

Endogenous Alkaline Phosphatase Activities in Rat Tissues

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Abstract

Endogenous ALP activities were examined in various tissues using different substrates, Nitroblue tetrazolium chloride/ 5-brom-4-chloro-3-indolyl phosphate (NBT/BCIP), Vector Blue, and Vector Red. Comparative analysis demonstrated marked differences of signal intensities depending on the substrates. Catalyzed NBT/BCIP products deposited heavily and proved to be sensitive for detecting signals, the Vector Blue the second, and the Vector Red less sensitive, although the three substrates yielded their own color precipitations. Endogenous ALP activities were observed in all tissues examined. The other factors such as histological procedures also affected endogenous ALP activities. Frozen tissues either fixed or unfixed exhibited well-preserved ALP activities, while the signals in paraffin-embedded materials were eliminated from most tissues, except for small intestine. An ALP inhibitor, or Levamisole, actually suppressed the enzyme activities in various tissues but not always successful nor perfect. The present study provokes cautious information necessary to control endogenous ALP activities in target tissues of interest when exogenous ALP is applied as a tool of signal detection.

Key words: Alkaline phosphatase, NBT/BCIP, Vector Blue, Vector Red,
Endogenous enzyme activity

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Introduction

Alkaline phosphatase (E.C. 3.1.3.1) (ALP) is frequently used to detect experimental signals in diverse arrays of contemporary research technologies. The experimentally applied ALP catalyzes the artificial substrates to insoluble products and visualizes their distinctive signals with high sensitivity. In mammals, however, endogenous ALPs are present in most, if not all, tissues and categorized into four isozymes; intestinal (IALP), germ cell (GALP), placental (PALP) and tissue non-specific ALP (TNALP). These ALPs (function as dimeric molecules) catalyze the hydrolysis of phosphomonoesters by releasing inorganic phosphate *in vivo*, and also catalyze the artificial substrates to insoluble precipitates as an exogenously applied ALP does. Of four isozymes, the IALP is known to be stable and have the strongest enzyme activity. Thus, the IALP is exclusively employed as a marker enzyme in various signal detection systems available from commercial sources.

Different ALP isozymes show different catalytic activities depending on the isozyme-specific properties, such as uncompetitive inhibition properties¹⁾, heat-stability²⁾ and allosteric properties³⁾. In certain experiments in which exogenous ALP is used, endogenous ALP activities are negligible and desired signals may be obtained in some cases, but unfortunately in the other cases may cause serious problems, even if the materials were processed alike. First, this may be ascribed to the differences of tissues, which contain different types and amounts of ALP isozymes. Second, the factors of procedures must be considered, because, if the same tissue was processed in different ways, the subsequent procedures may affect the endogenous ALP activities differently. This implies that the data created by the first method cannot be verified by the second compatible one. Third, the substrates for the enzyme should be considered as a factor affecting the resulting signals. The substrates used appear to determine the sensitivities of signals; that is, different substrates have their characteristic properties such as availability (yield of signals), color (contrast), and size of precipitates (sensitivity and contrast). Different ALPs may also prefer certain artificial substrates much to the others. The fourth factor is the inhibitors of the enzyme.

Some inhibitors are effective to certain isozymes of ALPs but not to the others, and *vice versa*. Many factors are involved in the activities of the endogenous as well as exogenous ALPs in a variety of experiments and medical examinations. Accordingly, the factors affecting experimental signals need to be controlled properly, even if a given IALP is employed as an exogenous marker enzyme.

The present study examined the enzyme activities of endogenous ALPs in various tissues, which were processed by histological ways most frequently used in experimental laboratories, and provides information useful for arranging experiments with ALP-mediated signal detection systems, in particular histological studies such as immunohistochemistry and *in situ* hybridization.

Materials and Methods

Male Sprague-Dawley rats (10 or 13-week-old, 350–450 g in body weight) were used in the present study. The rats were inbred and kept in an artificial condition of 12 hour-day/night cycle and at 25°C. The rats were anesthetized with pentobarbital sodium (Nembutal, 1 mL/kg body weight, i.p., Dinabott Inc.) before sacrificing. To know effects of fixative on endogenous ALP activities, rats were perfused with 150 mL 0.9% saline through the heart, followed by 400 mL 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), and postfixed in the same fresh fixative for 18–24 hours at 4°C. Fixed tissues were divided into two groups; one for frozen tissues and the other for paraffin-embedded tissues. The tissues for frozen sections were rinsed in phosphate-buffered 20% sucrose for another 18–24 hours at 4°C. The tissues for paraffin-embedding were processed routinely according to a standard histological protocol. Paraffin-embedded materials were cut serially at 10 μ m in thickness. For fresh frozen tissues, rats were irrigated transcardially with 150 mL 0.9% saline alone. Thereafter, for frozen (or cryostat) sections, tissues were frozen in powdered dry ice, and then kept at –80°C. Serial sections were cut on a cryostat at 15 μ m in thickness, thaw-mounted on slides and store at –40°C until use. For preservation of tissue textures, fresh frozen sections were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 minutes at room

temperature just prior to processing for ALP activities. The handling and treatment of the animals conformed to the guidelines of the Animal Study Committee of the Kagawa Prefectural College of Health Sciences.

For examining endogenous APL activities, sections were processed according to the user's manual of the detection kit for ALP, or Non-radioactive Labeling and Detection of Nucleic Acids (Roche Diagnostics, Germany). Sections processed in different ways (above) were incubated with different substrates at concentrations indicated, NBT/BCIP (Roche Diagnostics, Germany), Vector Blue and Vector Red (Vector Lab., CA), for 10, 30 or 60 minutes. The combination of BCIP and NBT is known as the most sensitive indicator for chlomogenic detection of ALP. ALP catalyzes the dephosphorylation of BCIP, generating 5-bromo-4-chloro-3-indolyl hydroxide, which dimerizes to the insoluble compound, 5,5'-dibromo-4,4'-dichloroindigo. This dimerization reaction triggers the reduction of one molecule of NBT into the insoluble purple dye, diformazan. The Vector Blue and Vector Red generate blue and red precipitates respectively, and appear useful for differential staining (color-contrast) when used in an appropriate situation. These sensitivities of the Vector's reagents are assumed to be compatible to the BCIP/NBT. Neither the contents nor the chemical reaction processes of the Vector's reagents are disclosed.

The effect of an ALP inhibitor or Levamisole (Vector Lab., CA) was examined by co-incubating with NBT/BCIP on sections for 30 minutes. Levamisole is known to suppress ALP activities except for the intestine-derived ALP. Levamisole was used at either a concentration indicated or two-fold high the concentration.

Results

Endogenous ALP activities were ubiquitously detected as catalyzed substrate precipitations *in situ* in either fixed or fresh (or unfixed) frozen tissues; hypophysis, tongue, parotid gland, submandibular gland, lung, heart, liver, pancreas, kidney, spleen, duodenum, jejunum, ileum, testis, epididymis, and skeletal muscles (intercostal and calf muscles). For fresh frozen materials, additional tissues were adopted for fur-

ther extensive search; trigeminal ganglion, thymus, oesophagus, fundic and pyloric regions of the stomach, adrenal gland, urinary bladder, and prostate. All tissues investigated exhibited variable signals of endogenous ALP activities.

Signals of ALP activities vary even in the same tissue in combination with different substrates, NBT/BCIP, Vector Blue and Vector Red. Sections incubated with these substrates resulted in variable amount of precipitations; that is, the substrates exhibited different sensitivities for detecting signals (Fig. 1). NBT/BCIP yielded a large amount of purple-brown precipitations and showed the superiority in signal intensity to the other two substrates (Fig. 1 A, B, C and D). The substrate Vector Blue formed blue precipitates but was slightly less sensitive in comparison with NBT/BCIP (Fig. 1 E, F, G and H). The substrate Vector Red was apparently less sensitive to produce proper red signals of the enzyme activities (Fig. 1 I, J, K and L).

Catalyzed substrate precipitations for endogenous ALP activities vary from one tissue to another in intensity as well as in cellular and subcellular localization. Intense signals of ALP activities were observed in the luminal halves of epithelial cells of the jejunum (Fig. 1 A and E) and of proximal urinary tubule cells of the renal nephrons (Fig. 1 B and F). Delicate precipitations for ALP activities were seen associated with cell membrane along the bile capillaries in the liver parenchyma (Fig. 1 C and G). The whole cell labeling was seen in myoepithelial cells of the submandibular gland (Fig. 1 D and H), as well as over acinar cells of the parotid gland and small intralingual serous glandular tissues. These structures described above are only representative examples of tissues investigated, in the remaining of which cells and subcellular structures also displayed distinctive signals of ALP activities.

The incubation time (10, 30 or 60 minutes) with NBT/BCIP showed to affect signal intensities of ALP activities in fixed frozen sections (Fig. 2 A vs C, 2 E vs G, and 2 I vs K). Signals of ALP activities in the kidney attained mostly to a highest level in 10-minute incubation. The prolonged incubation did not improve the signals effectively but rather made the resolution of signals obscure by overreacting (Fig. 2 A-B-C). In the submandibular gland and liver, signals of ALP ac-

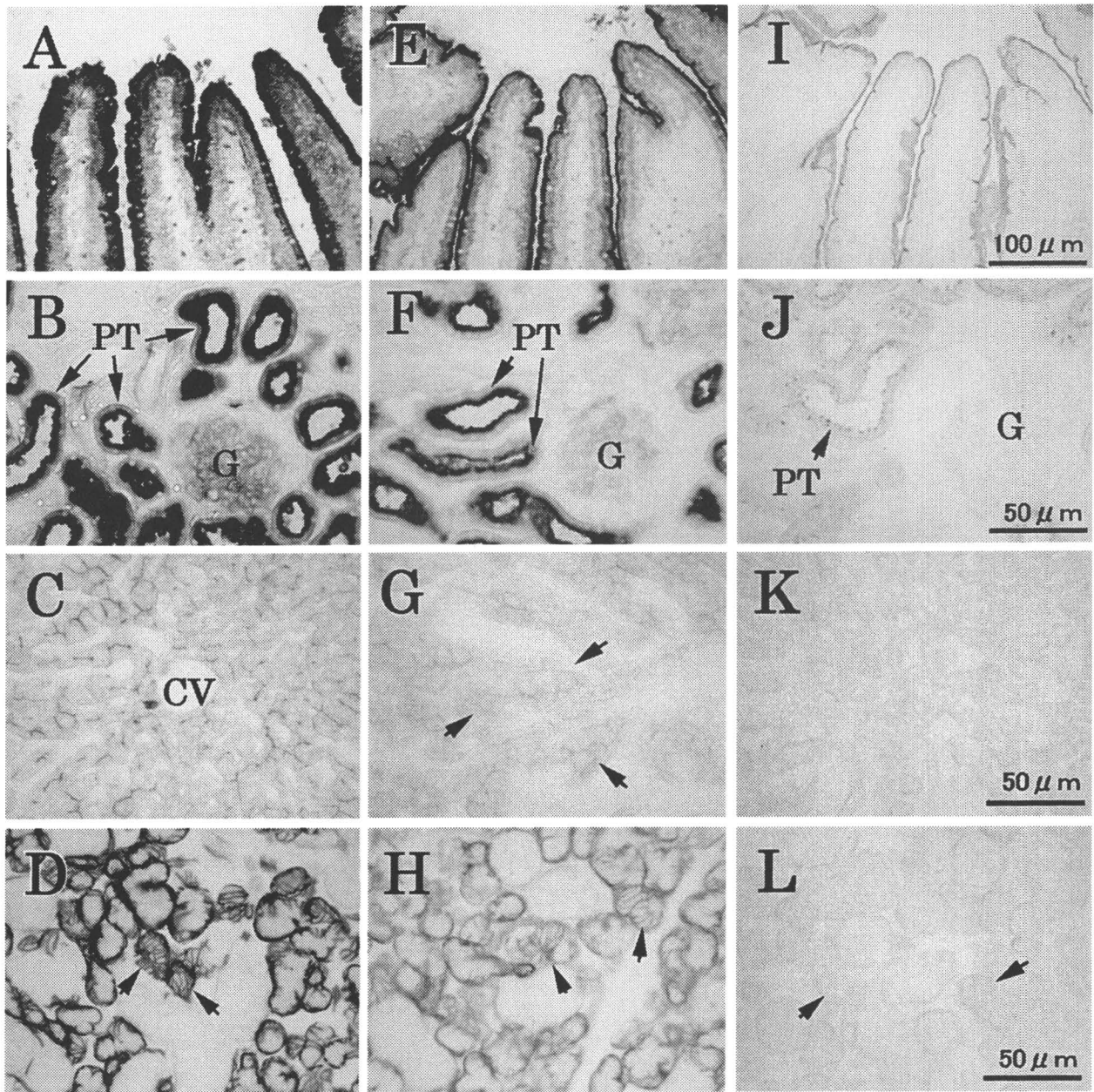


Fig. 1 Alkaline phosphatase (ALP) activities in fixed frozen sections in combination with different substrates, NBT/BCIP, Vector Blue or Vector Red (30-minute incubation). Precipitations of NBT/BCIP (A-D) are intense, Vector Blue (E-H) moderate, and Vector Red (I-L) scanty. In jejunum (A, E, I) and proximal urinary tubule of renal nephrons (B, F, J) ALP signals were localized in the luminal halves of epithelial cells. In liver (C, G, K) ALP signals were traced along bile capillaries (small arrows). In submandibular gland (D, H, L) myoepithelial cells (small arrows) are labeled with catalyzed precipitations. CV; central vein, G; glomerulus, PT; proximal urinary tubule, The scales indicated in the right panels (I, J, K, L) are common in each rows.

tivities were intensified with the increase of incubation time (Fig. 2 I-J-K and 2 E-F-G). Myoepithelial cells adherent to serous acinar cells of the submandibular gland became clear against the background in 60-minute incubation (Fig. 2 K). In the liver, at first (10-minute incubation) signals of ALP activities were feeble in intensity and small in number (Fig. 2 E). The

prolonged incubation apparently improved the signals which were able to be seen clearly along the courses of bile capillaries in the hepatic cell cords (Fig. 2 E-F-G). The last case, in contrast with the kidney, indicated that the longer incubations enhanced the signals to a desired level and made the signals of interest clearer.

Levamisole, or ALP activity inhibitor, suppressed

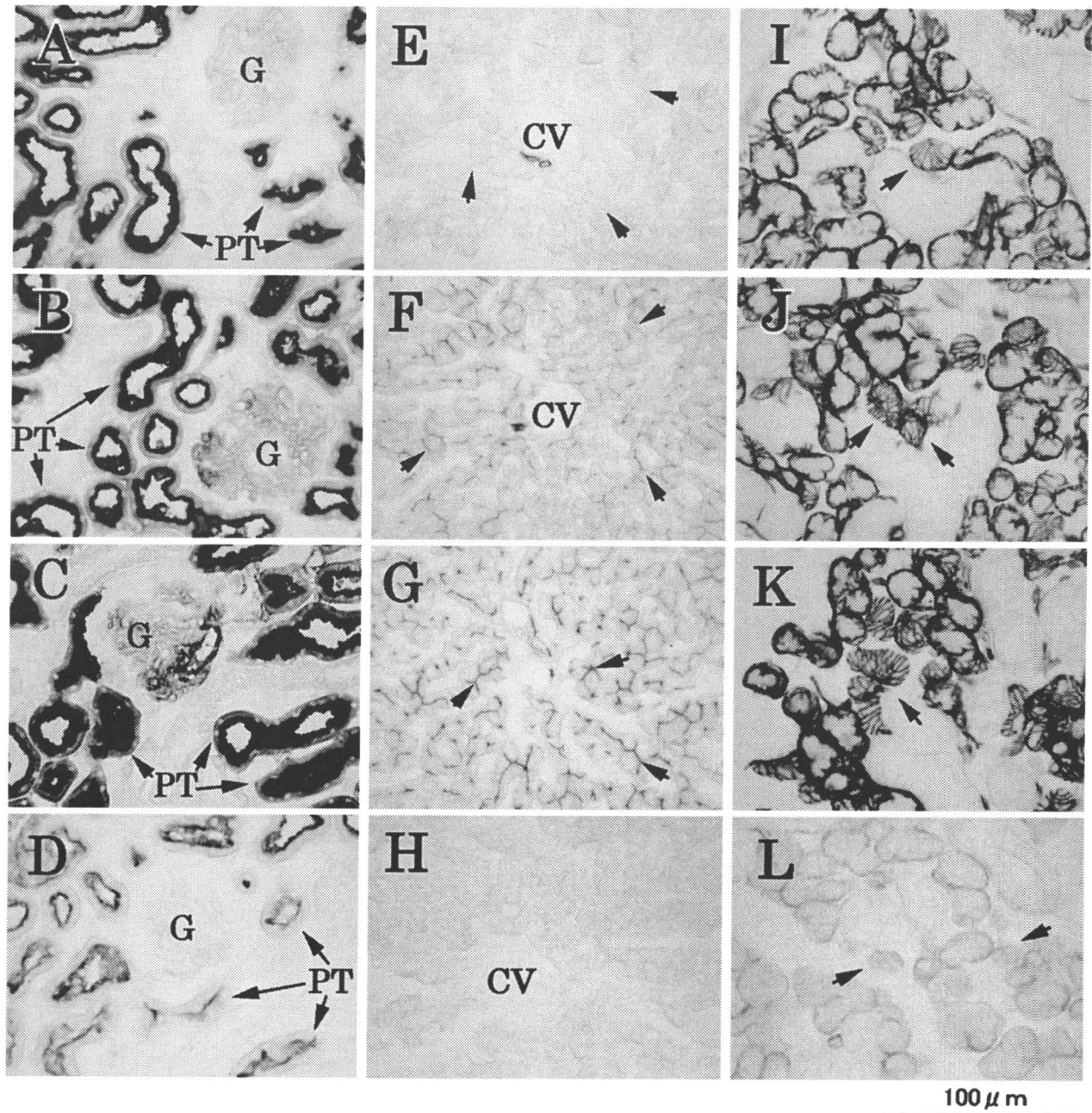


Fig. 2 Effects of incubation time on alkaline phosphatase (ALP) activities in fixed frozen sections using NBT/BCIP (10, 30 or 60 minutes). Signals of ALP activities in kidney (A-C) and submandibular gland (I-K) attained to higher levels in 10-minute incubation. The prolonged incubation appreciably intensified signals of myoepithelial cells (small arrows) of submandibular gland (I-K). ALP signals in liver (E-G) were improved by longer incubation; therefore, the signals were able to be traced along bile capillaries in the hepatic cell cords. Levamisole suppressed ALP activities in kidney (D) and submandibular gland (L) but not perfect. ALP signals in liver (H) were lost completely after co-incubation with Levamisole. CV; central vein, G; glomerulus, PT; proximal urinary tubule, The scale at the bottom is common to all panels (A-L).

endogenous ALP activities in liver and submandibular gland (Fig. 2 D and L). In the kidney strong signals of ALP activities still remained in the luminal surfaces of the proximal urinary tubules of nephrons (Fig. 2 D). Signals of myoepithelial cells adherent to serous acinar

cells of the submandibular glands were decreased drastically but not completely (Fig. 2 L). The delicate ALP signals along the bile capillaries were completely eliminated from the liver parenchyma (Fig. 2 H).

Various ways of tissue-processing in laboratory

works employ various organic and inorganic chemicals (including chelators and degenerating reagents), different pH, different osmolarity, heat, metalions and so forth, and become potential factors affecting the enzyme activities in tissues. In frozen materials, on the whole, sections from a variety of tissues exhibited stronger signals for ALP activities regardless of either fixed or unfixed. In fresh frozen tissues, however, the enzyme activities were well preserved but the cellular and subcellular structures are not always suitable for microscopic analysis in detail. In experiments to suppress endogenous ALP activities, Levamisole was less effective in fresh frozen tissues in comparison with

fixed tissues (Fig. 3 D, E and F). On the other hand, signals of ALP activities were mostly eliminated or reduced to an undetectable level in paraffin-embedded tissues, except for epithelial cells of the small intestine (Fig. 3 G, H and I).

Because of the strong enzymatic activity, the intestinal ALP (IALP) activity was able to be detected even in paraffin-embedded jejunum and ilium (Fig 4 A and B) and duodenum as well. The signals of ALP activities were, however, decreased in intensity, in particular in the ilium. Although Levamisole is known to be ineffective for the intestinal ALP, unexpectedly, signals of ALP activities were rather augmented in the

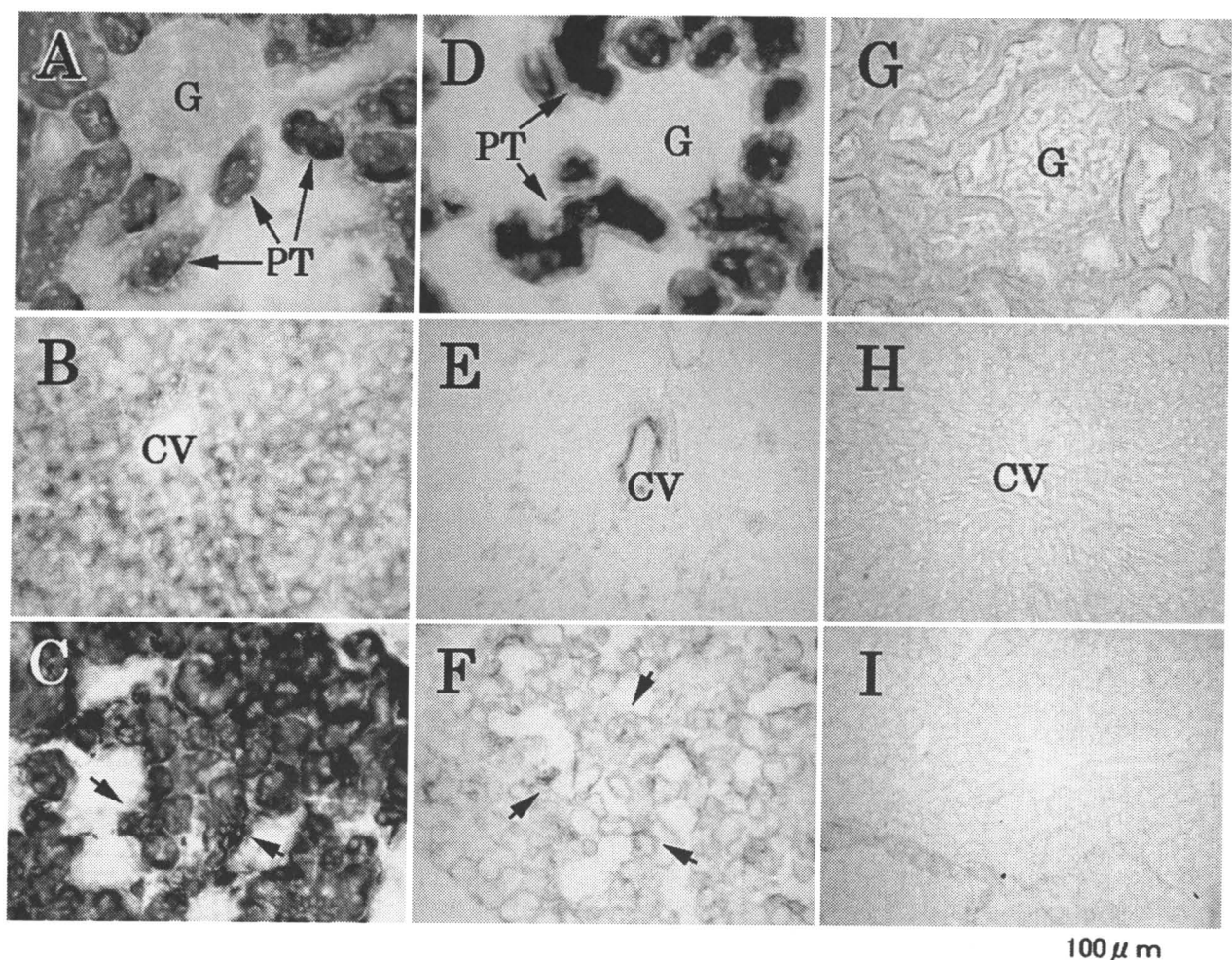


Fig. 3 Effects on alkaline phosphatase (ALP) activities in kidney (A, D, G), liver (B, E, H) and submandibular gland (C, F, I) processed in different ways. Fresh frozen sections well-preserved enzyme activities of ALP (A, B, C). The precipitations appear slightly diffuse compared with those of fixed ones. Levamisole suppressed ALP activities in most tissues investigated except for a couple of tissue (D vs E & F, also refer to Fig. 4). ALP signals were rather enhanced in proximal urinary tubules (D). In paraffin-embedded tissues, signals of ALP activities were eliminated, even from proximal urinary tubules of nephrons (G, H, I). CV; central vein, G; glomerulus, PT; proximal urinary tubule, The scale at the bottom is common to all panels (A-I).

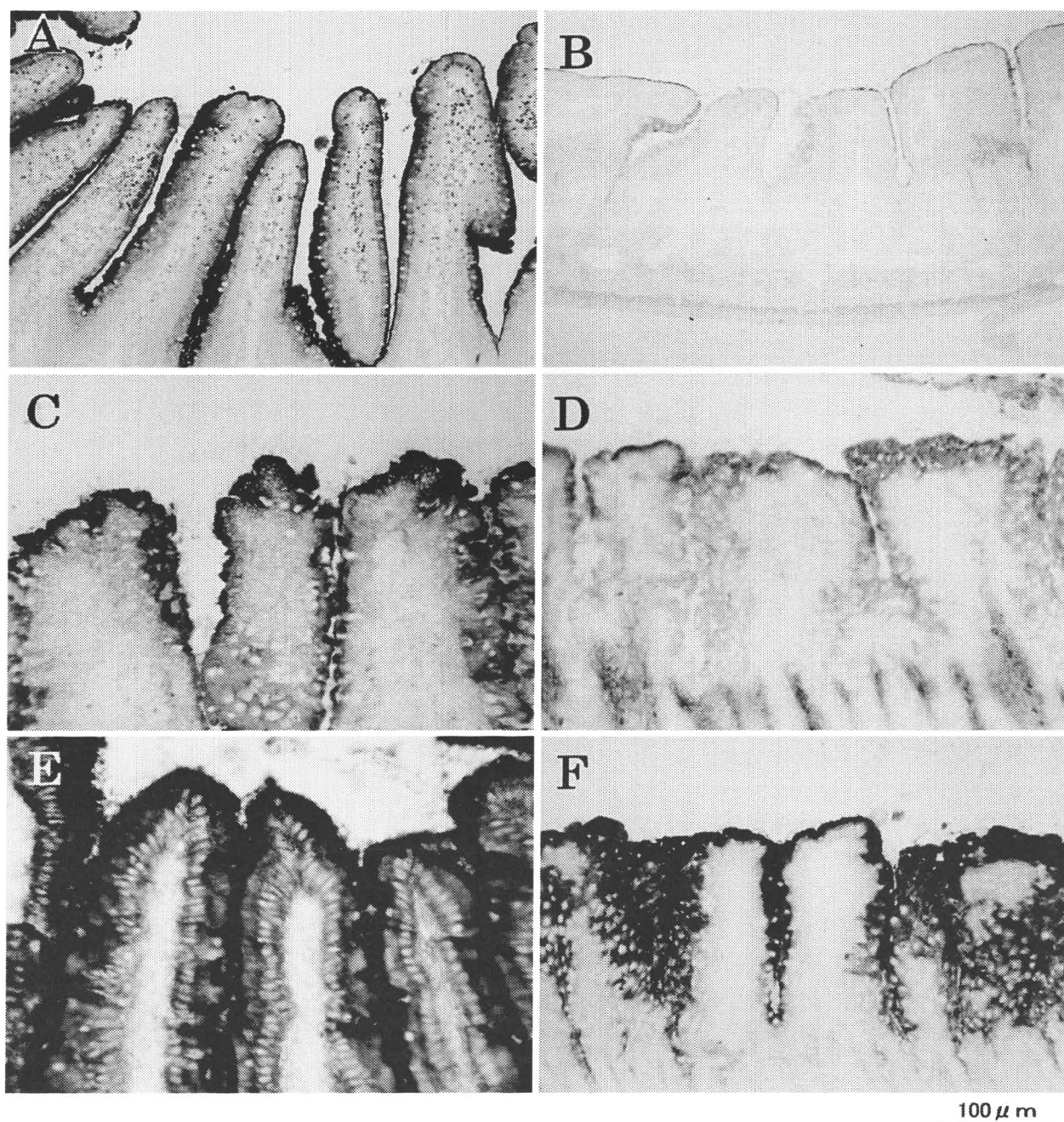


Fig. 4 Modified activities of intestinal alkaline phosphatase (ALP) in jejunum (A, C, E) and ileum (B, D, F) after processing for paraffin-embedding (A, B) or Levamisole (E, F). The routine histological processing for paraffin-embedding apparently reduced ALP activities (A, B) in comparison with fresh frozen tissues (C, D). On the other hand, co-incubation with an ALP activity inhibitor Levamisole resulted in enhanced signals of ALP activities in jejunum and ileum (E, F). The scale at the bottom is common to all panels (A-F).

epithelial cells of the jejunum and ileum following co-incubation with NBT/BCIP and Levamisole for 30 minutes (Fig. 4 C vs E, 4 D vs F). These phenomena were observed in cases where Levamisole was used at two-fold high the recommended concentration.

Discussion

Endogenous ALP

ALPs are ubiquitous enzymes seen in various body tissues. The genetic species of ALPs are categorized into 4 isozymes in mammals; therefore, three tissue specific ALPs are intestinal, germ cell and placental

ALPs (IALP, GALP and PALP), and the fourth is tissue non-specific (TNALP). These endogenous ALP isozymes function as homodimeric molecules in normal and have their own properties of uncompetitive inhibition properties¹⁾, heat-stability²⁾ and allosteric properties⁵⁾. The present study provides information that there are many factors affecting signals of endogenous ALP activities.

Different substrates for ALPs generate marked differences in sensitivities. When different artificial substrates were used, the colors of catalyzed substrates appear critical to determine the sensitivities of experimental protocols; that is, signals with high contrast are easy to detect. NBT/BCIP, Vector Blue, or Vector Red was used for adjacent serial sections in the present study. NBT/BCIP produced dense signals of dark purple-brown precipitations, signals of Vector Blue are light-blue in a moderate amount and Vector Red yielded a small amount of light-red deposits. Although the actual amounts of these precipitations were not measured biochemically, the signal versus noise (S/N) ratio of the ALP signals were apparently different under microscopic observation. The size of catalyzed deposits and the transparency may also be critical factors for optical analyses.

Chemical reagents and experimental protocols in routine laboratory works should be considered to obtain optimal results. A chemical factor (paraformaldehyde) and the subsequent procedures were compared in frozen sections, between fixed and unfixed. Unfixed or fresh frozen sections were also immersed briefly in buffered paraformaldehyde just prior to reaction for ALPs. The immobilization of proteins by brief immersion is used commonly for fresh frozen sections, in order to eliminate undesired protein actions such as proteinase, kinase and phosphatase and also to minimize the loss of water-soluble structures in the subsequent procedures. In both fixed and unfixed sections, the enzyme precipitations of endogenous ALPs exhibited the same tissue and cellular distribution with each other, except for sharpness/looseness depending on the tissue texture preserved. The signals of ALPs in fixed sections were well-preserved in consonance with tissue structures. Both fixed and unfixed sections exhibited the increased intensification of ALP signals (i.e., in liver) with the prolonged incubation, implying that paraformaldehyde

fixation does not adversely affect the activities of endogenous ALPs. On the other hand, sections processed for paraffin-embedding exhibited no signal except for the small intestine (i.e., duodenum and jejunum). The heat-stable ALP isozyme alone was able to maintain the decreased activity. The processing for paraffin-embedding appears to diminish the activities of ALP isozymes, as a large number of antigenic properties are ruined (Morita unpublished observations).

The inhibitions on ALP activities using Levamisole were compared between fixed and unfixed sections. The ALP activities in both the sections were drastically reduced or eliminated in most tissues, except for the small intestine and kidney. It is, however, intriguing in unfixed sections, but not fixed ones, that the ALP activities are augmented in the small intestine where IALP resides. Because Levamisole inhibits the activities of GALP, PALP and TNALP but not IALP, the augmented signals in the small intestine may depend on the increased availability of the substrates by IALP during the incubation.

The enzymatic activities of PALP and GALP are inhibited by L-phenylalanine (L-Phe) through an uncompetitive mechanism, whereas L-leucine (L-Leu) inhibits GALP 17-fold more strongly than it does PALP; that is, the germ cell ALP is selectively inhibited by L-Leu¹⁾. In human choriocarcinoma cells (malignant trophoblasts) GALPs comprise heterodimers and show the great sensitivity to EDTA and L-Leu⁴⁾. On the other hand, three human bone ALP isoforms (B/I; 126 kDa, B 1; 136 kDa and B 2; 141 kDa) are similar in freeze-thaw stability, solubility, heat inactivation, and inhibition by L-Phe, L-homoarginine, and Levamisole⁵⁾. The isoforms are also kinetically similar (i.e., maximal velocity and Km at pH 8.8 and pH 10.0). Desialylation with neuraminidase reduced the apparent sizes of B 1 and B 2 to 127 kDa (i.e., approximately to that of B/I).

The present study revealed different levels of enzymatic activities of endogenous ALPs in most tissues examined. When an exogenous ALP is employed in the experimental study, therefore, these endogenous ALP activities have to be controlled properly not to interfere the desired signals of experimental probes, such as ALP conjugated nucleic acids and IgG. Endogenous ALP activities does not cause serious problems in some

experiments, but in the other cases the specific signals of interest may be ruined. Particularly, for detecting feeble expression of target molecules, the longer incubation with substrate may result in unclear signals with high background (i. e., low S/N ratio).

Exogenous ALP

A large number of signal detection systems available from commercial sources may introduce the IALP into the secondary or tertiary molecular probes, although it can be conjugated to the primary molecular probe via a linker^{6, 7)}. Wherever the ALP is introduced into probes, the resulting signals appear satisfactory and the insert position of the enzyme does not appear to alter the essential enzymatic activity. Rather, different protocols and/or differently labeled molecules can be arranged either to obtain multiple signals or to intensify target signals.

The present study has shown an intriguing result of Levamisole inhibition experiment. After successful treatments of endogenous ALPs, it is likely that an exogenous IALP conjugated to experimental probes may generate the enhanced, specific signals of target molecules in co-incubation with Levamisole.

Clinical and Pathological Aspects of ALP

Endogenous ALPs are involved in a wide range of pathological conditions such as metabolic bone diseases and a variety of cancers. The IALP in the epithelial cells of the small intestine are stable and strong, and appears to work as a defense factor to diminish the toxicity of lipopolysaccharide (LPS). After the oral administration of LPS to rats, serum LPS content was increased within 2 hours, maintained for a couple of hours and declined by 6 hours⁸⁾. This phenomenon was augmented when L-Phe, or an inhibitor of IALP, was simultaneously administered with LPS. In addition, reduced LPS toxicity by treating with IALP in vitro was restored in the presence of L-Phe⁸⁾. Furthermore, human aortic endothelial cells pre-exposed to the IALP (50 nIU/mL) showed resistance (40-fold the counterpart) to the LPS toxicity⁸⁾. In rat hearts, ALP activity was detected at the extracellular surface of the capillary endothelial cells and in their caveolae. Two hours after LPS administration, the initial changes were observed as a remarkably increased number of enzymati-

cally positive capillaries, intensified cytochemical reaction of endothelial cells, and an increased number of caveolae⁹⁾. The initial cytochemical reaction was maintained during the first 24 hours after administration and then was restored to a normal level in one week⁹⁾. These two studies may imply that ALP isozymes are implicated in the pathological events through both the initial and the subsequent time-dependent reactivity.

The hyperexpression of four ALP genes in human has been observed in various tumors, and their serum levels are often used as tumor markers, particularly cancers of the testis and ovary¹⁰⁾. Cellular ALPs are also recognized as important markers for monitoring tumor cell behavior in malignancies; (1) Testis tumor showed positive staining for ALP, particularly PALP. (2) The intensity of ALP expression varies in tumor cell lines. (3) Exposure of tumor cells expressing EGF receptor to epidermal growth factor (EGF) led to a decreased ALP expression¹¹⁾.

In normal and H-ras-transformed fetus fibroblasts, PALP stimulates DNA synthesis and cell proliferation in synergism with insulin, zinc and calcium. PALP may promote fetus development as well as the growth of cancer cells which express oncogenic Ras¹²⁾. Evidences of PALP may indicate a role of the isozyme in cell division in normal and transformed cells^{11, 12)}.

Bone ALP (TNALP) circulates as a variable mixture of anchorless isoforms in vivo and the relative abundance of these bone ALP isoforms in serum may reflect various aspects of the metabolic bone disease. Hypophosphatasia is a rare inborn error of metabolism characterized by defective bone mineralization caused by a deficiency of liver-, bone- or kidney-ALP due to mutations in TNALP gene. The clinical expression of the disease is highly variable, ranging from stillbirth with a poorly mineralized skeleton to pathologic skeletal fractures which develop in late adulthood. An elevated level of the plasma TNALP in patients with liver disease can be an assessment for Paget's disease¹³⁾, whereas bone ALP is indistinguishable from normal. Antibodies generated against PALP were used to characterize marker ALPs of cancer of the ovary, testis, lung, and the gastrointestinal tract¹⁴⁾. The GALP is predominantly expressed in carcinoma-in-situ of the testis¹⁵⁾.

While ALPs are homodimeric molecules in normal, the expression in cancer cells of more than one ALP isozyme leads to release of heterodimeric enzymes into body fluids. The Kasahara ALP isozyme was identified in a variety of human cancer cell lines and cancer sera. Later, a study of HuG-1 cell line (stomach cancer) reported that the unusual ALP (HuG-AP) was heterodimers of IALP/PALP¹⁶⁾. Ovarian cancer cells express homodimeric PALP and GALP¹⁷⁾, and heterodimeric PALP/GALP⁴⁾. However, no heterodimers have ever been reported between any of the tissuespecific ALPs and TNALP. The heterodimers of ALP isoforms are not only associated with pathological events, but heterodimers of IALP and PALP are expressed in the intestine during human postnatal growth¹⁸⁾.

The fact that ALPs can form heterodimers is of substructural significance since ALPs are non-cooperative allosteric enzymes where the stability and the catalytic properties of each monomer are controlled by the conformation of the second subunit³⁾. In bovine intestine, up to seven IALP isozymes with different kinetic properties are co-expressed¹⁹⁾, and the formation of heterodimers can give rise to significant functional complexity and novel substrate specificities.

Conclusion

The present study focused on the correlation between the enzymatic activities and the factors affecting biological properties of endogenous ALPs in rat tissues, reporting that chemical reagents and some protocols used in routine laboratory works become potential inhibitory factors of the enzymatic activities. Because many contemporary technologies employ ALP as a sensitive tool in their protocols, the present data will be useful to obtain optimal signals of target molecules and also instructive to control the activities of endogenous ALPs for accurate evaluation of experimental signals. Furthermore, ALP molecules and activities are closely associated particular human metabolic diseases and cancers, so that biological, pathological and molecular aspects of ALP isozymes are also of importance in the medical examinations as well as experimental studies.

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