Immunocytochemical Localization of D-Amino Acid Oxidase in the Proximal Tubule Cell of Porcine Kidney

By

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Summary: The immunocytochemical localization of D-amino acid oxidase was demonstrated in the proximal tubule cells of porcine kidney. D-amino acid oxidase was localized predominantly on the cell membrane of the brush border which could be seen on the free surface of each cell. On the inner-membrane of the apical canaliculi and large vacuoles, reaction product showing the localization of D-amino acid oxidase was demonstrated. The positive reaction was also recognized on the plasma membrane of the lateral cell surfaces and the basal infoldings. It was suggested that D-amino acid oxidase was localized on the surface of cytoplasmic membrane which seemed to have a high activity of resorption of amino acids and various substances.

D-amino acid oxidase (E.C.1.4.3.3) is classified as flavin enzyme since it requires flavin adenine dinucleotide (FAD) as coenzyme. This enzyme acts only on members of the non-naturally occurring D-series of amino acid, but has broad specificity for a number of this group. Oxidative diamination of D-amino acids is catalyzed by the enzyme and produces α-ket-acid, NH₃ and H₂O₂. Biochemically this enzyme was mostly found in liver and kidney, especially in those of the carnivora (Krebs, 1951).

In the peroxisomes of liver cells and the cells of kidney tubules, D-amino acid oxidase was detected (Baudhuin et al., 1965, Arnold et al., 1979, Hand, 1979, Suzuki, 1980).

In a previous study on the localization of D-amino acid oxidase in the pig kidney by immunofluorescent technique (Takano et al., 1970), evidence that apical cytoplasm of the proximal convoluted tubule cells and the Bowman's capsular cells showed intense fluorescence was offered. Precise localization of the enzyme, however, could not be clarified under the light microscope.

This present study provided evidence that more precise localization of this enzyme in the cells of proximal tubule was shown by immunocytochemical method.

Materials and methods

Tissue preparation: Fresh samples from the kidney of 10 pigs were fixed by immersion in Zamboni’s solution (Zamboni and de

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Martino, 1967) containing 1% glutaraldehyde for 2–4 hours at 0–4°C. The tissue blocks were trimmed into in PBS containing 10% sucrose and quickly frozen in liquid nitrogen. The frozen specimens were cut into 8–10 μm with a cryostat kept at −25°C. After the immunocytochemical staining the section were post fixed with 1% osmium tetroxide in 0.05M cacodylate buffer pH 7.4 for 30 min., dehydrated in graded ethanols and embedded in Epon-Araldite mixture. Ultrathin sections were made, mounted on the mesh copper grids and observed with a Hitachi H-800 electron microscope without any electron staining.

Antigen and Antibody: Crystalline D-amino acid oxidase of hog origin was commercially obtained from Sigma Chemical Company (Lot. 12F-0266). Anti D-amino acid oxidase IgG was raised in the rabbit and purified with the method as described previously (Takano, et al. 1970)

Preparation and purification of conjugate: The conjugation of horseradish peroxidase to γ-globulin was carried out according to the method reported by Nakane and Kawaoi (1976). The conjugate was purified by the method of Yamashita et al. (1976) using DEAE cellulose column.

Immunocytochemical staining: The frozen sections, treated with periodic acid, were incubated with the peroxidase labelled antibody for 5 hours at room temperature. After the incubation, the sections were rinsed briefly with cooled PBS containing 10% sucrose (PBS-sucrose), and were fixed in 1% glutaraldehyde solution in 0.05M phosphate buffer pH 7.2 for 30 min. at 4°C. After rinsing with PBS-sucrose, sections were pretreated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl buffer at pH 7.6 for 30 min. at 4°C, and then treated with DAB-H₂O₂ complete solution (0.05% of DAB and 0.005% of H₂O₂ in 0.05M Tris-HCl buffer at pH 7.6) for 2 to 5 min. at 4°C.

Control tests: a) As an immunologic negative control, normal rabbit serum IgG labelled with the peroxidase was applied. b) Immunocytochemical staining was done after treatment with non-labelled anti D-amino acid oxidase IgG. c) To test the activity of endogenous peroxidase, sections were incubate with in the DAB-H₂O₂ complete solution for 5 min. at 4°C.

Results

Normal morphology: The apical portion of the cells showed a conspicuous brush border on its luminal surface. Just beneath the brush border, well developed apical cytoplasm appeared to be composed of exceedingly complicate shape. There were numerous apical canaliculi and some large clear vacuoles in the apical cytoplasm. These apical canaliculi and vacuoles had filamentous inner surface coats. Perinuclear cytoplasm was occupied by numerous mitochondria appeared to be in various shape and sizes. Microbodies were also found of 0.4 to 1.0 μm in size and each of them had finely granular matrix with moderate electron density containing needle like dense crystalloid.

Immunocytochemistry: According to direct immunocytochemical method with peroxidase labelled immunoglobulin, D-amino acid oxidase was localized predominantly in the brushborder of the Bowman’s capsule and the proximal tubule. Especially, in the proximal convoluted tubule, the DAB reaction products were localized exclusively in the brushborder and apical large vacuoles (Fig. 2). The inner surface coats of the apical canaliculi and the vacuoles situated in the apical cytoplasm (Fig. 1) displayed a positive immunocytochemical reaction for D-amino acid oxidase (Fig. 2). A positive reaction was also observed in the intercellular
matrix at the junction between cells and on the lateral cell surfaces (Fig. 3 and 4). Most of the large vacuoles showing a positive reaction are in contact with the lateral and apical cell surfaces (Fig. 3 and 4). The basal infoldings consisting of outward-directed cytoplasmic processes alternating with deep grooves also appeared to have a positive reaction on their surfaces of interfoliated plasma membrane and the interfoliated spaces (Fig. 5 and 6). Stronger reaction was shown in the microbodies scattered in the cytoplasm. No positive reaction was displayed in the mitochondria and other cell organelles (Fig. 3, 4 and 5).

**Discussion**

In the previous study by immunofluorescent technique (Takano, *et al*. 1970), the D-amino acid oxidase was demonstrated in the apical cytoplasm of the cells constituting the Bowman's capsule and the proximal convoluted tubule. Although the precise localization of the enzyme had not been shown distinctly, it was discussed that the place where D-amino acid oxidase was situated were coincident with the place where ammonia was eliminated. Biochemically, D-amino acid oxidase activity was demonstrated exclusively in the microbody isolated from rat liver (Baudhuin *et al.*, 1965) and pig kidney cortex (Suzuki, 1980). On the other hand, the enzyme was localized ultrastructurally not only in the microbody but also in the brushborder, apical canaliculi and on the lateral cell membrane, by means of immunocytochemical method (the present study) and cytochemical method using cerous chloride procedure (Miyazaki, *et al*. 1985). The cerium method for D-amino acid oxidase recently developed by Briggs *et al*. (1975) for the demonstration of NADH-oxidase. Veehuis and Bonga (1977) have demonstrated a cytochemical localization of D-amino acid oxidase in the microbodies of teleost kidney cell by the cerium method using D-alanine as a substrate. Cytochemically, D-amino acid oxidase was demonstrably enzyme when kojic acid was provided as inhibitor, a known competitive D-amino acid oxidase inhibitor, and when D-amino acids as substrate. It is well known that D-amino acid oxidase and other hydrogen peroxidase-producing oxidase are contained in microbody (peroxisome) as peroxisomal oxidases (Veenhuis and Bonga, 1976, Weimar and Neims, 1977, Arnold *et al*. 1979, Hand, 1979). However, subcellular localization of D-amino acid oxidase excepting in the microbody has been disputed. In the leukocyte the enzyme is found in the granule fraction (Cline and Lehrer, 1969) and it is in the soluble fraction and microsome fraction (Eckstein *et al*. 1971). On the other hand, Robinson *et al*. (1978) described that D-amino acid oxidase is localized on the cell surface and in the phagosome of human polymorphonuclear leukocytes, by using the cerium method. In the present study, D-amino acid oxidase was demonstrable on the surface of the brushborder, lateral membrane and basal infolding as shown in Fig. 3, 4, 5 and 6. Moreover, this immunocytochemical results were displayed in the same appearance in the mongolian gerbil kidney depending upon the cerium method (Miyazaki, *et al*. 1985). However, in the salivary acinar cells, lacrimal gland cells and sweat gland cells, no reaction product was found on the surface of cell membrane by the cerium method. Although the meaning of distribution of D-amino acid oxidase in the cell membrane is uncertain, it has been suggested that D-amino acid oxidase is localized in the cytoplasmic membrane which has a high absorbent activity of various substances, such as the surfaces of leukocyte membrane and phagosome membrane (Robinson, *et al*. 1978), or the brush-border and the basal infolding of kidney.
The localization of D-amino acid oxidase was displayed in different appearances depending on whichever was the method, either the cerium method or the ferricyanide method. However, the reason for the difference was not known (Robinson, et al. 1978). While in kidney there was no discrepancy between the cases where the cerium technique and the immunocytochemical procedure was used respectively. Then it seemed to be confirmed that D-amino acid was localized on the plasma membrane of the proximal tubule cells of pig and mongolian gerbil kidney. The present finding that D-amino acid oxidase is distributed on the cell surface of proximal tubule cells should be developed in further confirmation that the plasma membrane is involved in peroxide formation.

References

PLATES
Explanation of Figures

Bb: Brush border  
V: Vacuole  
N: Nucleus  
MB: Microbody  
M. Mitochondria  
BI: Basal infolding  
AC: Apical canaliculus  
ICS: Inter cellular space  
BL: Basement lamina

Plate I

Fig. 1. Normal structure of the apical portion of the proximal tubule cells. Well developed apical canaliculi and vacuole which have filamentous inner surface coats are recognized. X15,000
Plate II

Fig. 2 and 3. Localization of D-amino acid oxidase in the supra-nuclear cytoplasm. Dense reaction products are seen on the brushborder, inner surface of large vacuoles and inter cellular space.  
Fig. 2 $\times 15,800$, Fig. 3. $\times 12,500$
Plate III

Fig. 4. Immunocytochemical staining for D-amino acid oxidase localization. The apical canaliculi (arrows) and large vacuoles located near the lateral cell wall display a positive immunocytochemical reaction. Mitochondria have no positive reaction. X 18,000
Plate IV

Fig. 5. Localization of D-amino acid oxidase in the basal cytoplasm of proximal tubule cell. The microbody appear to have a strong positive reaction. Reaction products are also observed in the basal infolding. × 17,500

Fig. 6. The basal infolding showing dense reaction product. The spaces between the cell membranes of the infoldings occupy the positive immunocytochemical reaction products. × 22,500