocorticoids have revealed CIALP activity on the membrane of hepatocytes.

However, evaluation of enzyme kinetics, inhibition analysis, antigenic properties, and N-terminal amino acid sequences have confirmed that CIALP is a product of the intestinal ALP (IALP) gene. Corticosteroid-induced ALP differs from IALP in carbohydrate composition: CIALP is heavily glycosylated with sialic acid residues, whereas IALP is virtually devoid of sialic acid. In addition, results of in vitro experiments suggest that CIALP may be a reglycosylated form of IALP that is synthesized in the intestines.

After experimental administration of glucocorticoids, LALP and CIALP have differing kinetics with regard to appearance in serum and hepatic tissues. Serum and hepatic LALP activity begins to increase within 24 to 48 hours after glucocorticoid administration. In contrast, serum and hepatic CIALP activity do not begin to increase until 7 to 10 days after initiation of glucocorticoid treatment. The reason for this difference in kinetics is unknown, but it could be regulated at the level of gene transcription.

The objectives of the study reported here were to confirm that the liver can synthesize CIALP and that hepatic CIALP expression increases during glucocorticoid treatment in dogs. We also examined the kinetics of hepatic ALP isoenzyme mRNA expression and its correlation with serum and tissue LALP and CIALP activities.

Materials and Methods

Animals—Eight mature mixed-breed dogs were used in the study. Dogs were housed at the Laboratory Animal Facility at our institution. Use of the dogs was approved by the University of Illinois Laboratory Animal Care Advisory Committee.

Procedure—Two dogs were used for the initial cloning and sequencing of portions of the LALP and CIALP genes. One dog was administered prednisone (1 mg/kg, SC, q 24 h) for 21 days. The second dog was used as a source of untreated tissues. The dogs were euthanatized on day 32, and samples (1 to 2 g) of the liver, kidneys, and intestinal mucosa of the duodenum were harvested from both dogs, snap frozen, and stored in liquid nitrogen until further use.

Six dogs were used to study the kinetics of LALP isoenzyme transcription. Dogs were administered prednisone (1.0 mg/kg, SC, q 24 h) for 32 days. The first day of administration was designated as day 0. Liver biopsy specimens were...
obtained from each dog via laparoscopy on days 0, 2, 5, and 10. All biopsy specimens were snap frozen and stored in liquid nitrogen. Dogs were euthanatized on day 32, and hepatic tissues were harvested. Serum samples were obtained from each dog on each day that hepatic specimens were obtained.

**RNA extraction**—Total cellular RNA was extracted from tissues of the liver, kidneys, and intestinal mucosa of the duodenum that had been snap frozen and stored in liquid nitrogen. Extraction was accomplished by use of a guanidine thiocyanate-phenol-chloroform technique.11

Reverse transcription-polymerase chain reaction (RT-PCR) assay—Aliquots (1 μg) of total RNA from the liver, kidneys, and intestinal mucosa were reverse transcribed into cDNA by use of a commercial kit1 performed in accordance with the manufacturer's protocol. Normal hexamers supplied with the kit were used as the primers for reverse transcription. The resulting cDNA was diluted 1:2 with sterile, RNase-free water and used as a template for the PCR procedure.

The N-terminal amino acid sequences of LALP and CIALP were determined by Edman degradation of highly purified preparations of each isoenzyme.1 The upstream primers used for PCR were designed by use of the open reading frame of the N-terminal amino acid sequence of canine LALP and CIALP. Downstream consensus sequence primers were designed by use of known ALP gene sequences of other species.1,11 Each PCR assay contained 1× reaction buffer (50 mM KCl, 20 mM Tris-HCL [pH 8.4], 0.1% Triton X-100), 1.75 mM MgCl2, 0.2 mM of each dinucleoside triphosphate, 1 μM of the designed ALP primers, 1.25 units of Taq DNA polymerase,1 and 5 μl of diluted tissue-specific cDNA template. The thermocycle pattern included an initial denaturing step at 95 C for 3 minutes, which was followed by 35 cycles of denaturing at 95 C for 30 seconds, primer annealing at 60 C for 30 seconds, and primer extension at 72 C for 45 seconds. The final step was primer extension at 72 C for 7 minutes, which was followed by an end step at 4 C.

After completion of the PCR procedure, 10 μl of the reaction product was separated on a 1.5% agarose gel. Gels were stained with ethidium bromide in tris borate EDTA (TBE) buffer and electrophoresed at 80 V for approximately 1 hour.

Cloning of PCR products—The PCR products that resulted from LALP- and CIALP-specific primers were purified by use of a commercial PCR purification kit.1 The purified PCR products were ligated into a commercial vector performed in accordance with the manufacturer's protocol.1 Plasmids with the properly sized inserts were identified and sent to a commercial sequencing facility for DNA sequencing, which was accomplished by use of universal T7 and SP6 primers. Sequence results were compared with those of other species by use of a sequence-similarity search tool.

The LALP and CIALP sequences were extended by use of a RT-PCR procedure. Primers for the PCR assay were designed from canine sequences available from the original clones and from consensus sequences near the 3'-end of products from other species. An amplification product would result in an overlap of new sequence with a previously obtained sequence when the newly designed primers were used. The RT-PCR protocol was the same as described previously. Canine liver and intestinal RNA was used as a template, and the resulting PCR product was cloned and sequenced. The PCR products with suspected intron sequences were also cloned and sequenced.

Northern blot analysis—Aliquots (10 μl) of a loading dye containing formaldehyde and ethidium bromide were added to 1.0, 5.0, and 10.0 μg of total RNA from the liver, kidneys, and intestinal mucosa. Samples were denatured for 5 minutes at 65 C, loaded onto 1.0% denaturing-formaldehyde agarose gels, and separated by use of electrophores at 80 V for approximately 2 hours in 1× 3-[N-morpholino]propanesulfonic acid (MOPS) buffer. Following separation, the RNA was developed by use of UV light and examined for integrity by detection of 28S and 18S rRNA bands. Distance from the insertion point to the rRNA bands was measured. The RNA was hybridized with 32P-labeled DNA probes, which were prepared by use of Klenow fragment and random primer extension. The labeled probes were separated by use of a 10% polyacrylamide gel, and the DNA probe was visualized by use of autoradiography by exposure for 4 hours. The autoradiographs were scanned by use of a densitometer, and the RNA bands were quantitated. The RNA was denatured by use of UV light for approximately 1 minute. It then was wrapped in plastic and stored at –20 C until use. The mem-

Figure 1—Sequence of canine liver alkaline phosphatase (LALP) cDNA. Primers used for amplification of the 1,150-base pair (bp) LALP mRNA product are underlined, and the intron sequence is italicized.

Figure 2—Sequence of canine intestinal alkaline phosphatase (IALP) cDNA. Primers used for amplification of the 599-bp IALP mRNA product are underlined, and the intron sequences are italicized.
branes were treated by use of a prehybridization procedure in a plastic bag with 1.0 ml of a commercial hybridization solution/100 mm² of membrane. The prehybridization procedure lasted approximately 2 hours at 42 C in a rotating waterbath. Approximately 30 to 50 ng of LALP- or CIALP-purified PCR product was used as probes for northern blots. The DNA probes were labeled with ³²P-dCTP by use of a random prime labeling kit preformed in accordance with the manufacturer’s protocol. After labeling at 37 C for 2 hours, the probe was denatured at 95 C for 5 minutes and added to the hybridization bag by use of a syringe and needle. Hybridization was performed in a waterbath for 12 to 14 hours at 42 C with continuous rotation. The blot containing the serially diluted total RNA from the liver and kidneys was hybridized with the LALP probe, and the blot with total RNA from the intestinal mucosa was hybridized with the CIALP probe. Following hybridization, the membranes were washed in 50 ml of solution (0.1 SSC, 0.1% SDS) at 42 C for 2 minutes, and the supernatant was decanted and saved. The LALP and CIALP activities of dilutions of tissue extracts were performed as described previously. Briefly, 1 µl of diluted cDNA was used as a template for the multiplex PCR procedure and amplified for 30 cycles. Sterile water replaced cDNA template to serve as a negative-control sample. The PCR assay for each specimen was performed in 5 volumes of n-butanol was added to the homogenate, and samples were mixed by use of a vortexer at 10-minute intervals for 1 hour. Homogenates were centrifuged at 14,000 X g for 2 minutes, and the supernatant was decanted and saved. The LALP and CIALP activities of dilutions of tissue extracts were measured by use of an automated p-nitrophenylphosphatase assay on an automated serum chemistry analyzer. Serum LALP and CIALP activities were measured by use of the same automated assay system. Relative quantitation of ALP isoenzyme mRNA—Relative quantitation of mRNA transcripts in each liver biopsy specimen was performed by use of a commercially available multiplex PCR kit. Multiplex PCR is defined as the use of 2 or more primer sets in a single PCR procedure. One primer pair recognized the target of interest (ie, LALP and IALP), and the other primer pair was used to amplify an invariant endogenous control sample (18S rRNA). Amplification of 18S rRNA acts as a control sample for adjustment of the relative quantification of the target gene. Extraction and reverse transcription of total tissue RNA were performed as described previously. Briefly, 1 µg of total RNA from each biopsy sample was reverse transcribed. Then, 5 µl of diluted cDNA was used as a template for the multiplex PCR procedure and amplified for 30 cycles. Sterile water replaced cDNA template to serve as a negative-control sample. The PCR assay for each specimen was performed in 50 ml of solution (0.1X SSC, 0.1% SDS) at 42 C for 15 minutes. Blots were then washed in 50 ml of solution (0.1X SSC, 0.1% SDS) at 42 C for 30 minutes, washed again in 50 ml of solution (0.1X SSC, 0.1% SDS) at 55 C for 30 minutes, and finally washed twice in 50 ml of solution (0.1X SSC, 0.1% SDS) at 65 C.

Membranes were wrapped in plastic and exposed to radiographic film at –80 C for 24 hours.

Activity of ALP isoenzyme in tissues—Liver biopsy specimens were weighed and placed in 4 volumes of 0.05M acetate buffer (pH 4.9). Tissues were homogenized in acetate buffer by use of a small pestle in 2-ml microcentrifuge tubes. One volume of n-butanol was added to the homogenate, and samples were mixed by use of a vortexer at 10-minute intervals for 1 hour. Homogenates were centrifuged at 14,000 X g for 2 minutes, and the supernatant was decanted and saved. The LALP and CIALP activities of dilutions of tissue extracts were measured by use of an automated p-nitrophenylphosphatase assay on an automated serum chemistry analyzer. Serum LALP and CIALP activities were measured by use of the same automated assay system.
duplicate. An aliquot (10 µl) of each PCR product was subjected to electrophoresis through a 1.5% agarose gel. Ethidium bromide in TBE buffer was added, and gels were electrophoresed at 90 V for approximately 1.5 hours. Relative density of the resulting bands was measured by use of densitometry.

Statistical analysis—Mean values of all data from liver biopsy specimens were evaluated by use of a 1-way ANOVA and the Dunnett post hoc test for multiple comparisons. Significance was set at $P < 0.05$ for all tests.

Results

Canine ALP isoenzyme gene sequences—Total size of 1 of the cloned sequences was 2,246 base pairs (bp) of the canine LALP gene (Fig 1). Total size of the second cloned sequence was 1,338 bp of the canine CIALP-IALP gene (Fig 2). The gene segments were confirmed to be canine ALP genes on the basis of comparison with ALP DNA sequences of other species and consistent results with additional clones. There was substantial homology between canine LALP and CIALP-IALP cDNA sequences and those of other mammalian species. Homology of the canine LALP sequence was 91% with feline, 90% with bovine, and 89% with human TNS ALP. The CIALP-IALP sequence had homology of 85% with human, 82% with bovine, and 81% with rat IALP.

Identification of IALP transcripts in hepatic tissue of dogs—Northern blots of tissues from the liver
and kidneys of glucocorticoid-treated and control dogs had positive results when tested for LALP (Fig 3). The LALP transcripts were approximately 3.7 to 3.9 kilobases (kb). Intensity of the bands was greater in hepatic tissue from glucocorticoid-treated dogs than in hepatic tissue from untreated control dogs, which is consistent with increased expression of LALP mRNA in glucocorticoid-treated liver tissue. Intensity of the bands was nearly equal in renal tissues of glucocorticoid-treated dogs and control dogs. Intestines from glucocorticoid-treated and control dogs had negative results when tested for LALP transcripts. Use of the CIALP-IALP probe on the same blot revealed hybridization in the hepatic tissues increased in a nearly identical manner to that of serum activities. However, analysis of results of the study reported here suggests that CIALP is expressed in relatively low copy numbers in clinically normal dogs.

**Kinetics of LALP and CIALP mRNA transcripts in hepatic tissues**—Serum LALP activity for the glucocorticoid-treated dogs steadily increased from day 0 through day 32 (Fig 5). Serum CIALP activity began to increase on day 10 and then progressively increased through day 32 (Fig 6). The LALP and CIALP activities in the hepatic tissues increased in a nearly identical manner to that of serum activities.

Results for the relative quantitative multiplex RT-PCR procedure revealed a band of the appropriate size for LALP (1,150 bp) in liver biopsy samples (Fig 7). The 18S rRNA PCR product was of the predicted size (500 bp). Expression of LALP mRNA increased significantly by day 5. By day 10, mRNA concentrations began to plateau and then remained constant until the termination of the study (Fig 8). Results of the relative quantitative multiplex RT-PCR procedure in hepatic tissues did not reveal consistent evidence of expression until day 10 of treatment (Fig 9). Significant increases, compared with values for day 0, were not evident until day 32 (Fig 8).

**Discussion**

Results of the study reported here support the assumption that the liver is a site of CIALP synthesis and that tissues obtained from the intestines, liver, and kidneys of dogs express the same ALP isoenzymes, except for CIALP, as seen in other species. Furthermore, on the basis of other studies in which hepatic CIALP activity is associated with hepatocytes, we concluded that the cell responsible for CIALP synthesis is the hepatocyte. In another study, it was suggested that increases in CIALP activity following glucocorticoid administration could be attributable to reglycosylation of intestinal ALP isoenzyme by hepatocytes. However, analysis of results of the study reported here indicates that the liver can function as a site of de novo synthesis of CIALP.

The study reported here also revealed some additional characteristics regarding ALP expression in dogs. For example, northern blot analysis of intestinal mucosa from dogs revealed a single IALP transcript. Although this is similar to several mammalian species, it differs from humans, in which multiple transcripts of IALP result from 4 polyadenylation sites at the 3' noncoding regions. These findings suggest that in humans and rats there are 2 separate genes for IALP. In contrast, the findings of the study reported here suggest that there is only 1 gene for canine IALP and that multiple polyadenylation sites do not exist.

This study also confirms a suspicion mentioned in other studies (ie, that the kinetics of hepatic LALP and CIALP synthesis differ following glucocorticoid treatment of dogs). In another study, it was documented that increases in serum and tissue LALP activity are evident shortly after glucocorticoid treatment is initiated, whereas increases in serum and tissue CIALP activity are delayed for several days. Analysis of findings of the study reported here indicates that this difference in expression kinetics between LALP and CIALP is correlated with increased tissue concentrations of LALP and CIALP mRNA and, hence, the differences in onset of induction of synthesis of each enzyme.

We also found that concentrations of LALP mRNA transcripts did not continue to increase after reaching a plateau concentration shortly after glucocorticoid treatment was started. A similar phenomenon has been observed with other genes expressed in hepatocytes and other cell types in response to glucocorticoids. However, despite a plateau in concentrations of LALP mRNA, the LALP activity measured in the hepatic tissue...
steadily increased over time. Hence, the increase in serum and tissue ALP activity cannot be explained by increased transcription alone. A plateau in concentrations of ALP mRNA with continually increasing ALP activity has also been described in an osteosarcoma cell line that was exposed to vitamin D. In that study, it was determined that vitamin D increased stability of ALP mRNA, possibly through an inducible stabilizing protein. Although stability of ALP mRNA was not measured in the study reported here, increased stability of LALP mRNA mediated through an inducible protein may be a viable mechanism to explain the results we observed.

The plateau in LALP expression in hepatic tissue was not evident for CIALP. Rather, concentration of CIALP mRNA paralleled CIALP activity in the hepatic tissues over the entire treatment period. This suggests that the regulation of CIALP synthesis differs from that of LALP. Although a parallel increase in expression and activity has been documented for other genes and proteins, a parallel response has not been described for ALP genes. Another difference evident between LALP and CIALP was the delay in onset of appearance of CIALP transcripts, compared with that of LALP transcripts. In fact, increased expression of CIALP mRNA in hepatic tissues was not significantly different from values for day 0 until day 32 of treatment. A delay in appearance of CIALP activity in the serum and hepatic tissues of glucocorticoid-treated dogs has been described. On the basis of results of the study reported here, it appears that delayed expression of the CIALP gene in hepatic tissue following glucocorticoid treatment is the reason for the delayed appearance of CIALP activity in serum and tissue. Examples of delayed expression of ALP genes in response to glucocorticoids are abundant. However, none of those studies describes such a prolonged delay (approx 10 days) as was seen here for hepatic CIALP. The mechanism responsible for this delayed response is not clear, although it may involve an effect of a secondary protein or a synergistic response. For example, it has been observed with other genes that a delayed response in expression can be attributed to the need of a mediator protein that facilitates induction of a secondary gene. Such a mediator protein must reach a critical concentration before transcription exceeds baseline amounts hence the delayed response. It is possible that delayed expression of the CIALP gene following glucocorticoid treatment in dogs is the result of the need to induce expression of an unknown protein (or proteins) to facilitate CIALP transcription. As was described earlier, an inducible protein may be responsible for LALP accumulation in the liver after administration of glucocorticoids. Therefore, it is likely that an inducible mediator protein, operating via different mechanisms, is necessary for overall expression of ALP mRNA in the liver of glucocorticoid-treated dogs.

References


