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Widespread cellular distribution of aldehyde oxidase in human tissues found by immunohistochemistry staining

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Summary. Aldehyde oxidase (EC 1.2.3.1) is a xenobiotic metabolizing enzyme that catalyzes a variety of organic aldehydes and N-heterocyclic compounds. However, its precise pathophysiological function in humans, other than its xenobiotic metabolism, remains unknown. In order to gain a better understanding of the role of this enzyme, it is important to know its exact localization in human tissues. In this study, we investigated the distribution of aldehyde oxidase at the cellular level in a variety of human tissues by immunohistochemistry. The enzyme was found to be widespread in respiratory, digestive, urogenital, and endocrine tissues, though we also observed a cell-specific localization in the various tissues studied. In the respiratory system, it was particularly abundant in epithelial cells from the trachea and bronchium, as well as alveolar cells. In the digestive system, aldehyde oxidase was observed in surface epithelia of the small and large intestines, in addition to hepatic cells. Furthermore, the proximal, distal, and collecting tubules of the kidney were immunostained with various intensities, while glomerulus tissues were not. In epididymus and prostate tissues, staining was observed in the ductuli epididymidis and glandular epithelia. Moreover, the adrenal gland, cortex, and notably the zona reticularis, showed strong immunostaining. This prevalent tissue distribution of aldehyde oxidase in humans suggests some additional pathophysiological functions besides xenobiotic metabolism. Accordingly, some possible roles are discussed.

Key Words: Aldehyde oxidase, Human, Immunohistochemistry, Tissue distribution

Introduction

Aldehyde oxidase (EC 1.2.3.1) is a xenobiotic metabolizing enzyme that catalyzes a variety of organic aldehydes and N-heterocyclic compounds, with a substrate specificity similar to that of xanthine oxidase (Beedham, 1985). We have previously described the histochemical localization of this enzyme in various rat tissues (Moriwaki et al., 1996). However, histochemical localization studies of aldehyde oxidase have not been performed thus far in human tissues, except for the spinal cord by in situ histochemistry (Berger et al., 1995), as its activity in humans is very low compared to rats, thus rendering it difficult to be purified and to raise specific antibodies. Although aldehyde oxidase has been suggested to be involved in biogenic amine metabolism (Beedham et al., 1995), its pathophysiological role in humans remains unknown. Recently, the primary amino acid sequence of human aldehyde oxidase was determined (Wright et al., 1993; Terao et al., 1998). In the present study, we developed a specific polyclonal antibody against human aldehyde oxidase using a synthetic peptide sequence, and then investigated the distribution of aldehyde oxidase in various human tissues, using immunohistochemical staining procedures, to obtain information about its precise localization and gain a better understanding of the role of this enzyme in various human tissues.

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Materials and methods

Tissues

Tissue cytoplasmic protein (human adult normal tissue protein) was purchased from BioChain Institute Inc. (Hayward, CA, USA). Paraffin-embedded normal human tissue samples were obtained from Novagen Inc. (Madison, WI, USA) and then subjected to immunohistochemical staining. Some of the tissue samples were obtained from corpses as soon as possible after death, and then fixed in 4% paraformaldehyde-PBS (0.01M phosphate-buffered saline, pH 7.4), embedded in paraffin at 60 °C, and subjected to immunohistochemical staining.

Preparation of polyclonal antibody against human aldehyde oxidase

KLH-peptide conjugate was employed as an antigen

to construct a human aldehyde oxidase antibody. The peptide sequence, Cys-Ser-Thr-Leu-Lys-Try-Gln-Asn-Ile-Gly-Pro-Lys-Gln-His-Pro-Glu-Asp-Pro-Ile, located at amino acid position 562 to 580 of the human aldehyde oxidase, was synthesized by a peptide sequencer and purified using an HPLC. The synthetic polypeptide was conjugated with a carrier protein by means of the KLH by MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester) method. Two hundred micrograms of KLH-peptide conjugate with Freund's complete adjuvant was subcutaneously injected into a male white rabbit. Thereafter, immunizations with 100 μg of KLH-peptide conjugate with Freund's incomplete adjuvant were repeated five times within a two-week period. Seven days after the final immunization, blood was withdrawn, separated by centrifugation at x1700g for 10 min, and warmed to 56 °C for 30 min. Solid $(NH_4)_2SO_4$ was then slowly added with gentle stirring to the resultant serum to obtain a final concentration of 30%. The suspension was allowed to stand for 30 min, and then fractionated by centrifugation at x5000g for 30 min. The pellet was discarded and additional $(NH_4)_2SO_4$ was added to the supernatant to obtain a 50% saturation. After a second centrifugation at x5000g for 30 min, the supernatant was discarded and the pellet was resuspended and dialyzed against PBS, affinity purified with a Tresyl column (TOSOH, Tokyo, Japan) and then used as a polyclonal antibody against human aldehyde oxidase.

Specificity testing of the antibody against human aldehyde oxidase

The specificity of the polyclonal antibody for human aldehyde oxidase was fully confirmed by immunoblot analysis. After electrophoresis, cytoplasmic protein samples from the respective tissues were transferred electrophoretically from a polyacrylamide gel to a PVDF membrane. The PVDF membrane was blocked overnight at 4 °C in Block Ace (Dainippon Pharmaceutical Co. Ltd, Tokyo, Japan). After being rinsed with TBS (0.05M Tris-buffered saline, pH 7.4) containing 0.05% Tween 20 (TBST), the PVDF membrane was incubated for 1 hour at room temperature with 4 μg/mL of Tresyl column affinity purified polyclonal antibody against human aldehyde oxidase in 25% Block Ace. The PVDF membrane was washed three times with TBST, 10 min each time, and then incubated at room temperature for 30 min with a labeled polymer (ENVISION +, Dako Corporation, Carpinteria, CA, USA). The membrane was washed again three times with rinse buffer, for 10 min each time. Aldehyde oxidase protein was then visualized by use of a Liquid DAB substrate-chromogen system (Dako Corporation).

Immunohistochemical procedures

Immunolocalization of aldehyde oxidase in the tissue sections was performed according to labeled polymer techniques. The tissue sections were deparaffinized, hydrated, and treated with proteinase K (Dako Corporation) for 6 min. Endogenous peroxidase activity in the tissues was eliminated by preincubation with 3% H_2O_2 in methanol for 10 min. After three washes with PBS for 5 min, the tissue sections were placed in a blocking reagent (Protein Block Serum-Free, Dako Corporation) for 10 min to reduce nonspecific staining of the bridging antibody, and then incubated overnight with the Tresyl column affinity purified polyclonal antibody against human aldehyde oxidase (4 μg/mL) in 25% Block Ace in PBS at 4 °C. After being washed 3 times for 5 min in PBS, the sections were incubated at room temperature for 30 min with ENVISION +. Following washing with PBS 3 times for 5 min, the sections were placed in a Liquid DAB substrate-chromogen system for 5 to 10 min. The intensity of the staining was controlled while viewing through a microscope. Counterstaining was performed with Mayer hemalum solution (Diagnostica Merck, Darmstadt, Germany). Finally, after a wash in PBS, the sections were dehydrated in a graded ethanol series and mounted in resin-based permanent mounting medium (Shandon/Lipshaw Co., Pittsburgh, PA, USA). As control immunostainings, sections were incubated in the same manner, except that the primary antibody was replaced by normal rabbit IgG (Rabbit immunoglobulin fraction, negative control, Dako Corporation).

Results

Specificity of anti-aldehyde oxidase polyclonal antibody and expression of aldehyde oxidase protein in human tissues by immunoblot analysis

The specificity of the polyclonal antibody against human aldehyde oxidase was checked by immunoblot analysis using normal human liver cytoplasmic extract. As illustrated in Fig. 1, lane 1, a single major band of MW at approximately 150kd was detected in the liver cytoplasmic extract. In addition, the liver tissues showed a significant proportion of an aldehyde oxidase fragment of proteolytic origin, which was probably due to the high concentration of protease normally present in this organ. The amount of aldehyde oxidase protein in various human tissues was also determined by immunoblot analysis. A faint to moderate single band at the molecular weight of 150 kd corresponding to aldehyde oxidase protein was observed in specimens from the prostate (Fig. 1, lane 9), adrenal gland (Fig. 1, lane 11), and testis tissues (Fig. 1, lane 12). In contrast, no protein band corresponding to aldehyde oxidase was detectable in the lung, kidney, large intestine, small intestine, cerebrum, heart, pancreas, or stomach tissues (Fig. 1, lane 2- 8, 10).

Expression of aldehyde oxidase protein in human tissues by immunohistochemistry

The tissue distribution of human and rat aldehyde

oxidase protein is summarized in Table 1. The distribution of reactivity observed in various tissues was as follows.

Digestive tissue

Hepatocytes and sinusoidal lining cells displayed diffuse staining for aldehyde oxidase. However, the reactivity for aldehyde oxidase was unevenly distributed, as it was more intense in the pericentral hepatocytes around the central vein than in the periportal hepatocytes around the portal triad, which was similar to the results found for the rat liver (Fig. 2a). In addition, the bile duct showed a moderate immunostaining (Fig. 2a, arrow). In the esophagus, faint immunostaining was observed at the basal layer of the surface epithelia (data not shown), while stomach specimens were not immunostained (Fig. 2c). In the small and large intestines, a faint reaction was recognized on the surface epithelia. However, no reaction was observed in the respective glandular cells (Fig. 2e,g). The lamina propria in the small intestine was also immunostained (Fig. 2e). In contrast, control sections treated with normal rabbit IgG did not show staining (Fig. 2b,d,f,h).

Cardiovascular tissue

Cardiac and skeletal muscle cells, as well as those from the aorta, were not immunostained for aldehyde oxidase (data not shown).

Respiratory tissue

Surface epithelia of the bronchium were moderately stained for aldehyde oxidase, while alveolar cells were strongly immunostained (Fig. 3a,c). Cartilage cells were also stained, but those of the bronchial glands were not (Fig. 3a, arrow). Control sections treated with normal rabbit IgG did not show staining (Fig. 3b,d).

Urinary system tissue

In the kidney, the proximal, distal, and collecting tubules were immunostained for aldehyde oxidase with

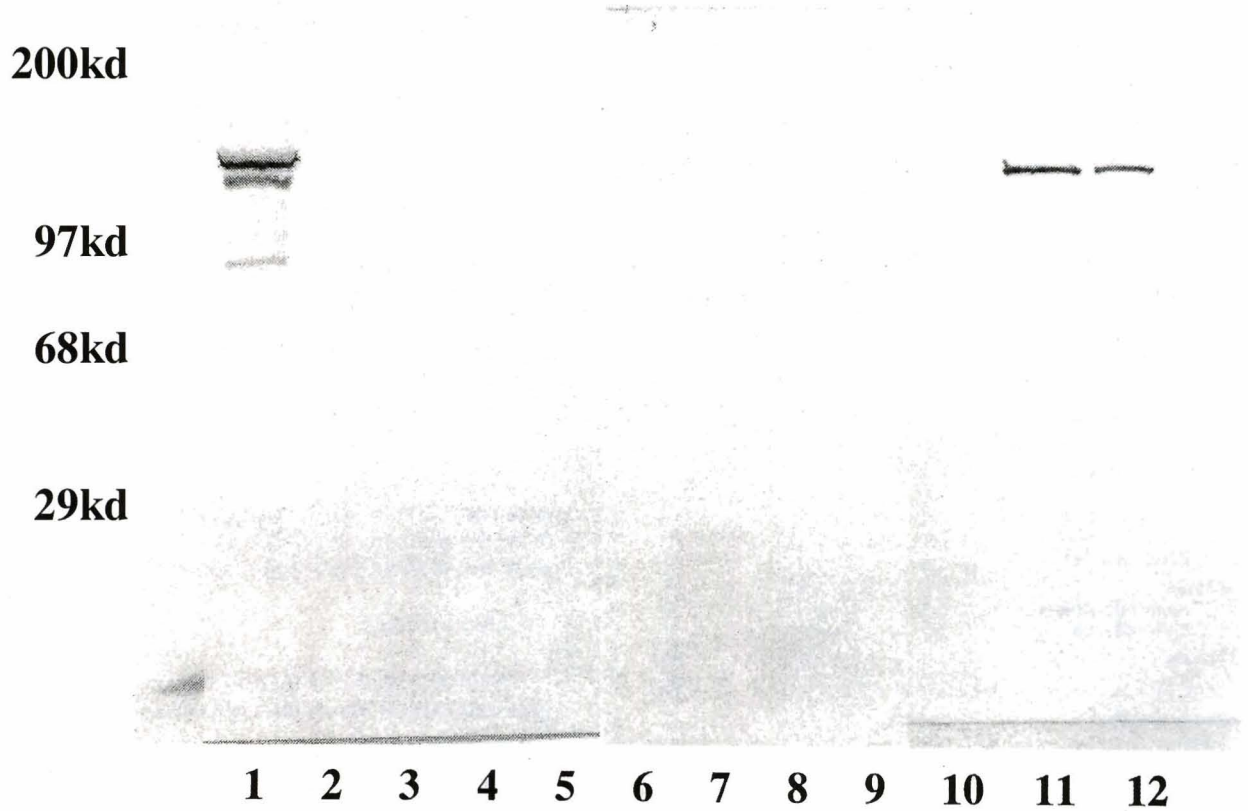

Fig. 1. Immunoblot analysis of aldehyde oxidase from various human tissues. Cytoplasmic proteins (25 μg per lane) were loaded onto a 7.5% SDS-polyacrylamide gel and subjected to immunoblot analysis. After transfer, the PVDF membranes were hybridized with the anti-human aldehyde oxidase polyclonal antibody. Immunoreactive bands were visualized with the use of DAB, after incubation with a second antibody linked to horseradish peroxidase. Molecular markers are indicated in the left column (200 kd, myosin H-chain; 97 kd, phosphorylase b; 68 kd, ovalbumin; 29 kd, carbonic anhydrase). Lane 1, liver; lane 2, lung; lane 3, kidney; lane 4, large intestine; lane 5, small intestine; lane 6, cerebrum; lane 7, heart; lane 8, pancreas; lane 9, prostate; lane 10, stomach; lane 11, adrenal gland; and lane 12, testis. A single band at the molecular weight of 150 kd corresponding to aldehyde oxidase protein is observed in specimens from the adrenal gland (moderate), testis (moderate), and prostate (weak), while the liver (strong) shows a significant proportion of an aldehyde oxidase fragment of proteolytic origin, probably due to the high concentration of protease present in this organ, in addition to a single band at the molecular weight of 150 kd.

Table 1. Summary of aldehyde oxidase distribution in human and rat tissues.

TISSUE	IMMUNOSTAINING	
	Human	Rat
Respiratory tissues		
Throat	-	ND
Lung		
Trachea	±	±~++
Bronchium	++	++
Alveolus	+	+
Urinary tissues		
Kidney		
Glomerulus	-	-
Proximal tubule	+~++	±
Distal tubule	+~++	+
Collecting tubule	+~++	+
Bladder		
Transitional cells	-	ND
Prostate		
Glands	+++	+
Digestive tissues		
Liver		
Pericentral hepatocyte	++	++
Periportal hepatocyte	+	+
Bile dcut	++	++
Pancreas		
Acinus	-	+
Duct	-	ND
Islet of Langerhans	-	ND
Parotid gland	-	+
Esophagus		
Epithelium, basal layer	±	+
Epithelium, intermediate layer	-	++
Epithelium, superficial layer	-	+
Glands	-	ND
Muscle	-	ND
Stomach		
Surface mucous cell	-	+
Gastric glands	±	+
Small intestine		
Columnar epithelium	±	±
Goblet cells	-	+
Intestinal glands	-	-
Lamina propria	+	-
Endocrine tissues		
Adrenal		
Zona glomerulosa	++	-
Zona fasciculata	++	-
Zona reticularis	+++	-
Testis		
Spermatogonium	++	+
Spermatozoa	++	-
Ovary	-	ND
Thyroid	-	ND
Neural tissues		
Brain	-	-~++
Spinal cord	-	-
Cardiovascular tissues		
Heart	-	-~±
Aorta	-	-
Reproductive tissues		
Epididymus	+++	ND

-: no staining; ±: equivocal staining; +: low staining; ++: moderate staining; +++: high staining; ND: not described. Results of tissue distribution of rat aldehyde oxidase are cited from Histochem. Cell Biol. 1996; 105: 71-79 (Moriwaki et al.).

various intensities, while glomerulus specimens were not (Fig. 4a). These staining patterns were very similar to those of rat kidney. Control sections treated with normal rabbit IgG did not show staining (Fig. 4b).

Endocrine tissue

Strong immunostaining was observed in the adrenal gland samples. There was a staining gradient observed across the specimens, with the zona reticularis having the highest level (Fig. 4c). These staining patterns were strikingly different from those of the rat adrenal gland, which showed no staining, though they resembled rat xanthine oxidase. In contrast, neither the thyroid nor pancreas specimens had any staining for aldehyde oxidase (data not shown). Control sections treated with normal rabbit IgG did not show staining (Fig. 4d).

Reproductive tissue

In the prostate and epididymus, strong immunostaining was observed in the tubulo-alveolar prostatic gland epithelia (Fig. 5a) and ductuli epididymidis (Fig. 5c), while seminal vesicles were intensely immunostained for aldehyde oxidase (data not shown). Furthermore, the longitudinal folds of the fallopian tube mucosa showed staining, though the ovary specimens did not (data not shown). Control sections treated with normal rabbit IgG did not show staining (Fig. 5b,d).

Lymphoid tissues

Lymphoid tissues from the thymus, lymph node, tonsil, and spleen were not labeled for aldehyde oxidase (data not shown).

Neural tissues

Neural tissues from the medulla oblongata, corpus callosum, spinal cord, peripheral nerve, cerebral cortex, mesencepharon, cerebellum, pons, and hippocampus were not stained for aldehyde oxidase (data not shown).

Other tissues

Salivary gland, skin, and uterus specimens were not immunostained for aldehyde oxidase (data not shown).

Discussion

Purification of aldehyde oxidase has been performed using liver specimens from rabbits (Stell et al., 1989), guinea pigs (Yoshihara and Tatsumi, 1985), mice (Yoshihara and Tatsumi, 1997a,b), hogs (Li Calzi et al., 1995), and humans (Johns, 1967). However, aldehyde oxidase activity in the human liver is much lower when compared to that in rat, rabbit, or guinea pig livers. As a result, it is difficult to purify human aldehyde oxidase to

the point of homogeneity and to raise an antibody using the purified enzyme. Recently, the primary amino acid sequence of human aldehyde oxidase was determined (Wright et al., 1993; Terao et al., 1998), which has provided an important tool for the detection of aldehyde oxidase in human tissues at the protein level as well as at the mRNA level.

The present study was undertaken to: 1) determine

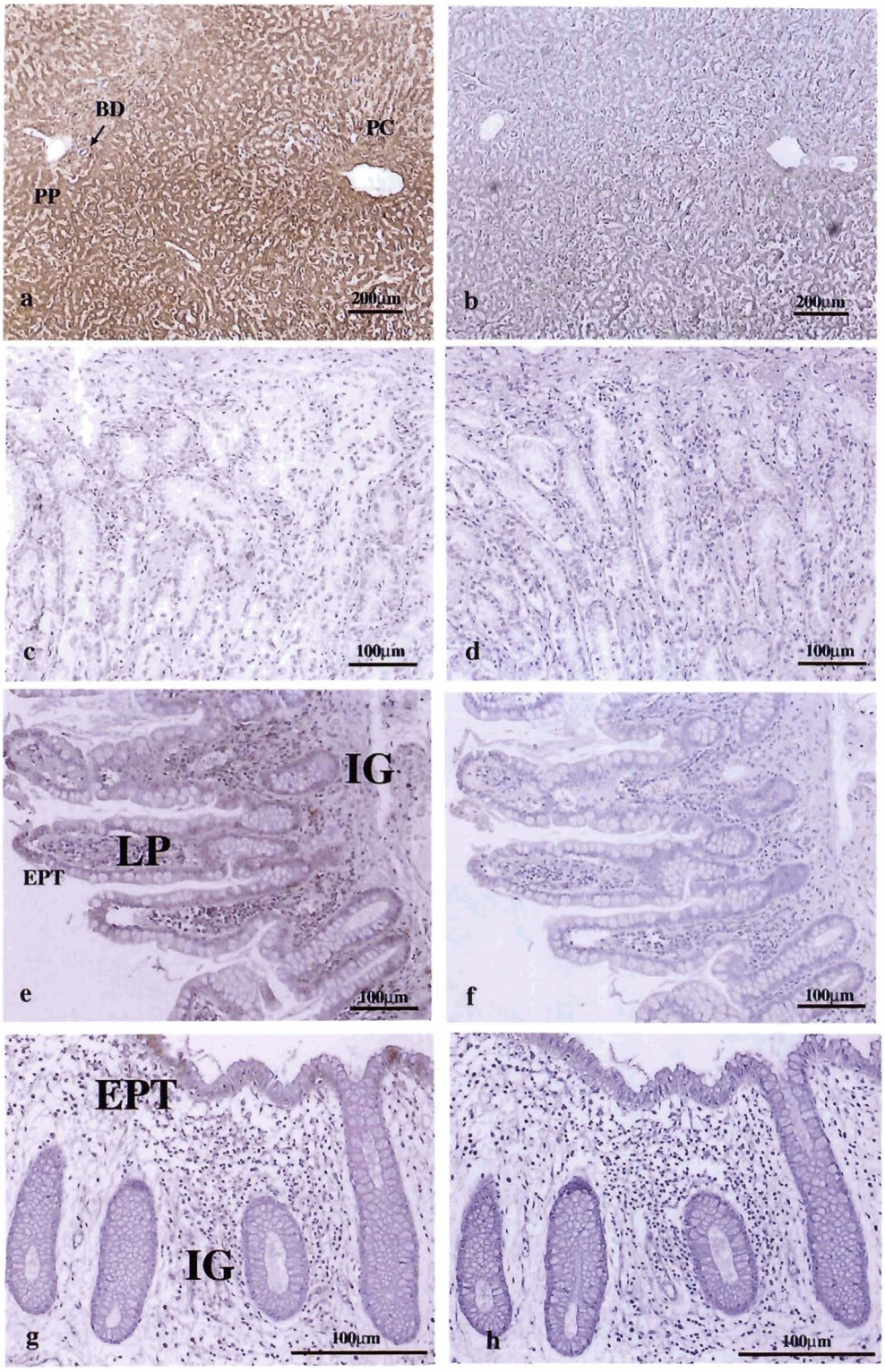

Fig. 2. Immunohistochemical staining of aldehyde oxidase protein in human digestive system tissues with the aldehyde oxidase polyclonal antibody. In the liver, diffuse but uneven immunostaining is observed in the hepatocytes and sinusoidal lining cells. The reactivity for aldehyde oxidase is more intense in the pericentral area (PC) than in the periportal area (PP) **(a)**. In addition, the bile duct (BD) shows a moderate immunostaining **(a, arrow)**. Stomach samples are not immunostained **(c)**. Epithelia (EPT) of the small and large intestines are weakly immunostained. However, their respective glandular cells (IG) are not immunostained against aldehyde oxidase **(e, g)**. Lamina propria (LP) of the small intestine is also immunostained **(e)**. Control sections treated with normal rabbit IgG do not show staining **(b, d, f, h)**. 5-μm paraffin sections.

the distribution of human aldehyde oxidase protein in various tissues; 2) compare the level of aldehyde oxidase protein in the liver with that of other tissues; and 3) compare the distribution of aldehyde oxidase protein in various human tissues with that of rat tissues.

The present method of immunohistochemistry, using a sensitive and specific polyclonal antibody, enabled us to map the cellular distribution of aldehyde oxidase protein in a variety of human tissues. Our results were similar to those of Moriwaki et al. (1996) and Kurosaki et al. (1999), who studied aldehyde oxidase protein and mRNA levels, respectively, in rats and mice.

Aldehyde oxidase activity has been exclusively observed in hepatic tissues and, since its initial discovery, the enzyme has been considered to be xenobiotic. However, the importance of its widespread tissue distribution other than hepatic tissue remains unclear, though several pathophysiological functions of this protein, other than xenobiotic metabolism, have been suggested (Moriwaki et al., 1997). The significance of aldehyde oxidase has been implicated in microbicidal activity (Badwey et al., 1981), and its localization in epithelium may suggest a role in the defense mechanism. Furthermore, it has been proposed that aldehyde oxidase is also involved in the metabolism of biogenic amine (Beedham et al., 1995), alcohol toxicity (Mira et al., 1995), pheromone degradation (Rybczynski et al., 1989, 1990), and re-expansion pulmonary edema (Jackson et al., 1988). Moreover, the discovery of this enzyme in the intestines suggests that it may be involved in protection against toxic plants, as suggested previously for terrestrial gastropods (Large and Connock, 1993).

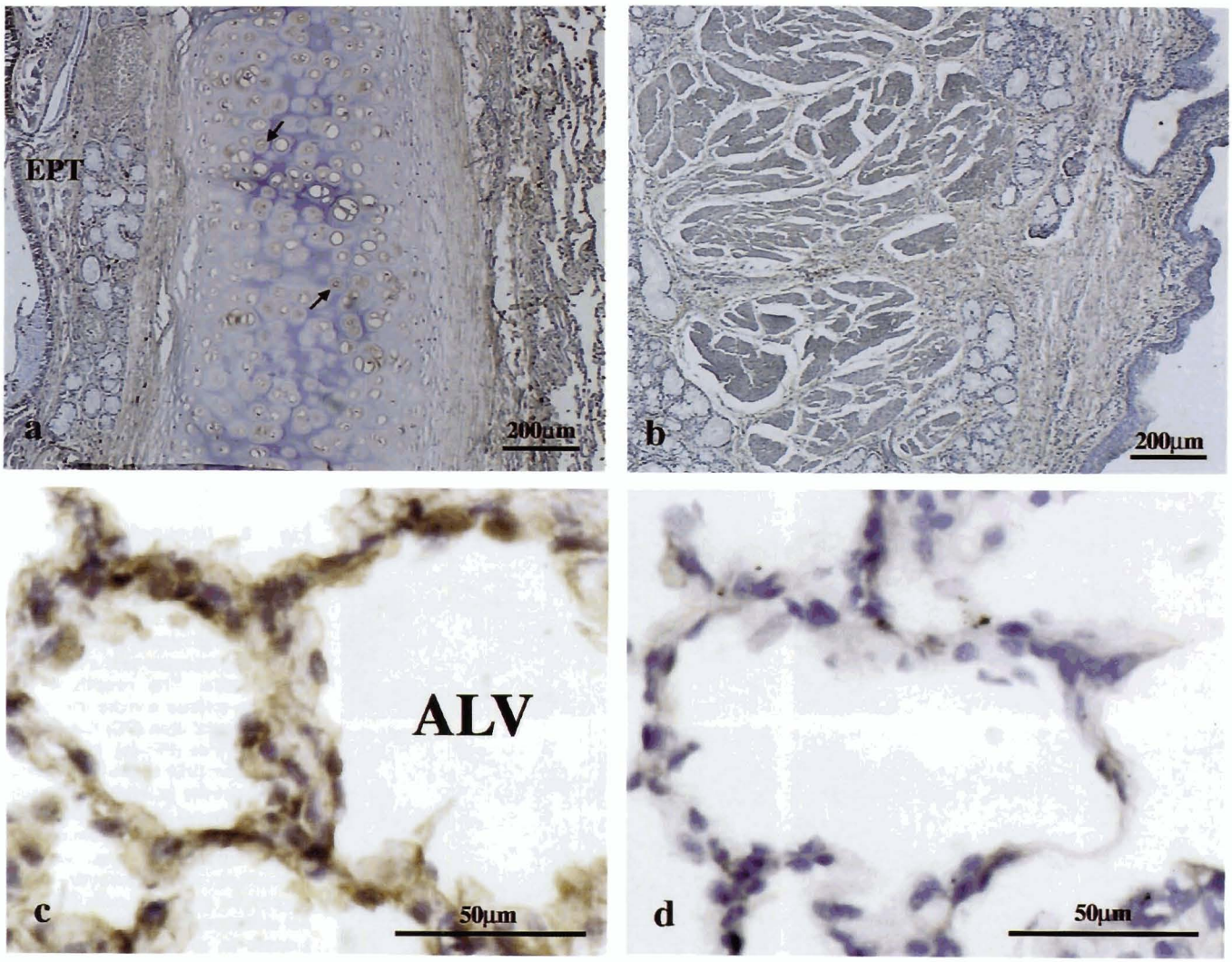

Fig. 3. Immunohistochemical staining of aldehyde oxidase protein in human respiratory system tissues with the aldehyde oxidase polyclonal antibody. In the trachea and bronchium, strong immunostaining is observed in the epithelial cells (EPT) **(a)**. In addition, cartilageous cells are also moderately immunostained **(a, arrow)**. Strong immunostaining is recognized in the alveoli (ALV) **(c)**. Control sections treated with normal rabbit IgG do not show staining **(b, d)**. 5-μm paraffin sections.

The most intriguing finding in the present study was the localization of aldehyde oxidase in adrenal gland, epididymus, prostate, and testis specimens, as shown by immunoblot analysis and immunohistochemistry results. A strong staining intensity gradient was detected across the adrenal gland specimens, with the zona reticularis having the highest level of staining. This strong staining of aldehyde oxidase in the zona reticularis of the adrenal gland, as well as in the renal tubules, may suggest a possible role in electrolyte and organic ion transport. It has previously been demonstrated that aldehyde oxidase activity is regulated by testosterone (Kurosaki et al., 1999) and growth hormone (Yoshihara and Tatsumi, 1997a,b). Thus, in the present study it is noteworthy that aldehyde oxidase was observed in samples from the testis, epididymus, and prostate, which are the target organs of testosterone.

Recent studies have demonstrated that aldehyde oxidase is involved in familial recessive type of amyotrophic lateral sclerosis (Berger et al., 1995; Bendotti et al., 1997; Wright and Repine, 1997). Furthermore, aldehyde oxidase mRNA has been observed in mouse and human spinal cords (Berger et al., 1995; Bendotti et al., 1997), though its subcellular localization was different, in that it was observed in large motor neurons in the mouse spinal cord as in the glial, but not in the motor neurons in the anterior horn neuron of the human spinal cord. In the present study, aldehyde oxidase protein was not observed in the spinal cord.

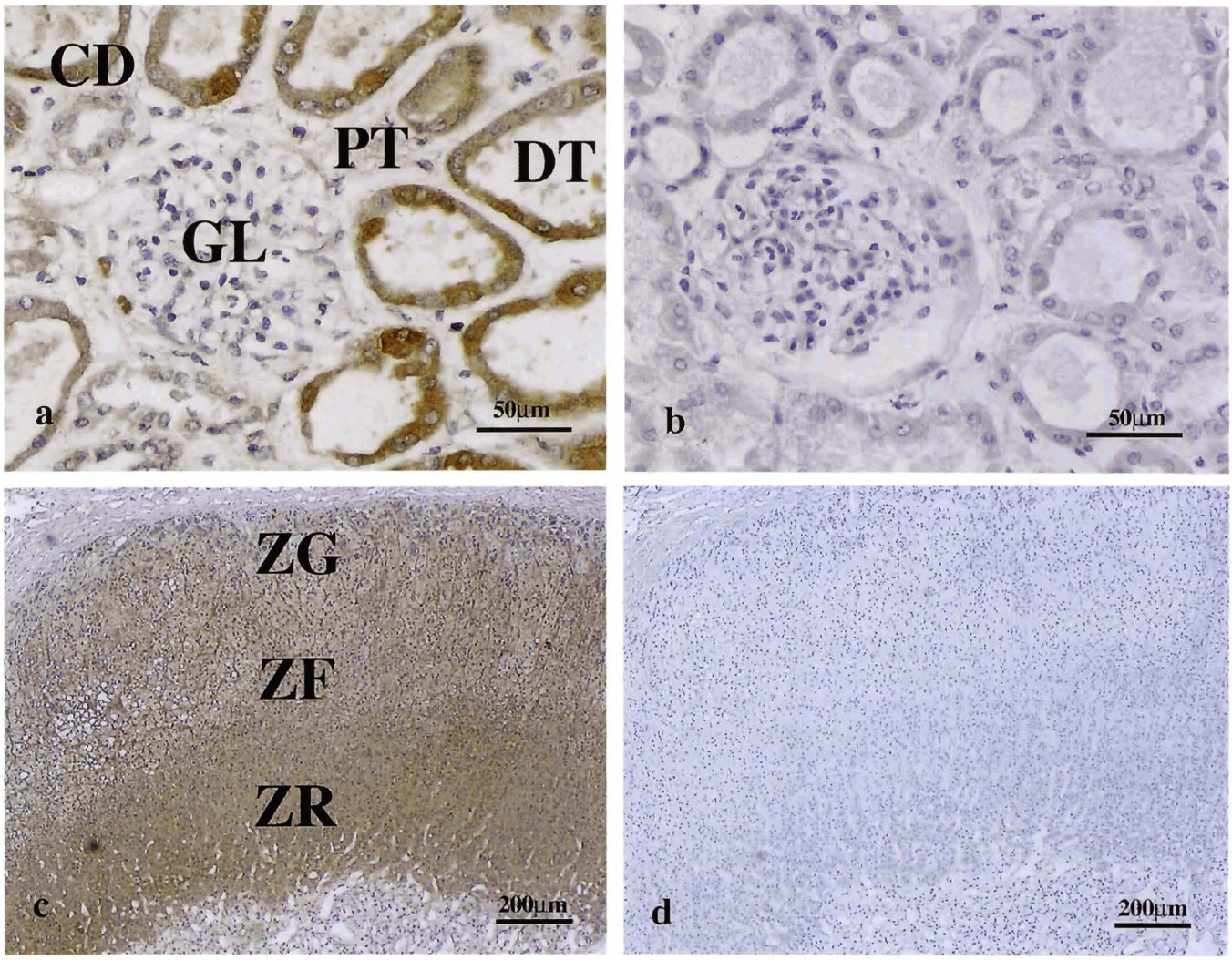

Fig. 4. Immunohistochemical staining of aldehyde oxidase protein in human urinary and endocrine system tissues with the aldehyde oxidase polyclonal antibody. In the kidney, strong but uneven immunostaining is detected in the proximal (PT), distal (DT), and collecting tubules (CT), whereas the glomeruli (GL) only has background levels of immunostaining **(a)**. A strong staining intensity is demonstrated in the adrenal gland **(c)**. There is an aldehyde oxidase staining gradient across the adrenal gland specimen, with the zona reticularis (ZR) having the highest level. Control sections treated with normal rabbit IgG do not show staining **(b, d)**. 5-μm paraffin sections.

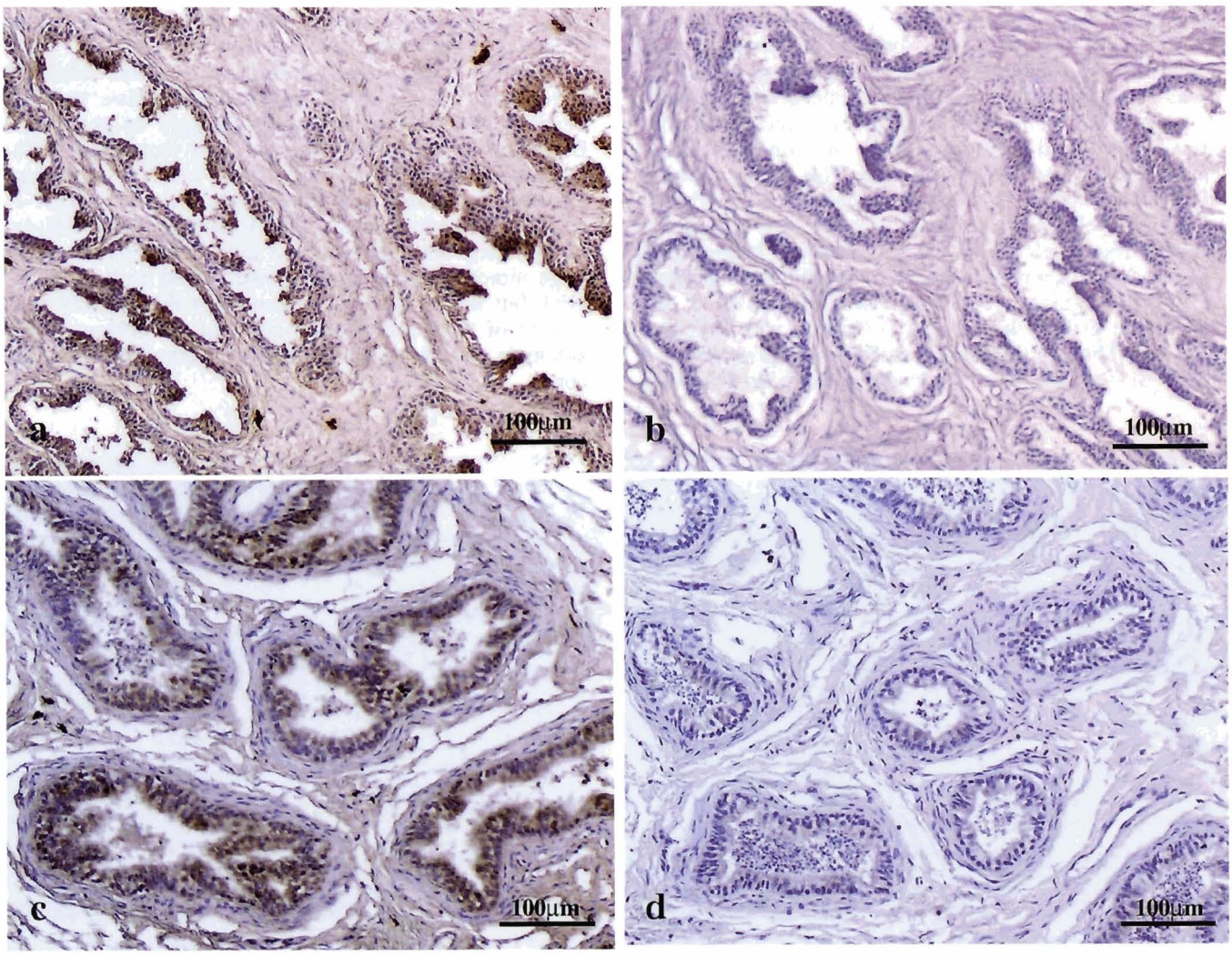

Fig. 5. Immunohistochemical staining of aldehyde oxidase protein in human reproductive system tissues with the aldehyde oxidase polyclonal antibody In the prostate, the glandular epithelium is strongly stained **(a)**. In the epididymus, strong immunostaining is observed in the ductuli epididymidis **(c)**. Control sections treated with normal rabbit IgG do not show staining **(b, d)**. 5-mm paraffin sections.

A deficiency of aldehyde oxidase has not yet been recognized clinically, though a combined deficiency of xanthine oxidase and aldehyde oxidase was suggested in an in vivo study using N-methylnicotinamide (Reiter et al., 1990). Additionally, previous studies have suggested that aldehyde oxidase is identical to retinal oxidase (Tomita et al., 1993; Huang and Ichikawa, 1994). Since retinal oxidase plays an important role in the synthesis of retinoic acid, which is essential for reproduction, normal embryogenesis, morphogenesis, neural development, and cellular differentiation (Thaller and Eichele, 1987; Levine and Hoey, 1988), it may be speculated that a deficiency of aldehyde oxidase is likely to affect these crucial processes. Studies on aldehyde oxidase deficiency using knock-out mice will be required to elucidate the role of aldehyde oxidase in these processes.

In conclusion, the widespread tissue distribution of aldehyde oxidase in humans does not provide an immediate clue to its biological functions. However, its expression in these selective sites may be of significance and could provide evidence regarding its action. Therefore, the present study may serve to improve the understanding of the pathophysiological role of aldehyde oxidase in humans, though the significance of its prevalent distribution, other than the liver, seems to be a matter for future research.

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