Immunohistochemical Localization of D-Aspartate in Islets of Langerhans

Miki HIASA and Yoshinori MORIYAMA*

Laboratory of Membrane Biochemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences; Okayama 700–8530, Japan.
Received February 15, 2006; accepted March 7, 2006; published online March 14, 2006

D-Aspartate is a putative modulator of neuroendocrine functions, and is present in various neuroendocrine cells as well as the central nervous system. Here we show that the islet of Langerhans is a D-aspartate-containing endocrine organ. Immunohistochemical analysis with specific antibodies against D-aspartate indicated that D-aspartate is present in all islet cells, and predominantly present in α cells and a subpopulation of F-cells. Since these cells are glutamatergic in nature, it is possible that D-aspartate is involved in the glutamate signaling pathways in the islets.

Key words D-aspartate; islets of Langerhans; α cell; β cell; insulin

Although the L-enantiomers of amino acids are predominant in living organisms, substantial levels of D-amino acids have been detected in mammals [reviewed in 1]. In particular, D-aspartate is present in the central nervous system and various neuroendocrine cells.[1,2] Specific antibodies against D-aspartate revealed the localization of the amino acid in a subset of satellite and basket cells in the cerebellum, adrenal chromaffin cells, pituitary glands, Leydig cells and pinealocytes.[2—10] D-Aspartate is believed to modulate neuroendocrine functions in either an intercellular or intracellular manner. In pinealocytes, D-aspartate is synthesized, *de novo*, localized in the cytoplasm, and released through a Na^+-dependent glutamate/aspartate transporter at the plasma membrane.[6,10] Then, D-aspartate may act as an intercellular messenger and inhibit melatonin synthesis in a receptor-mediated manner.[10] Pheochromocytoma PC12 cells also synthesize D-aspartate *de novo*.[5] Then, PC12 cells store D-aspartate in dopamine-containing secretory granules, and secreted it through exocytosis.[11] In Leydig cells, D-aspartate may act as an intracellular messenger, and stimulate testosterone synthesis through modulation of steroidogenesis.[3,12] In pituitary cells, internal D-aspartate is metabolically converted to *N*-methyl-D-aspartate (NMDA). The resultant NMDA in turn stimulates the secretion of hypothalamic releasing hormones.[8] Thus, the modes of action of D-aspartate in neuroendocrine cells seem to be diverse in nature but remains little understood.

To elucidate the overall signaling pathway for D-aspartate in mammals, we have explored the novel site of D-aspartate. Using immunohistochemical approaches involving D-aspartate specific antibodies, we found that the islets of Langerhans, a miniature endocrine organ for blood glucose-regulating hormones, contain D-aspartate.

MATERIALS AND METHODS

Antibodies Antibodies against D-aspartate were prepared according to Schell *et al.* as described.[2,6,11] The antiserum was further purified by affinity chromatography on CNBr-activated Sepharose 4B conjugated with D-aspartate. The purified antibodies were divided into small portions and frozen at −80 °C.[11] The specificity of the purified antibodies was examined by enzyme-linked immunosolvent assay. The antibodies did not cross react with L-aspartate or L-glutamate, or other related amino acids, the details being given previously.[11] Monoclonal antibodies against glucagons, insulin, somatostatin and pancreatic polypeptide were obtained from Sigma, Cymbus Biotechnology Ltd., Chemicon, and Linco Research, respectively.

Immunohistochemical Analysis Immunohistochemical analysis was performed as described[11,13] with a slight modification. Male Wistar rats were used at 7 postnatal weeks in the study. Rats were anesthetized with ether and then perfused intracardially with saline, followed by 0.2% picric acid and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Then, their both pancreases and pineal glands were isolated, and then the frozen sections were prepared.[13] Specimens were incubated in phosphate-buffered saline containing bovine serum albumin at 1% and goat serum at 2% containing 0.2% saponin to permeabilize the cellular and organelle membranes for 15 min. Then, specimens were reacted with antibodies at 0.7 μg/ml diluted in phosphate-buffered saline containing 0.5% bovine serum albumin for 1 h at room temperature. Specimens were washed four times with phosphate-buffered saline and then reacted with the second antibodies for 1 h at room temperature, and washed seven times with phosphate-buffered saline. The second antibodies used were Alexa Fluor 568-labeled anti-mouse IgG at 2 μg/ml, Alexa Fluor488-labeled anti-rabbit IgG at 4 μg/ml, FluoroLink Cy3-labeled goat anti-rat IgG at 0.5 μg/ml, FluoroLink Cy3-labeled goat anti-rabbit IgG at 2 μg/ml, or FluoroLink FITC-labeled goat anti-guinea pig IgG at 10 μg/ml. These second antibodies were obtained from Molecular Probes. Finally, immunoreactivity was examined under either an Olympus FV300 confocal laser microscope or an Olympus BH-2 fluorescence microscope.

RESULTS AND DISCUSSION

Using immunohistochemical approaches, we have examined novel D-aspartate-containing neuroendocrine cells. We found that significant immunoreactivity is present in the islets of Langerhans (Fig. 1A). D-Aspartate immunoreactivity is predominantly present in the peripheral region. Weak signals were obtained in the mantle region of the islets. The exocrine part of the pancreas exhibits only a background level of D-aspartate immunoreactivity (Fig. 1A). The immunoreactivity was not observed when preabsorbed antibodies were

* To whom correspondence should be addressed. e-mail: moriyama@pheasant.pharm.okayama-u.ac.jp © 2006 Pharmaceutical Society of Japan

used (Fig. 1B). The D-aspartate immunoreactivity in the pineal gland was also shown, as a positive control (Figs. 1C, D)

Islets of Langerhans constitute at least four major types of endocrine cells, glucagon-secreting α cells, insulin-secreting β cells, somatostatin-secreting δ cells and pancreatic polypeptide-secreting F cells. Double-labeling immunohistochemistry revealed that D-aspartate immunoreactivity is associated with glucagon, but less with insulin or somatostatin (Figs. 2A—C). D-Aspartate immunoreactivity is also partly associated with pancreatic polypeptides (Fig. 2D). These results indicated that D-aspartate is predominantly present in α cells and a subpopulation of F cells.

Recent increasing evidence shows that excitatory amino acids are involved in the regulation of hormonal secretion in islets [reviewed in ref. 14]. Islets of Langerhans use L-glutamate as a primary intercellular messenger to regulate the secretion of glucagon, followed by regulation of blood glucose[13—16]: L-glutamate is stored in glucagon-containing secretory granules in α cells, and co-secreted with glucagon upon low glucose stimulation. L-Glutamate is also present in pancreatic polypeptide-containing secretory granules in a subpolulation of F cells.[13,17] Then, the released L-glutamate

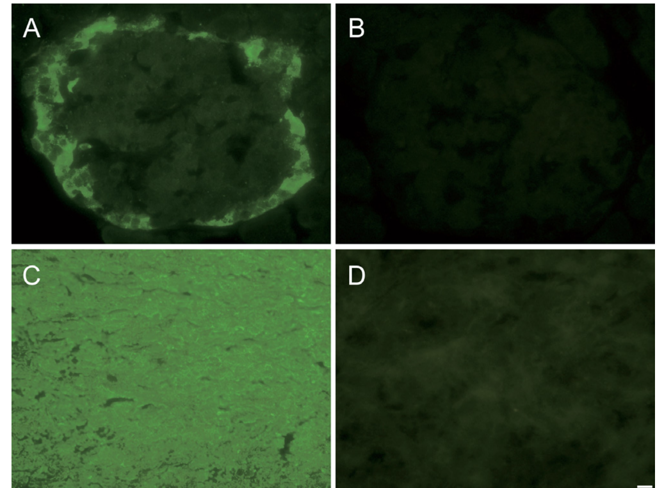

Fig. 1. Immunohistochemical Detection of D-Aspartate in Islets of Langerhans

Sections of pancreas (A, B) or pineal glands (C, D) were immunostained with antibodies against D-aspartate (A, C) or preabsorbed antibodies (B, D). The D-aspartate-specific antibodies (20 μg protein) were absorbed in the presence of 1 mM D-aspartate. Bar=10 μm.

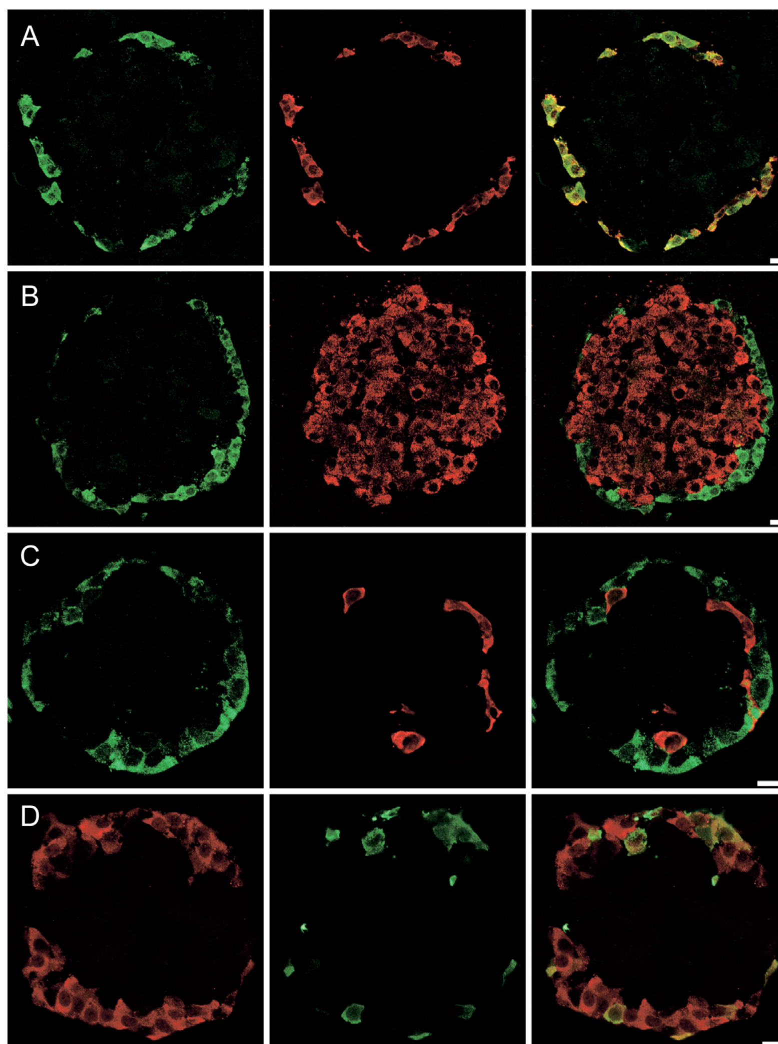

Fig. 2. Double Labeling Immunofluorescence Microscopy Revealed That D-Aspartate Is Present in α Cells and a Population of F-Cells in Islets of Langerhans

Sections of pancreas were doubly immunostained with antibodies against (A) D-aspartate (green) and glucagon (red), (B) D-aspartate (green) and insulin (red), (C) D-aspartate (green) and somatostatin (red), or (D) D-aspartate (red) and pancreatic polypeptide (green). Merged pictures are also shown. Bar=10 μm.

binds to ionotropic glutamate receptors on neighboring β and δ cells, causing the secretion of γ-aminobutylate and somatostatin, respectively.[15,16] γ-Aminobutylate (GABA) and somatostatin act as secondary intercellular transmitters in islets, and inhibit glucagon secretion in a receptor-mediated manner.[13,15] α cells also receive L-glutamate signals by way of a metabotropic glutamate receptor, which causes inhibition of glucagon secretion.[16] In the present study, we have shown that that α cells and a subpolulation of F-cells contain D-aspartate (Fig. 2). Since D-aspartate is excitatory in nature, it is possible that D-aspartate is also involved in L-glutamate-mediated intercellular- or intracellular signaling pathway in islets. It is interesting to examine whether α cells secrete D-aspartate, and whether endogenous D-aspartate inhibits glucagons secretion as in the case with L-glutamate. Such studies are currently under way in our laboratory.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1) Hashimoto A., Oka T., *Prog. Neurobiol.*, **52**, 325—353 (1997).
2) Schell M. J., Cooper O. B., Snyder S. H., *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 2013—2018 (1997).
3) D'Aniello A., Di Cosmo A., Di Cristo C., Annunzaito L., Petrucelli L., Fisher G., *Life Sci.*, **59**, 97—104 (1996).
4) Imai K., Fukushima T., Hagiwara K., Santa T., *Biomed. Chromatogr.*, **9**, 106—109 (1995).
5) Moriyama Y., Yamada H., Hayashi M., Oda T., Yamaguchi A., *Neurosci. Lett.*, **248**, 57—60 (1998).
6) Ishio S., Yamada H., Hayashi M., Yatsushiro S., Noumi T., Yamaguchi A., Moriyama Y., *Neurosci. Lett.*, **249**, 143—146 (1998).
7) Hayashi M., Itabashi T., Moriyama Y., *Neurosci. Lett.*, **267**, 37—40 (1999).
8) D'Aniello G., Tolino A., D'Aniello A., Errico F., Fisher G. H., Di Fiore M. M., *Endocrinology*, **141**, 3862—3870 (2000).
9) Lee J. A., Long Z., Nimura N., Iwatsubo T., Imai K., Homma H., *Arch. Biochem. Biophys.*, **385**, 242—249 (2001).
10) Ishio S., Yamada H., Craft C. M., Moriyama Y., *Brain Res.*, **850**, 73—78 (1999).
11) Nakatsuka S., Hayashi M., Muroyama A., Otsuka M., Kozaki S., Yamada H., Moriyama Y., *J. Biol. Chem.*, **276**, 26589—26596 (2001).
12) Nagata Y., Homma H., Lee J.-A., Imai K., *FEBS Lett.*, **444**, 160—164 (1999).
13) Hayashi M., Otsuka M., Morimoto R., Hirota S., Yatsushiro S., Takeda J., Yamamoto A., Moriyama Y., *J. Biol. Chem.*, **276**, 43400—43406 (2001).
14) Moriyama Y., Hayashi M., *Trends in Pharmacol. Sci.*, **24**, 511—517 (2003).
15) Muroyama A., Uehara S., Yatsushiro S., Echigo N., Morimoto R., Morita M., Hayashi M., Yamamoto A., Koh D.-K., Moriyama Y., *Diabetes*, **53**, 1743—1753 (2004).
16) Uehara S., Muroyama A., Echigo N., Morimoto R., Otsuka M., Yatsushiro S., Moriyama Y., *Diabetes*, **53**, 998—1006 (2004).
17) Hayashi M., Morimoto R., Yamamoto A., Moriyama Y., *J. Histochem. Cytochem.*, **51**, 1375—1390 (2003).