Biosynthesis of D-aspartate in mammalian cells

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Abstract In this communication, we demonstrate that D-aspartate (D-Asp) is synthesized in pheochromocytoma cells (PC12). To our knowledge this is the first report of biosynthesis of D-Asp in mammalian cells. Synthesis of D-Asp was demonstrated by its time-dependent accumulation in the cell culture, and by the fact that this accumulation was proportional to the number of inoculated cells. D-Asp in PC12 cells was identified by (i) co-elution with authentic D-Asp on two different HPLC columns, an octadesyl silica column and a Pirkle-type chiral column, (ii) reversed elution order of D-Asp and L-Asp on another Pirkle-type chiral column with an opposite configuration, and (iii) sensitivity to D-Asp oxidase. In the cells the amount of D-Asp was approx. 12–14% of total Asp and no other investigated D-amino acid was detected. The amount of D-Asp did not increase during the culture of mouse 3T3 fibroblasts and human neuroblastoma NB-1 cells. Immunocytochemical staining with anti-D-Asp antiserum demonstrated that D-Asp synthesized is present in the cytoplasm of the cells.

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Key words: D-Amino acid; D-Aspartate; Biosynthesis; D-Serine; PC12

1. Introduction

Recent reports have demonstrated the existence of various D-amino acids in the mammalian body [1,2]. The transient appearance of D-aspartate (D-Asp) was demonstrated during the development of a variety of tissues. Quantitative analysis by HPLC indicated that the D-Asp level was transiently increased during the development of the cerebral hemisphere of the rat brain [3,4], and of the retina and the brain of the chick embryo [5]. In humans, high concentrations of D-Asp (approx. 60% of total Asp is D-form) were found in the prefrontal cortex at gestational week 14, but this level rapidly decreased to a trace level at delivery [6]. Immunohistochemical study with anti-D-Asp antiserum prepared previously in this laboratory demonstrated that D-Asp was localized in specific types of cells in various endocrine tissues such as testis [7], adrenal [8], pineal [9] and pituitary glands [10] (Lee et al., unpublished observation) at distinct periods of development. The periods of maximal appearance of D-Asp corresponded well with those of the morphological and functional maturation in the respective tissues [4,7,11]. Concerning its physiological function(s), D-Asp is reported to have an affinity for the glutamate (Glu) binding site of the *N*-methyl-D-aspartate (NMDA) receptor [12–15] and to be involved in testosterone synthesis in rat testis [16]. These lines of evidence suggest that D-Asp is related to the regulation of the maturation and differentiation of the cells in the brain and endocrine tissues, although the details remain to be clarified.

It is clear that D-amino acids found in bacteria [17], plants [18] and the lower animals such as insects [19], prawns [20,21], crayfish [20] and bivalves [22] are synthesized by racemases or D-amino acid transaminases. However, it is not known whether D-Ser and D-Asp are synthesized in mammals, since these D-amino acids in diet can be absorbed by the intestine and transported to various tissues in mammals [1,2,23]. Neidle and Dunlop [5] observed that the amounts of D-Asp increased significantly in fertilized chicken eggs during incubation. Since the egg is a closed system without dietary supplementation, this result indicates biosynthesis of D-Asp in chicken embryos and the biosynthesis could also reasonably occur in the mammalian body. However, direct evidence of biosynthesis of D-Asp has not yet been established in mammals. Because of the predominant presence of D-Asp in medullar cells of the rat adrenal gland [4,8,10] and the transient appearance of D-Asp during ontogeny of the rat brain [3,4], we investigated the biosynthesis of D-Asp in a cell line of PC12 cells. These cells are derived from the rat adrenal medulla and can be differentiated into neuronal cells upon stimulation with various growth factors (e.g. nerve growth factor (NGF)). Hence, they possess the features of both adrenal medullar cells and of neurons. To our knowledge this is the first report demonstrating biosynthesis of D-Asp in mammalian cells. The present observation should contribute to our understanding of the D-Asp synthetic pathway and the physiological functions of D-Asp in mammalian cells.

2. Materials and methods

2.1. Materials

Mycoplasma-free PC12 cells were obtained from Rikken Cell Bank (Tsukuba, Japan) and NB-1 cells were from Health Science Research Resources Bank (Tokyo, Japan). Swiss 3T3 cells were kindly donated by Prof. T. Katada (Univ. Tokyo). A purified preparation of D-Asp oxidase from *Cryptococcus humicolus* UJ1 was obtained as described previously [24]. NGF (2.5S), trypsin, penicillin and streptomycin were obtained from Sigma (St. Louis, MO, USA). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was from Wako (Osaka, Japan). The reverse-phase column (ODS-M80 250×4.6 mm) and Pirkle-type chiral columns, Sumichiral OA2500S and OA2500R, were kindly supplied by Tosoh (Tokyo, Japan) and Sumika Chemical Analysis Service (Osaka, Japan), respectively. [4-^{14}C]-D-Asp was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Sera used in cell culture were checked to make sure that they were free of mycoplasma.

2.2. Cell culture

PC12 cells were cultured on collagen (type I)-coated polystyrene plates (Iwaki Glass, Tokyo, Japan) at 37°C under 5% CO_2 and 100% humidity. Cultured cells were passaged with 0.1% trypsin in Hanks' balanced salt solution (Gibco-BRL, New York, NY, USA). Growth culture medium consisted of Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL) containing 5% of heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) and 5% heat-inactivated horse serum (Gibco-BRL). Differential culture medium, TIP/DF, is composed of a mixture of DMEM and F12 (Gibco-BRL) (1:1) containing 1% N1 medium supplement (Sigma) and 50 ng/ml of NGF. All the media additionally contained 100 units/ml of streptomycin and 100 units/ml of penicillin.

Swiss 3T3 cells were cultured in DMEM containing 5% FBS and NB-1 cells in the mixture of RPMI-1640 (Gibco-BRL) and minimum essential medium (Gibco-BRL) (1:1) containing 10% FBS. Both cells were inoculated on 6-well polystyrene plates and the other conditions were the same as those of PC12 cells.

2.3. Sample preparation

Amino acids in the culture media were extracted with four volumes of methanol. One ml of methanol was added to the cells on the plate, followed by 10 min sonication in order to extract cellular amino acids. These methanol extracts were centrifuged at $5000 \times g$ for 10 min, and supernatants were evaporated to dryness, followed by fluorescent derivatization with NBD-F according to the method described in our
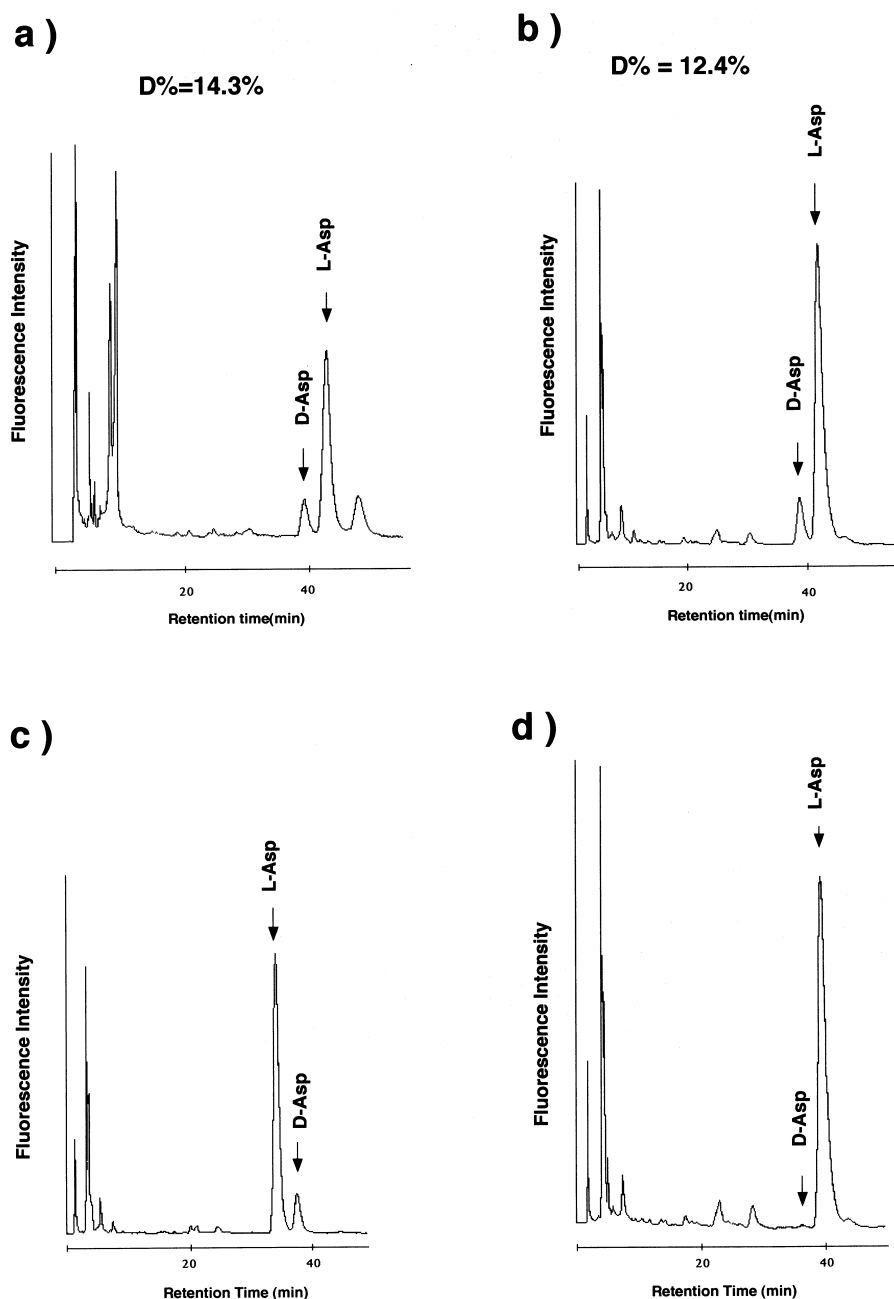

Fig. 1. Enantiomeric separation and identification of D-Asp in PC12 cells. The Asp fraction in cultured PC12 cells was isolated by reverse-phase HPLC as described in Section 2 and enantiomeric separation of the fractions was carried out by HPLC on a Pirkle-type chiral column (Sumichiral OA2500S). The cells (1.0×10^6 cells) were cultured in DMEM for 5 days (a) and in TIP/DF medium for 7 days (b). c: A fraction of b was subjected to HPLC on a OA2500R column. d: An amino acid preparation of b was pretreated with D-Asp oxidase before isolation and enantiomeric separation. Thirty-seven mU of D-Asp oxidase in 0.2 ml of phosphate buffer was used for the residue of 0.2 ml methanol extracts of the cells.

previous report [11]. Quantification of the amino acids was carried out by HPLC as described below.

In the experiment to investigate the effect of exogenous D-Asp on cellular D-Asp levels, PC12 cells were cultured in the presence of [4-^{14}C]-D-Asp. After removing the medium, cells were then washed two times with 1 ml of cold Hanks' balanced salt solution, and amino acids were extracted as described above. Portions of the extracts were used both for liquid scintillation counting and for D-Asp determination.

Enantioselective enzymatic oxidation of D-Asp was done in 100 mM sodium phosphate buffer, pH 7.5, containing 0.1% bovine serum albumin and 50 μM FAD. After the reaction, amino acids were extracted and derivatized as described above and the samples were subjected to HPLC analysis.

2.4. HPLC analysis

The determination of D,L-amino acid contents was carried out by a combination of reverse-phase HPLC and HPLC with a Pirkle-type chiral column as described in our previous report [11]. Briefly, the isolation and quantification of individual amino acids were performed by reverse-phase HPLC with fluorometric detection. Subsequently, enantiomeric separation of individual amino acids was carried out by HPLC with Sumichiral 2500S. A chiral column of an opposite chiral center (Sumichiral OA2500R) was also used for the identification of D-Asp.

2.5. Immunocytochemical staining

PC12 cells were mounted on collagen-coated glass slides. After cultivation for 2 days, cells were fixed with Karnovsky solution (2.5% glutaraldehyde, 2% paraformaldehyde and 0.1 M sodium cacodylate, pH 7.2) for over 30 min at 4°C. The fixed cells were treated with 0.5% NaBH$_4$ in PBS for 20 min, blocked with 3% BSA and 0.1% Tween 20 for 30 min and incubated overnight with anti-D-Asp antibody at room temperature. The stereospecific anti-D-Asp antibody was previously prepared in this laboratory [7,9]. The immunoreactivity (IR) was visualized by the peroxidase-antiperoxidase method using the PAP complex (Dako Japan, Tokyo, Japan). The slides were observed under a light microscope after the diamino-benzidine-H$_2$O$_2$ reaction and counterstaining with hematoxylin.

2.6. Statistic analysis

Results were expressed as mean ± standard deviation. Significant differences between groups were determined by Duncan's multiple-range test.

3. Results

3.1. D-Asp synthesis in PC12 cells

The Asp fractions isolated from PC12 cells cultured either in growth medium (Fig. 1a) or in differentiation medium (Fig. 1b) were subjected to enantiomeric separation on a chiral column. D-Asp contents were 14.3% and 12.4% of total Asp (D+L), respectively. The same fraction was also separated on another chiral column of an opposite configuration. The elution order of D,L-Asp was reversed, as shown in Fig. 1c, confirming that the peak was that of D-Asp, and not that of other possible contaminants. In addition, this peak was found to be sensitive to D-Asp oxidase treatment, an oxidative deamination enzyme with high stereospecificity for D-Asp but not for L-Asp. Fig. 1d shows that the D-Asp peak was considerably diminished to a very low level after the treatment with this enzyme, further confirming that it was that of D-Asp. No other D-amino acids such as D-alanine, -Ser, -Glu, -threonine and -proline were found in significant quantities (data not shown).

The time course of D-Asp increase was examined in the cells and the media during culture (Fig. 2). The level of D-Asp increased with the culture time, irrespective of the type of culture medium used. On the contrary, the D-Asp levels in the cultures of mouse fibroblast Swiss 3T3 cells and in human
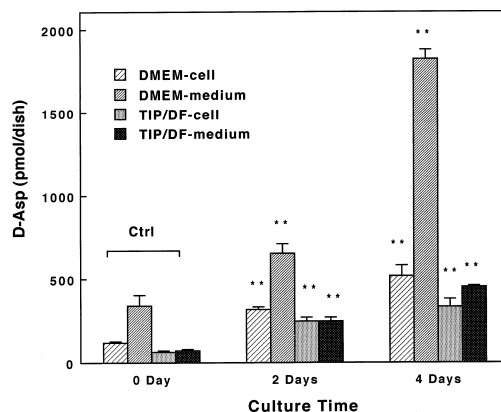

Fig. 2. Time-dependent D-Asp accumulations in the cultures of PC12. PC12 cells (7×10⁵ for DMEM, 5×10⁵ for TIP/DF) were inoculated on (6-well) collagen-coated polystyrene plates and were precultured for 1 day. Cellular contents at this stage were taken as the controls and arbitrarily defined as those of 0 day. After culture for 2 and 4 days, D-Asp contents in the cells and the media were determined. Bar: S.D. for experiments in triplicate. **: Significantly different compared to control (0 day), $P < 0.01$, $n = 3$. L-Asp levels in the cells cultured in DMEM were 6.01 ± 0.66 (0 day), 10.00 ± 0.86 (2 days) and 3.33 ± 0.65 nmol/dish (4 days), respectively, and D% (D-Asp/total Asp) in the cells were 1.95 (0 day), 3.10 (2 days) and 13.6% (4 days), respectively.

neuroblastoma NB-1 cells remained practically unchanged (D-Asp levels in 3T3 cells were 40.2 ± 6.3 (1 day), 33.8 ± 1.1 (2 days) and 28.7 ± 1.8 pmol/dish (3 days), respectively, and those in the media were 296 ± 3.5 (1 day), 253 ± 0.4 (2 days) and 251 ± 26.4 pmol/dish (3 days), respectively; data of NB-1 cells not shown).

In Fig. 3, the net increase in D-Asp in cells and media was evaluated three days after inoculation of the cultures with various numbers of PC12 cells. The amount of D-Asp in both the cells and media increased significantly during culture in TIP/DF medium, regardless of the presence of NGF. A three day incubation of cell-free medium that had previously served to cultivate PC12 cells did not result in any change in the amount of D-Asp. These lines of evidence indicate that D-Asp is synthesized in the culture of PC12 cells, since this amino acid is not supplemented during the culture.

It was possible that D-Asp is synthesized outside PC12 cells (e.g. by contaminating microorganisms) and then taken up into the cells. However, the results shown in Fig. 4 excluded this possibility. We cultivated PC12 cells in the presence of various amounts of radiolabeled D-Asp in order to investigate the effects of exogenous D-Asp on its intracellular level. Exogenous D-Asp up to the concentration of 1 μM did not affect the intracellular D-Asp level (Fig. 4). After the culture, only about 1.3–6 pmol of exogenous D-Asp was recovered from the cells, whereas an increase of more than 150 pmol of D-Asp was observed in the cells (Fig. 4). These results further indicate that biosynthesis of D-Asp takes place in PC12 cells, not outside the cells.

3.2. Localization of D-Asp in the cytoplasm of PC12 cells

The localization of D-Asp in PC12 cells was investigated by an immunocytochemical method using anti-D-Asp antiserum prepared previously in this laboratory [7–9]. Fig. 5 demonstrates that D-Asp immunoreactivity (IR) is mainly observed in the cytoplasm of PC12 cells. Although IR was apparent
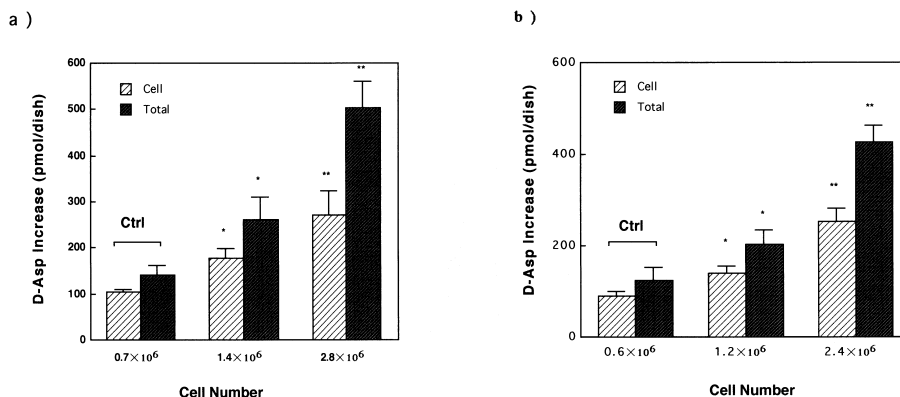
a)

b)

Fig. 3. Cell number-dependent D-Asp increase in PC12 cell cultures. Various numbers of PC12 cells were cultured for 3 days in TIP/DF medium in the presence (a) and absence (b) of NGF (50 ng/mg). The net increase of D-Asp contents in the cells and cells plus medium (total) during 3 days were presented. Bar: S.D. for experiments in triplicate. *: Significantly different compared to control, $P < 0.05$, $n = 3$; **: $P < 0.01$, $n = 3$.

over the entire cytoplasm, the intensity between the cells was somewhat different (Fig. 5). Preabsorption of antiserum with D-Asp hapten abolished IR and preimmune serum revealed no IR (data not shown).

4. Discussion

In this study, we have provided the first indication that D-Asp is actually synthesized in mammalian cells. The amount
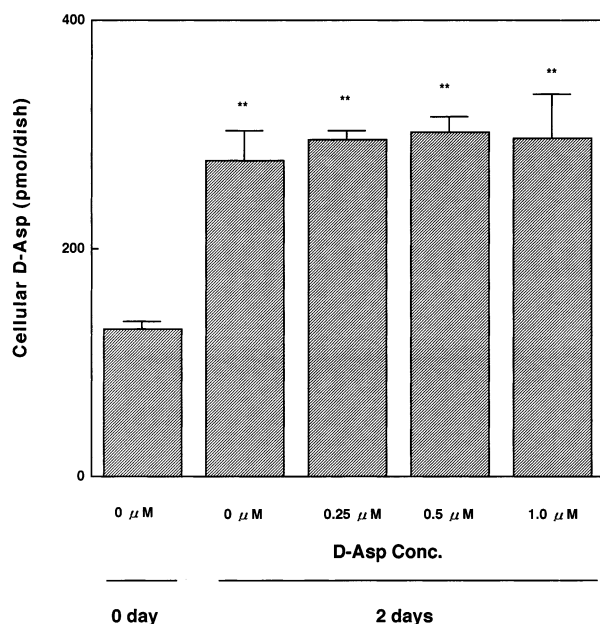

Fig. 4. Exogenous D-Asp is not taken up into the cells and has no effect on the cellular levels of D-Asp in PC12 cell cultures. PC12 cells (1×10^6 cells) were precultured in DMEM for 1 day. Cellular contents at this stage were taken as control and arbitrarily defined as those of 0 day. Culture was continued for 2 days after the media had been changed to TIP/DF containing various concentrations of radiolabeled D-Asp (0, 0.25, 0.5, 1.0 μM). The fresh medium contained approx. 25 nM D-Asp. D-Asp contents and radioactivities in cells were determined by HPLC and liquid scintillation counting as described in Section 2. After the culture, only approx. 1.3–6 pmol of the exogenous D-Asp (corresponding to about 0.3% of the radioactivity added) was recovered from the cells. Bar: S.D. for experiments in triplicate. **: Significantly different compared to control (0 day), $P < 0.01$, $n = 3$.

of D-Asp increased with the culture time of PC12 cells, and this increase was proportional to the size of the cell inoculum. D-Asp in PC12 cells was identified according to the following criteria: (i) co-migration with authentic D-Asp on two different HPLC columns, an octadesyl silica column and a subsequent Pirkle-type chiral column; (ii) reversed elution order of the D and L forms of Asp from a chiral HPLC column with an opposite chiral center; (iii) sensitivity to treatment with enantioselective D-Asp oxidase; (iv) in addition, positive immunocytochemical staining using an anti-D-Asp antiserum. Radiolabeled D-Asp in the medium was not incorporated into PC12 cells (Fig. 4). This is consistent with the previous reports that glutamate/aspartate transporter activity is not present in PC12 cells [25,26], since this transporter has an affinity for D-Asp in addition to L-Glu and L-Asp [27,28]. Fig. 4 demonstrates that a significant amount of D-Asp increased in the cells, whereas exogenous D-Asp was not taken up into PC12 cells. This result indicates that biosynthesis of D-Asp takes place in PC12 cells, not outside the cells.

The result showing that D-Asp was not increased in mouse 3T3 fibroblasts or in human neuroblastoma NB-1 cells implies that D-Asp synthesis is not ubiquitous but specific to PC12 cells. Our recent investigation indicates that D-Asp is not syn-
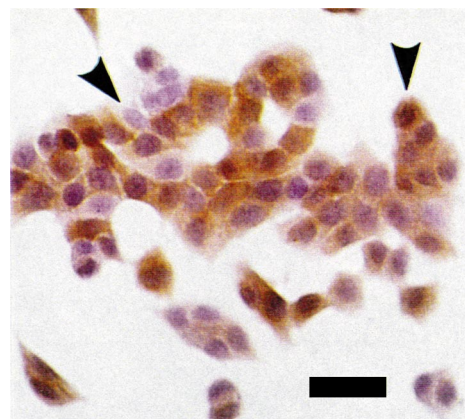

Fig. 5. Immunocytochemical staining of PC12 cells with anti-D-Asp antibody. PC12 cells cultured in DMEM for 3 days were probed with anti-D-Asp antiserum by the method described in the text. The arrows indicate two cells with different intensities. Bar: 25 μm.

thesized in the primary culture of rat pinealocytes, parenchymal cells of the pineal gland which contain significant amounts of D-Asp (Takigawa, Y. et al., unpublished observation). Since no other investigated D-amino acid was detected, PC12 cells would appear to produce D-Asp selectively. On the other hand, the present data also shows that D-Asp synthesis in PC12 cells is not affected by NGF, indicating that D-Asp is not a consequence of cell differentiation.

Immunostaining reveals that D-Asp is localized in the cytoplasm, consistent with the observations in the rat pineal [9,10], adrenal [8,10], testis [7] and brain [10]. The staining intensity of D-Asp is different between the cells as shown in Fig. 5, although details of the regulation of the cellular D-Asp level will require further investigation.

As described above, it has been reported that cloned subtypes of L-Glu transporters have affinities for D-Asp in addition to L-Glu and L-Asp [27,28]. Indeed, D-Asp administered intraperitoneally [16] or intravenously [29] is incorporated into a variety of organs such as testis, pituitary, adrenal and pineal glands, possibly via the transporters on the plasma membranes of the cells. The present data (Figs. 2 and 3) have revealed that significant amounts of the D-Asp produced in PC12 cells are released into the medium, although the details on the release mechanism remain to be investigated. We therefore presume that D-Asp is produced in certain tissue(s) of the mammalian body and transferred to other tissues via the vascular system.

As regards the synthetic pathway(s) of D-amino acids, they can be produced by racemases or D-amino acid aminotransferase (see Section 1). Recent reports [30–33] suggest that D-Ser is synthesized in the mammalian body. However, since most of these reports concern in vivo studies the exact synthetic pathway(s) of D-Ser require further clarification.

As for the synthetic pathway(s) of D-Asp in mammals, little information is available. Aspartate racemase genes have been cloned both from the archaebacterium *Desulfurococcus* strain SY [34], and from the eubacterium *Streptococcus thermophilus* [35]. Despite the evolutionary distance that separates them, both display good homology, implying that D-Asp racemase might be a common enzyme in living organisms. In this study, we demonstrated the biosynthesis of D-Asp in a mammalian cell line which should provide a useful model for the elucidation of the synthetic pathway and for genetic analysis.

References

[1] Hashimoto, A. and Oka, T. (1997) Prog. Neurobiol. 52, 325–353.
[2] Imai, K., Fukushima, T., Santa, T., Homma, H., Hamase, K., Sakai, K. and Kato, M. (1996) Biomed. Chromatogr. 10, 303–312.
[3] Dunlop, D.S., Neidle, A., McHale, D., Dunlop, D.M. and Lajtha, A. (1986) Biochem. Biophys. Res. Commun. 141, 27–32.
[4] Hashimoto, A., Oka, T. and Nishikawa, T. (1995) Eur. J. Neurosci. 7, 1657–1663.
[5] Neidle, A. and Dunlop, D.S. (1990) Life Sci. 46, 1517–1522.
[6] Hashimoto, A., Kumashiro, S., Nishikawa, T., Oka, T., Takahashi, K., Mito, T., Takashima, S., Doi, N., Mizutani, Y., Yamazaki, T., Kaneko, T. and Ootomo, E. (1993) J. Neurochem. 61, 348–351.
[7] Sakai, K., Homma, H., Lee, J.-A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T. and Imai, K. (1998) Arch. Biochem. Biophys. 351, 96–105.
[8] Sakai, K., Homma, H., Lee, J.-A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T. and Imai, K. (1997) Biochem. Biophys. Res. Commun. 235, 433–436.
[9] Lee, J., Homma, H., Sakai, K., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T., Yoshikawa, M. and Imai, K. (1997) Biochem. Biophys. Res. Commun. 231, 505–508.
[10] Schell, M.J., Cooper, O.B. and Snyder, S.H. (1997) Proc. Natl. Acad. Sci. USA 94, 2013–2018.
[11] Hamase, K., Homma, H., Takigawa, Y., Fukushima, T., Santa, T. and Imai, K. (1997) Biochim. Biophys. Acta 1334, 214–222.
[12] Monahan, J.B. and Michel, J. (1987) J. Neurochem. 48, 1699–1708.
[13] Ogita, K. and Yoneda, Y. (1988) Biochem. Biophys. Res. Commun. 153, 510–517.
[14] Olverman, H.J., Jones, A.W., Mewett, K.N. and Watkins, J.C. (1988) Neuroscience 26, 17–31.
[15] Ransom, R.W. and Stec, N.L. (1988) J. Neurochem. 51, 830–836.
[16] D'Aniello, A., Di Cosmo, A., Di Cristo, C., Annunziato, L., Petrucelli, L. and Fisher, G. (1996) Life Sci. 59, 97–104.
[17] Walsh, C.T. (1989) J. Biol. Chem. 264, 2393–2396.
[18] Ogawa, T. and Fukuda, M. (1973) Biochem. Biophys. Res. Commun. 52, 998–1002.
[19] Srinivasan, N.G., Corrigan, J.J. and Meister, A. (1965) J. Biol. Chem. 240, 796–800.
[20] Fujita, E., Okuma, E. and Abe, H. (1997) Comp. Biochem. Physiol. 116A, 83–87.
[21] Fujita, E., Okuma, E. and Abe, H. (1997) Fish. Sci. 63, 440–445.
[22] Matsushima, O., Katayama, H., Yamada, K. and Kado, Y. (1984) Mar. Biol. Lett. 5, 217–225.
[23] D'Aniello, A., D'Onofrio, G., Pischetola, M., D'Aniello, G., Vetere, A., Petrucelli, L. and Fisher, G.H. (1993) J. Biol. Chem. 268, 26941–26949.
[24] Yamada, R., Ujiie, H., Kera, Y., Nakase, T., Kitagawa, K., Imasaka, T., Arimoto, K., Takahashi, M. and Matsumura, Y. (1996) Biochim. Biophys. Acta 1294, 153–158.
[25] Ramachandran, B., Houben, K., Rozenberg, Y.Y., Haigh, J.R., Varpetian, A. and Howard, B.D. (1993) J. Biol. Chem. 268, 23891–23897.
[26] Zheng, S., Ramachandran, B., Haigh, J.R., Palos, T.P., Steger, K. and Howard, B.D. (1996) Oncogene 12, 555–562.
[27] Kanai, Y. and Hediger, M. (1992) Nature 360, 467–471.
[28] Pines, G., Danbolt, N.C., Bjørås, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E. and Kanner, B.I. (1992) Nature 360, 464–467.
[29] Imai, K., Fukushima, T., Santa, T., Homma, H., Suguhara, J., Kodama, H. and Yoshikawa, M. (1997) Proc. Jpn. Acad. Ser. B 73, 48–52.
[30] Wood, P.L., Hawkinson, J.E. and Goodnough, D.B. (1996) J. Neurochem. 67, 1485–1490.
[31] Iwama, H., Takahashi, K., Kure, S., Hayashi, F., Narisawa, K., Tada, K., Mizoguchi, M., Takashima, S., Tomita, U. and Nishikawa, T. (1997) Biochem. Biophys. Res. Commun. 231, 793–796.
[32] Takahashi, K., Hayashi, F. and Nishikawa, T. (1997) J. Neurochem. 69, 1286–1290.
[33] Dunlop, D.S. and Neidle, A. (1997) Biochem. Biophys. Res. Commun. 235, 26–30.
[34] Yohda, M., Endo, I., Abe, Y., Ohta, T., Iida, T., Maruyama, T. and Kagawa, Y. (1996) J. Biol. Chem. 271, 22017–22021.
[35] Yohda, M., Okada, H. and Kumagai, H. (1991) Biochim. Biophys. Acta 1089, 234–240.