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Immunohistochemical Localization of Xanthine Oxidase in Human Tissues

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We evaluated the immunohistochemical localization of xanthine oxidase in various human tissues. Xanthine oxidase was purified from cadaver liver. Polyclonal antibody against xanthine oxidase was raised in a rabbit. Immunoblot analysis showed that the raised antibody reacted specifically with one band whose position corresponded with that of the purified enzyme. Immunostaining of paraffin-embedded tissue sections showed intense reactivity in the following tissues: surface epithelium of tongue, esophagus, and trachea, sweat glands, and mammary glands. Weak, but positive, reactivity was observed in other tissues, such as glandular cells of the small and large intestine and renal tubules, skeletal muscle, gastric epithelial cells, alveoli of the lung, spleen, and liver cytoplasm. Xanthine oxidase staining was observed in infiltrating lymphocytes (probably T-lymphocytes but not in B-lymphocytes) in inflammatory lesions of the small and large intestine. Its ubiquitous localization suggests that xanthine oxidase is involved in cell proliferation/differentiation, the defense mechanisms, and in the pathogenesis of reperfusion tissue injury.

Key words: Xanthine oxidase, Immunohistochemical localization, Polyclonal antibody, Human tissue

I. Introduction

Xanthine oxidoreductase, a molybdo-flavoprotein, catalyzes the oxidation of hypoxanthine to xanthine and, subsequently, to uric acid. This enzyme exists in tissues primarily as an NAD^+-dependent dehydrogenase form (EC 1.1.1.204): under ischemic conditions, it is converted to an O_2-dependent oxidase form (EC 1.2.3.2) that produces superoxide radicals with the oxidation of purines in the tissue. Xanthine oxidase has been implicated in the pathogenesis of postischemic reperfusion tissue injury [16]. This enzyme also appears to be involved in cell differentiation [23], cell proliferation [21], defence against microorganisms [26-28] and metabolism of xenobiotics [15] *etc.* The previous studies were mainly performed in laboratory animals or *in vitro*. To clarify the pathophysiological role of xanthine oxidase in humans, it is important to investigate the localization of xanthine oxidase in human tissues. The immunohistochemical localization of xanthine oxidase has been demonstrated in only a limited number of tissues of humans, such as endothelial cells [5, 19, 25], and cardiac and skeletal muscle cells [9]. We used paraffin-embedded sections to determine the distribution of xanthine oxidase in several specimens of human tissues, including cancerous tissue.

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II. Materials and Methods

Tissues

The liver obtained from cadavers was frozen as soon as possible after death and stored at −70°C until use to provide a source of xanthine oxidase. Tissue specimens were fixed in 4% paraformaldehyde-PBS (0.01 M phosphate-buffered saline, pH 7.4), embedded in paraffin at 60°C, and subjected to immunohistochemical treatment.

Reagents

Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Xanthine, hypoxanthine, ammonium sulfate, folic acid, 1-ethyl-3-(3-dimethylaminoprophyl)-carbodiimide hydrochloride (WSC), hydroxyapatite, acrylamide, bis-acrylamide, ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine, and sodium dodecyl sulfate (SDS) were obtained from Wako Pure Chemical Industries (Osaka, Japan). 3,3′-diaminobenzidine (DAB) tetrahydrochloride was ob-

tained from Dojin Laboratories (Kumamoto, Japan). DEAE-cellulose (DE 52 microgranular) was purchased from Whatman Paper Ltd. (Maidstone, UK) and ECH Sepharose 4B from Pharmacia LKB (Uppsala, Sweden). MPL™+TDM emulsion was purchased from Ribi Immunochem. Research Inc. (Hamilton, MT, USA). Biotinylated goat anti-rabbit IgG and Vectastain ABC *Elite* kit were purchased from Vector Labs. (Burlingame, CA, USA). Block ace was obtained from Dainippon Pharmaceutical Co. Ltd. (Tokyo, Japan). Tween 20 and nitrocellulose membrane (Trans-Blot Transfer Medium) were from Bio-Rad Lab. (Richmond, CA, USA). Anti-human B-cell mouse monoclonal antibody (MB-1) and anti-human T-cell mouse monoclonal antibody (MT-1) were purchased from Bio-Science Products (Emmenbrucke, Switzerland). Other chemicals, unless specifically stated otherwise, were from Wako Pure Chemical Industries.

Methods

1) Preparation and specificity testing of antibody

Xanthine oxidase was purified from the liver of cadavers according to a previously described method [19]. Enzyme purity was verified by SDS-polyacrylamide gel electrophoresis (PAGE): a single band was observed on SDS-PAGE. Antibody against purified xathine oxidase was raised in a rabbit: A sterile solution of purified human

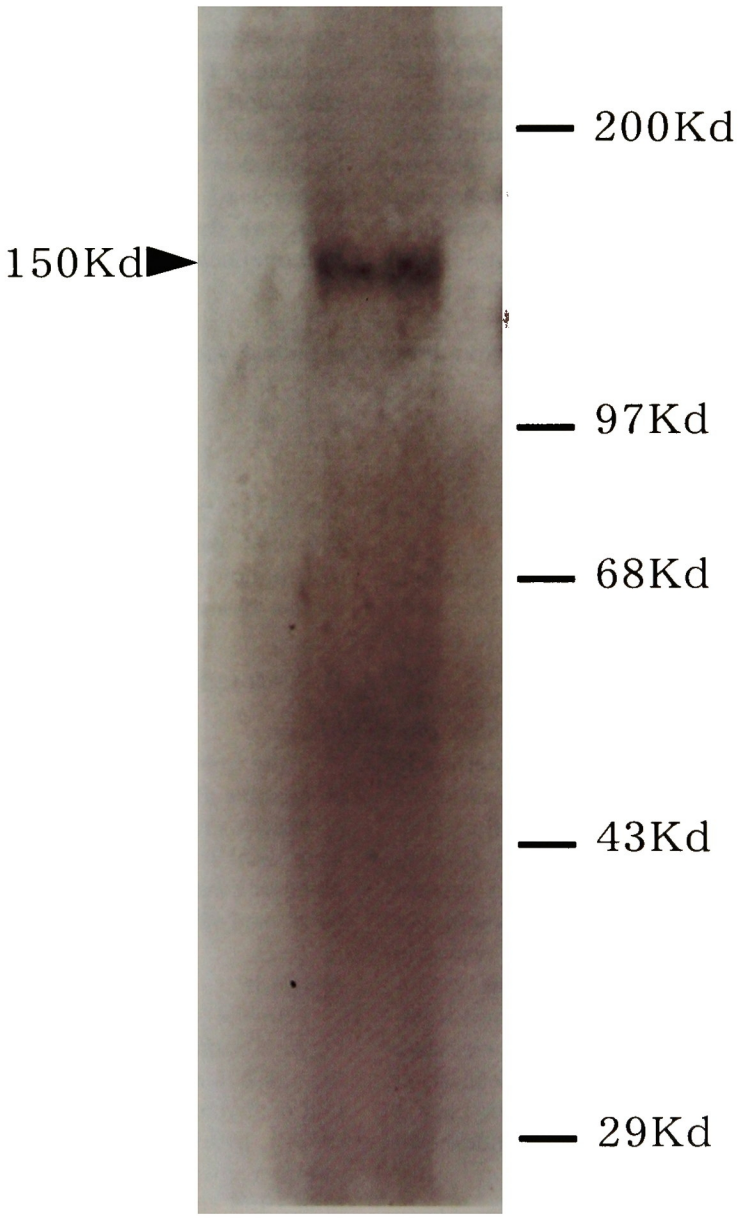

Fig. 1. Immunoblotting of crude extract from human liver. Molecular weight of xanthine oxidase protein, estimated by comparison with a molecular weight standard, is marked at the left.

liver xanthine oxidase (1 mg), emulsified with an equal volume of MPL™+TDM emulsion, was injected intracutaneously into a male New Zealand white rabbit. Starting 2 weeks later, equal volumes of the mixture were injected intracutaneously three times as boosters over a 6-week period. 3 days after the last booster injection, blood was drawn from an ear vein, serum was separated, and the complement was inactivated at 56°C for 30 min. To test the specificity of the antibody, immunoblot analysis was performed, as described previously [29]. After electrophoresis, xanthine oxidase protein was transferred electrophoretically from polyacrylamide gel slab to nitrocellulose membrane. The nitrocellulose membrane was blocked overnight at 4°C in PBS containing 1% BSA. After being rinsed with PBS containing 0.05% Tween 20, the nitrocellulose membrane was incubated for 1 hr at room temperature with a 1 : 1,000 dilution of a rabbit antibody against xanthine oxidase in PBS containing 1% BSA. The nitrocellulose membrane was washed four times, 10 min each time, in PBS containing 0.05% Tween 20 and was

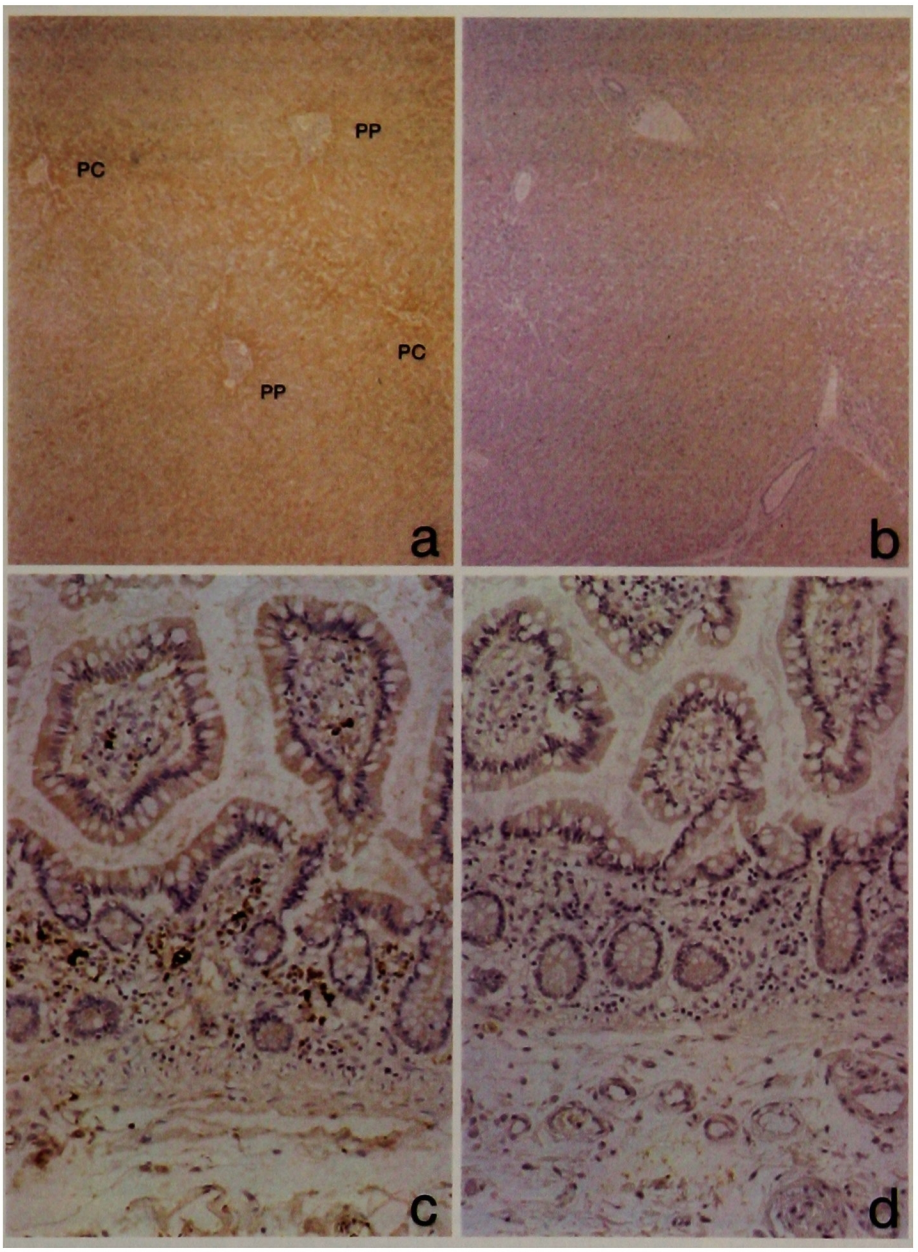

Fig. 2. Immunostaining of the liver and small intestine. Liver (**a, b**); Uneven positive immunostaining was found in the hepatocytes. Both the pericentral and periportal areas stained more intensely than the mid-zonal area (**a**). The bile duct was also stained. No reaction products were present in control sections (**b**). pc: pericentral area; pp: periportal area × 10 (in original). Small intestine (**c, d**); In the small intestine, the surface epithelium, including goblet cells, was weakly stained (**c**). Immunostaining was also positive in infiltrating lymphocytes. No specific reaction was seen in control sections (**d**). × 10 (in original).

then incubated for 1 hr with a 1 : 200 dilution of biotinylated goat anti-rabbit IgG in PBS containing 1% BSA. The membrane was washed three times, 10 min each time, with rinse buffer. Xanthine oxidase protein was visualized by use of DAB and the avidin-biotin-peroxidase complex (ABC) technique.

2) Immunohistochemical techniques

Immunoreactivity against xanthine oxidase in human tissues was detected by the ABC technique. Deparaffinized sections (4 μm) were treated for 30 min with 50 mM Tris-buffered saline, pH 7.4, (TBS) containing 1% H_2O_2 to block endogenous peroxidase activity in the tissues. Sections were blocked with Block ace for 60 min, then incubated overnight at 4°C with anti-xanthine oxidase serum diluted 1 : 1,000 in TBS containing 0.05% Tween 20 and 0.5% BSA (TBSBT). After being washed three times with TBS containing 0.05% Tween 20 (TBST), the sections were incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG (1 : 200 diluted with TBSBT). Sections were then rinsed three times with TBST and incubated for 45 min at room temperature with an avidin-biotin-peroxidase complex (Vectastain ABC *Elite* kit). After several TBST rinses, the immunohistochemical reaction was visualized by incubation with 50 mM Tris-Cl buffer (pH 7.6) containing 0.05% DAB and 0.01% H_2O_2. The reaction was terminated by immersion of the slides in TBS and rinsing in distilled water. After being counterstained with Mayer hematoxylin stain solution for 30 sec, the sections were washed, dehydrated, coverslipped and examined with an Olympus photomicroscope.

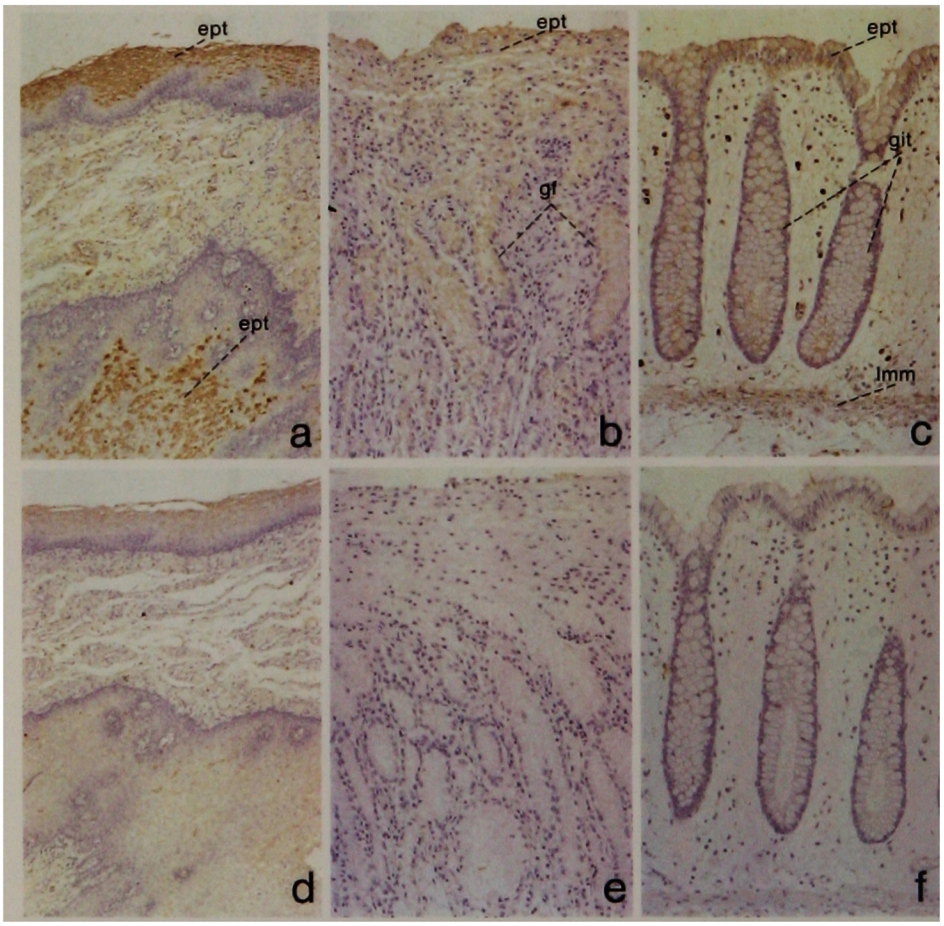

Fig. 3. Immunostaining of the esophagus, stomach, and large intestine. Esophagus (**a, d**); The epithelium, especially the keratinized layer of the esophagus, was intensely stained (**a**). Esophageal muscle cells were moderately stained. Faint reactivity was found in control sections, despite prior incubation with a blocking agent (**d**). ept: epithelium × 25 (in original). Stomach (**b, e**); Both surface mucous cells and gastric glands were weakly stained (**b**). No reaction products were present in control sections (**e**). ept: epithelium; gf: fundic gland × 50 (in original). Large intestine (**c, f**); Surface epithelium, intestinal glands, and lamina muscularis mucosa in the sigmoid colon were weakly stained (**c**). No reaction products were present in control sections (**f**). ept: epithelium; git: intestinal gland; lmm: lamina muscularis mucosa × 50 (in original).

III. Results

1) Specificity of antibody

The immunological specificity of the antisera was tested by immunoblot analysis. A single band of 150 kd, that corresponded to xanthine oxidase protein, was observed (Fig. 1).

2) Immunohistochemical staining

As expected from the biochemical studies, immunostaining was detected in the liver and small intestine.

Liver

Dark brown reaction products were observed in the cytoplasm of hepatocytes and sinusoidal cells in the liver. The staining for xanthine oxidase was unevenly distributed. Both the pericentral and periportal areas stained more intensely than the mid-zonal area (Fig. 2a). The bile duct was also stained.

Small intestine

In the small intestine, surface epithelia of the villi and goblet cells were weakly stained. Infiltrating lymphocytes were also stained (Fig. 2c). Various degrees of immunoreactivity to xanthine oxidase antibody were detected in several tissues, as described below.

Esophagus and gastrointestinal tract

Intense reactivity to xanthine oxidase antibody was found in esophageal epithelial cells, especially in keratinized epithelial cells (cornified and desquamating layer), with a moderate degree of reactivity found in muscle (Fig. 3a). Control sections showed the formation of a faint reaction product, despite prior incubation with a blocking agent (Fig. 3d). Both surface mucous cells and gastric glands were faintly stained for xanthine oxidase (Fig. 3b). In the

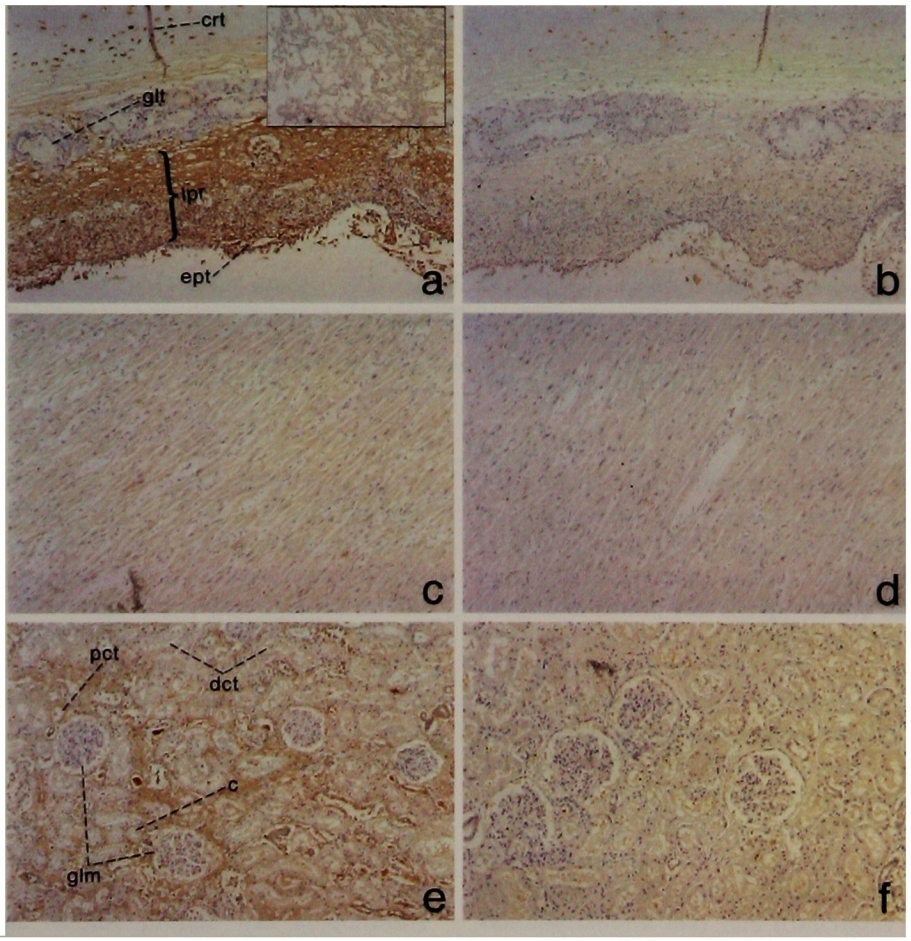

Fig. 4. Immunostaining of the lung, heart, and kidney. Lung (**a, b**); The tracheal epithelium and lamina propria were intensely stained (**a**), while alveoli were only faintly stained (**a**, inset). Cartilaginous cells were also immunostained (**a**). No reaction products were present in control sections (**b**). crt: tracheal cartilage; ept: epithelium; lpr: lamina propria; glt: tracheal gland ×25 (in original). Heart (**c, d**); Heart muscle cells were weakly stained (**c**). No reaction products were present in control sections (**d**). ×25 (in original). Kidney (**e, f**); In the kidney, proximal, distal and collecting tubules exhibited positive immunostaining of various intensities, but immunostaining was not observed in the glomeruli (**e**). ×25 (in original). No reaction products were present in control sections (**f**). ×33 (in original). c: collecting duct; dct: distal convoluted tubules; glm: glomerulus; pct: proximal convoluted tubules.

large intestine, surface epithelia, including goblet cells and lamina muscularis mucosa, were stained (Fig. 3c). Infiltrating lymphocytes were also stained (Fig. 3c).

Respiratory, circulatory, and excretory systems

Intense reactivity to xanthine oxidase antibody was found in tracheal epithelial cells and lamina propria (Fig. 4a), whereas alveoli were only faintly stained (Fig. 4a inset). Cartilaginous cells were also stained. Heart muscle cells were faintly stained for xanthine oxidase (Fig. 4c). In the kidney, both proximal and distal convoluted tubules reacted variably against xanthine oxidase antibody, but glomeruli were not stained at all (Fig. 4e).

Skin, tongue, and mammary gland

In the skin, sweat glands (Fig. 5a inset) were strongly stained, as was the desquamating epithelial layer of the tongue (Fig. 5c). Muscle cells were also stained (Fig. 5c). While the mammary glands were stained, the reactivity varied (Fig. 5e).

Lymph nodes

Immunoreactive lymphocytes were scattered in the perifollicular area of lymph nodes (Fig. 6a).

Cancerous tissues

Breast cancer metastazed to the liver did not stain for xanthine oxidase (Fig. 7).

The localization of xanthine oxidase in other tissues is summarized in Table 1.

IV. Discussion

The histochemical tissue localization of xanthine ox-

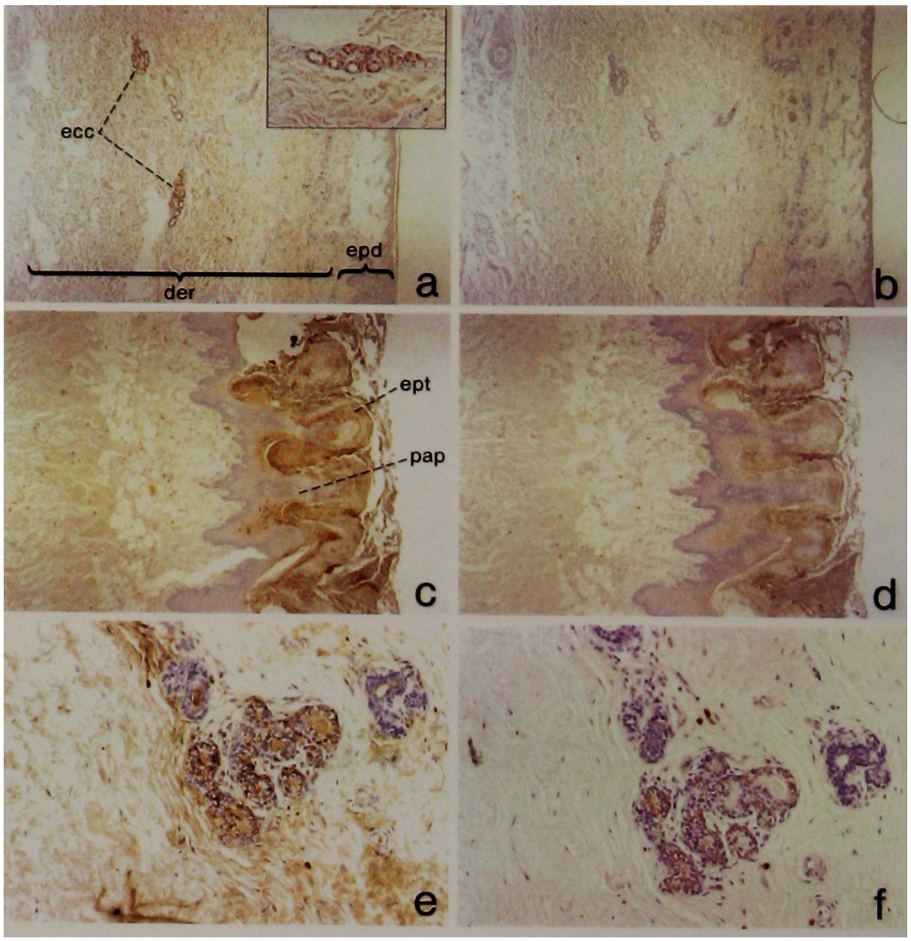

Fig. 5. Immunostaining of the skin, tongue and mammary gland. Skin (**a, b**); Sweat glands in the skin were intensely stained (**a**, inset). Connective tissues in the dermis were also stained, but only weakly (**a**). No reaction products were present in control sections (**b**). der: dermis; ecc: eccrine gland; epd: epidermis × 50 (in original). Tongue (**c, d**); In the tongue, the desquamating epithelial layer was intensely stained (**c**). Muscle cells were also immunostained, but unevenly (**c**). Control reactions showed moderate immunoreactivities in the desquamating layer of the tongue, despite prior incubation with a blocking agent (**d**). ept: epithelium; pap: papilla × 10 (in original). Mammary gland (**e, f**); Strong immunostaining was evident in mammary gland epithelial cells (**e**). However, not all mammary glands showed positive immunostaining and the existence of functional or immunological heterogeneity among these organelles is postulated. Control reactions showed moderate immunoreactivities in the mammary gland, despite prior incubation with a blocking agent (**f**). × 50 (in original).

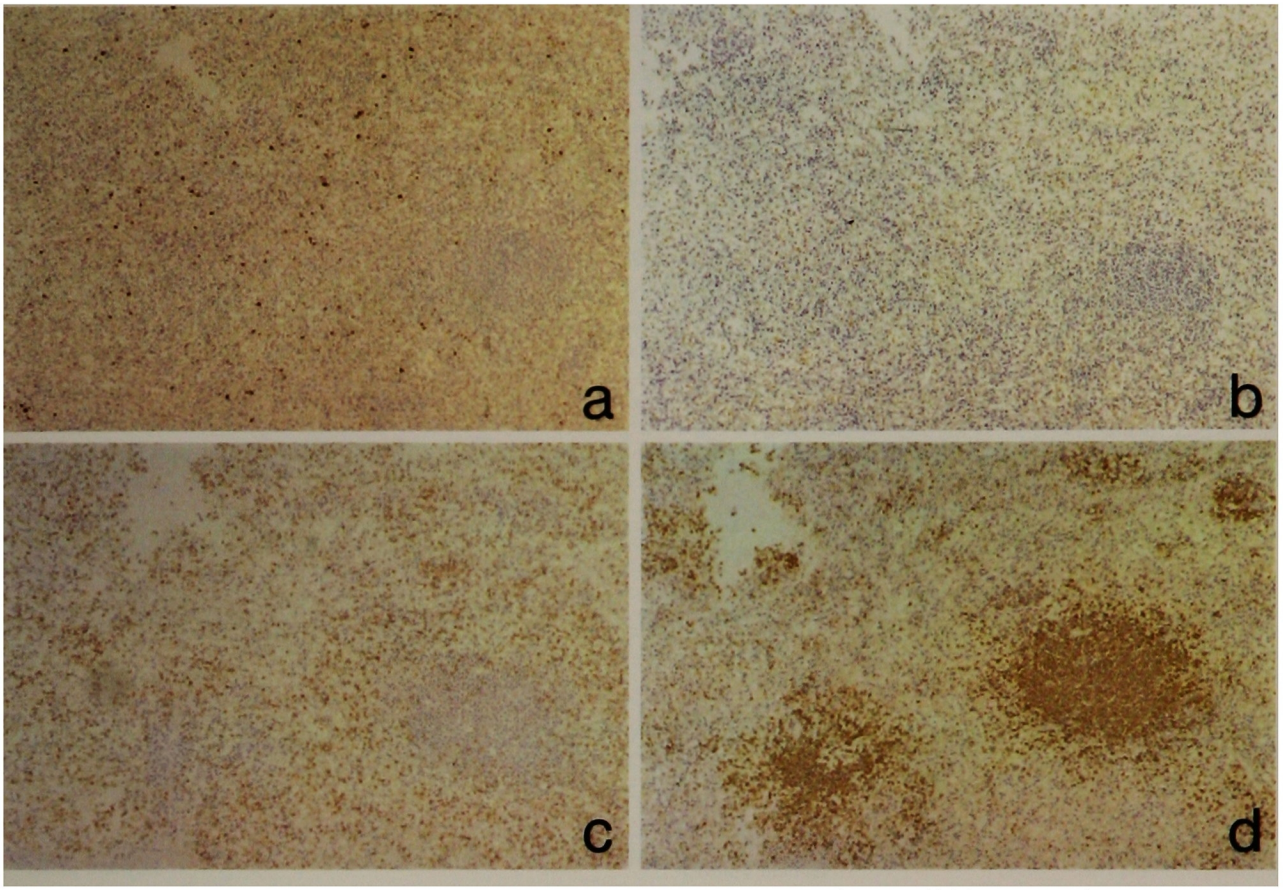

Fig. 6. Immunostaining of lymph nodes (**a**). Lymph nodes (**a, b, c, d**); Some lymphocytes in the perifollicular area of lymph nodes were immunostained, whereas those in the follicular area were not (**a**). No specific reaction was seen in the control sections (**b**). Immunostaining with anti-human T-cell mouse monoclonal antibody was positive in the perifollicular area (**c**), while immunostaining with anti-human B-cell mouse monoclonal antibody was positive in the follicular area (**d**). × 50 (in original).

idase has been performed in various animals, such as domestic fowl [8, 17], hog [12], rat [2] and hamster [10]. According to these studies, xanthine oxidase is localized in epithelial cells of kidney, intestine, and mammary gland, goblet cells, pancreatic acinar cells, and skeletal muscle cells, as well as in endothelial cells from various organs and liver cells. However, immunohistochemical localization of xanthine oxidase in human tissues has not been extensively examined, except for a few studies on a limited number of tissues [1, 5, 9, 19, 25]. We determined the immunohistochemical localization of xanthine oxidase in various human tissues.

The specificity of the polyclonal antibody used in the present study was confirmed by immunoblot analysis. We found that xanthine oxidase protein was ubiquitously distributed in various human tissues, such as the digestive, respiratory, urinary and genitoreproductive tracts, in addition to the endothelial lining cells of capillaries, as reported previously for various animal species. The presence of xanthine oxidase in liver cytoplasm was expected, since this site is the source of the antigen; this finding was in agreement with previous biochemical data. The present study demonstrated the localization of xanthine oxidase in liver cytosol, and also that its distribution in the liver was zonal rather than uniform. Xanthine oxidase was distributed mainly around the pericentral and periportal zones, rather than in the mid-zone of the liver lobule, a localization pattern somewhat different from that reported for the rat liver [20]. The distribution of xanthine oxidase in other tissues did not correspond to that of the activity staining reported by Kooij *et al.* [14], who demonstrated its existence only in the liver and duodenum. This disparity may be ascribed to: 1) a low sensitivity of activity staining as compared with immunohistochemical staining; van den Munckohof *et al.* [28] suggested that the optimal substrate concentration for xanthine oxidase may vary from tissue to tissue, with some tissues requiring a higher concentration of the substrate or a longer incubation time for positive activity staining. 2) Xanthine oxidase may exist in an inactive form, a desulfo or demolybdo form of protein, in tissues with negative activity staining [11], except for the liver and duodenum. In such tissues, when an external insult, such as ischemia, infection, or inflammation is imposed, xanthine oxidase may

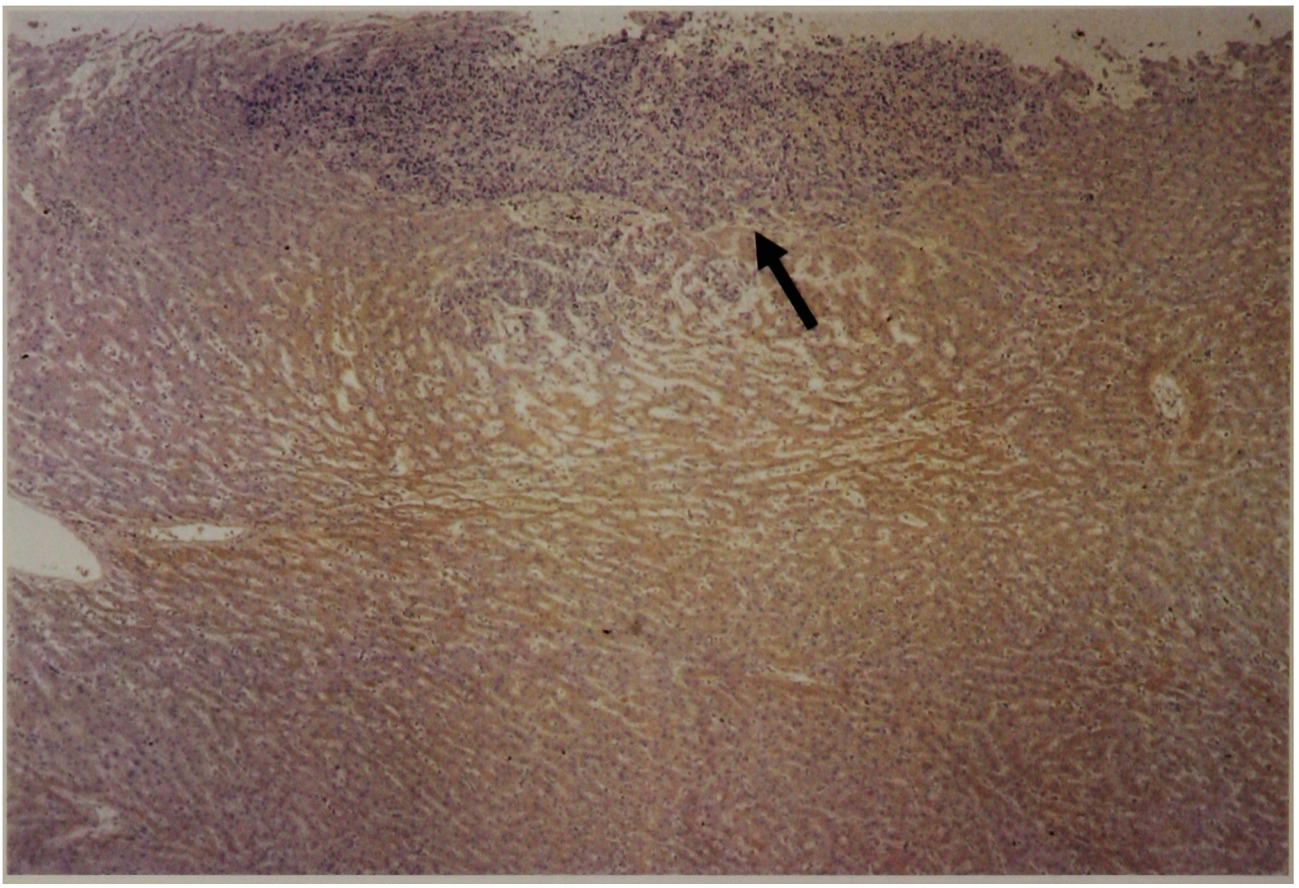

Fig. 7. Immunostaining of the liver with breast cancer metastasis. Liver with breast cancer metastasis; No immunostaining was observed in breast cancer tissue (arrow). ×25 (in original).

be converted from the inactive to an active form, probably sulfo protein, displaying activity. In tissues such as the mammary gland, where immunostaining was observed even in control reactions, this phenomenon may be ascribed to a naturally occurring antibody against xanthine oxidase [6].

The significance of the localization of xanthine oxidase in various tissues can be discussed in the context of the production of superoxide radical and/or uric acid. The superoxide radical is known to possess antimicrobial activity [4, 26, 27]. Therefore, the presence of xanthine oxidase in epithelial tissue, which is continuously exposed to external microorganisms, may serve as a defense mechanism by generating superoxide radicals [28]. It is possible that xanthine oxidase induces the process of cell differentiation/proliferation through the generation of superoxide radical, since previous studies have suggested the occurrence of superoxide radical-induced cell differentiation [24] or proliferation [21]. Thus, as shown in the present study, the extensive distribution of xanthine oxidase in human epithelial renewal tissues may, in part, support the putative role of this enzyme in the defense mechanism and in the process of differentiation/proliferation, in addition to its role in recirculation injury.

Uric acid has been studied as an antioxidant agent [3]. The localization of xanthine oxidase in epithelial cells suggests that uric acid also functions as such in the airway surface [22], which is rich in oxidative stress. This view may also pertain to all the external and internal body surfaces in which xanthine oxidase is present.

Xanthine oxidase is also widely distributed in exocrine glandular cells of various organs, such as the gastrointestinal tract, skin, breast, and kidney. In the kidney, renal tubules differed strikingly from glomeruli in staining for xanthine oxidase. Although it is difficult to interpret the significance of these localizations and to explain the biological functions of xanthine oxidase in glandular tissues solely on the basis of an immunohistochemical study, the findings suggest that xanthine oxidase is involved in secretory and/or absorptive processes, in addition to the above-mentioned functions.

Hellsten-Westing [9] observed the presence of xanthine oxidase in human macrophages and mast cells. In the present study, we observed the presence of xanthine oxidase in lymphocytes (mostly T-lymphocytes). These results suggest a possible role of the enzyme in the inflammatory process. Further investigation is required to clarify the role of xanthine oxidase in lymphocytes.

Table 1. *Distribution of xanthine oxidase in various human tissues*

Tissue	Immunostaining for XO	Tissue	Immunostaining for XO
Liver		Trachea	
hepatocyte		epithelium	++
zone 1 (periportal zone)	++	lamina propria	++
zone 2 (mid-zone)	+	muscle layer	+
zone 3 (pericentral zone)	++	cartilaginous cells	+
sinusoidal cell	+	Lung and Bronchus	
bile duct	+	alveolus	+
Tongue		bronchial epithelium	++
epithelium	+	Heart	
basal layer	−	myocytes	+
intermediate layer	+	coronary artery	+
superficial layer	+	Aorta	−
desquamating layer	++	Kidney	
muscle	+	glomerulus	−
Salivary gland	+	tubules	+
Esophagus		proximal tubules	+
epithelium	+	distal tubules	+
basal layer	−	collecting tubules	+
intermediate layer	+	Adrenal gland	
desquamating layer	++	cortex	
muscle	+	zona glomerulosa	−
Stomach		zona fasciculata	−
surface mucous cell	+	zona reticularis	−
gastric glands	+	medulla	−
Small intestine		Urinary bladder	−
columnar epithelium	+	transitional cells	±
goblet cells	+	Uterus	
intestinal glands	+	cervical epithelium	+
Large intestine		muscle	+
superficial epithelium	+	Ovary	−
goblet cells	+	Prostate	
lamina muscularis mucosa	+	glands	+
Pancreas		Striated muscle (diaphragm)	
acinar cells	−	muscle fiber	+
ducts	+	Skin	
islet of Langerhans	−	dermis	±
Spleen		sweat glands	++
follicle	−	Thyroid gland	−
perifollicular zone	+	Mammary gland	+
Lymph node		Cerebrum	NE
follicle	−	Cerebellum	NE
perifollicular zone	+	Spinal cord	NE
Mesentery		Lymphocytes	
mesothelial cells	−	T-lymphocytes	+
		B-lymphocytes	−

−: negative staining; ±: equivocal staining; +: positive staining; ++: strongly positive staining; NE: not examined

Results are conflicting concerning the xanthine oxidase activity in cancerous tissue. Some researchers have suggested that the activity of xanthine oxidase increases in cancerous tissue [13], whereas others have claimed that it decreases [7, 18]. This disparity may be due to the different histological types of cancerous tissue studied. In this immunohistochemical study, xanthine oxidase was not detected in breast cancer metastasis in the liver, suggesting

that absence of xanthine oxidase activity is an adaptive response allowing cancer cells to grow and proliferate, since the purine salvage pathway is accelerated in cancer tissues lacking xanthine oxidase activity.

In conclusion, although the present immunohistochemical study using polyclonal antibody against xanthine oxidase may not discriminate between the active and inactive forms of this protein, the demonstration of ubiquitous localization of xanthine oxidase in various human tissues may contribute to an understanding of the heretofore unknown pathophysiological role of this enzyme in human tissues.

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