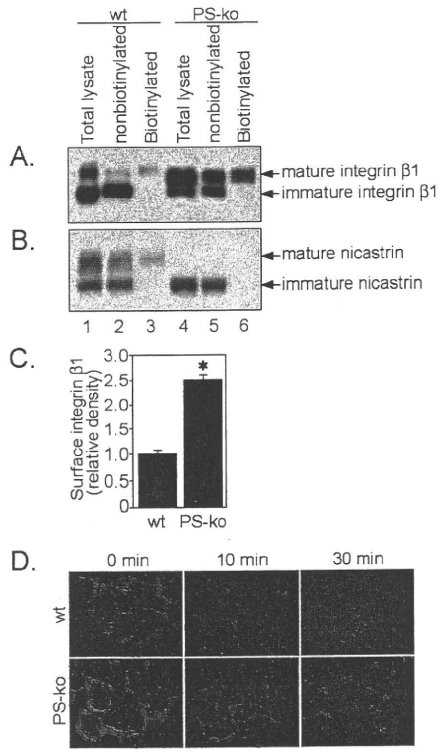

FIGURE 5: Presenilin deficiency promotes integrin β1 cell surface delivery and has no effect on integrin β1 internalization. Lysates of surface-biotinylated wt and PS-ko cells were incubated with streptavidin-agarose. Total lysate (lanes 1 and 4), nonbiotinylated (streptavidin-agarose nonbound, lanes 2 and 5), and biotinylated proteins (streptavidin-agarose bound, lanes 3 and 6) were analyzed by immunoblotting with antibodies against integrin β1 (A) and nicastrin (B). Only mature integrin β1 and nicastrin were delivered to the cell surface. The cell surface expression level of integrin β1 significantly increased in the PS-ko cells, and the relative density of integrin β1 on the cell surface was calculated (C). Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, Bonferroni/Dunn test. Living wt and PS-ko cells were labeled on ice with an antibody to integrin β1, washed, and incubated at 37 °C to initiate internalization. After 0, 10, or 30 min of incubation at 37 °C, the cells were permeabilized and stained with rhodamine-coupled goat antimouse IgG. Confocal images were taken with a Zeiss LSM 510 confocal system (D).

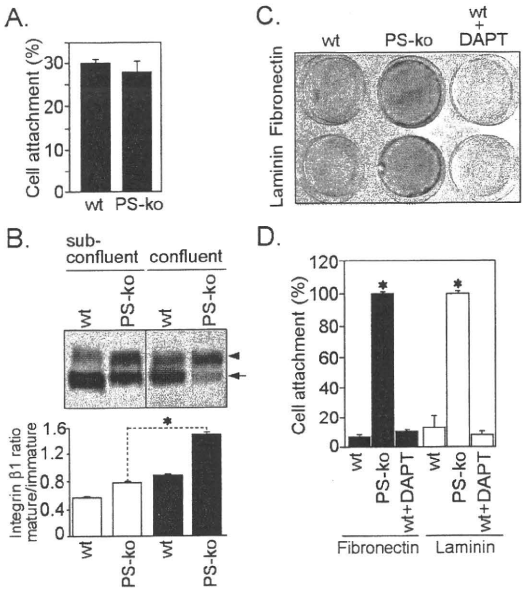

FIGURE 6: Enhanced cell adhesion to integrin β1 ligands, fibronectin and laminin in confluent PS-ko cells. The single wt and PS-ko fibroblasts prepared from subconfluent cultures were plated on fibronectin-coated 96-well plates. The plates were incubated for 30 min at 37 °C in 5% (v/v) CO_2. Unbound or loosely bound cells were removed by aspiration and gentle washing in HBS. The bound cells were fixed and stained. The percentage of bound cells was measured and calculated (A). Lysates of the subconfluent or confluent wt and PS-ko fibroblasts were subjected to SDS−PAGE and Western blotting. The confluent PS-ko fibroblasts showed enhanced integrin β1 maturation compared with the subconfluent PS-ko fibroblasts. Arrowhead, mature integrin β1; arrow, immature integrin β1; □, subconfluent fibroblasts; ■, confluent fibroblasts. Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, confluent PS-ko cells vs subconfluent PS-ko cells, Bonferroni/Dunn test (B). The wt and PS-ko fibroblasts were plated on fibronectin- or laminin-coated 6-well plates with or without 5 μM DAPT after reaching confluence, and adhesion ability was estimated. The cells were incubated with 1 mM EDTA for 30 min at room temperature. Detached cells were washed out with HBS buffer, and attached cells were stained with 0.1% (w/v) crystal violet (C). The percentage area occupied by the attached cells was measured. ■, fibronectin-coated; □, laminin-coated. Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, PS-ko cells vs wt or wt + DAPT cells, Bonferroni/Dunn test (D).

measure the ability of cell adhesion to integrin-β1-ligand-coated dishes. Resuspended wt and PS-ko single cells prepared from subconfluent cultures showed similar attachment strengths to fibronectin-coated dishes (Figure 6A). In their subconfluent states, the PS-ko cells showed a moderate increase in the expression level of mature integrin β1 compared with the wt cells. However, in their confluent states, the new PS-ko cells showed a significantly increased expression level of mature integrin β1, with the expression level ratio of mature integrin β1/immature integrin β1 increasing 2-fold that in the subconfluent PS-ko cells (Figure 6B). We then examined whether PS-ko cells exhibit stronger attachment in their confluent states. The wt and PS-ko cells were plated on fibronectin- or laminin-coated 6-well dishes and cultured until the cells reached confluence. After treatment with EDTA, detached cells were washed out, and adhering cells were stained with crystal violet. The PS-ko cells exhibited a significantly stronger attachment to the fibronectin- or laminin-coated dishes than the wt cells. DAPT, a γ-secretase inhibitor, did not enhance the adhesion of the wt cells, indicating that the stronger attachment of the PS-ko cells to fibronectin- and laminin-coated dishes was a γ-secretase-independent effect (Figure 6C and D). These results suggest that cell-surface-mature integrin β1 with an increased expression level in PS-ko cells works as a receptor of fibronectin and laminin and that this increased expression level induces a strong adhesion of the cells to the ligands of integrin β1.

Because the increased expression level of integrin β1 on the surface of PS-ko cells seems to generate multiple functional integrin heterodimers, which enhance cell adhesion, we determined whether PS regulates the maturation of integrins α1-α7, αV, and αL and whether PS1 associates with integrin β1. No integrin α1, α4, α6, α7, or αL was detected in these cells by Western blotting (data not shown). The total expression level of integrin α2 was downregulated by PS deficiency (Figure 7A). Integrin αV expression level remained unchanged in the PS-ko cells and increased in the PS1(−/−) and PS2(−/−) cells; however, PS deficiency had no effect on the maturation of integrins α2 and αV (Figure 7A). Interestingly, similar to integrin β1, integrins α3 and α5 in the PS-ko, PS1(−/−), and PS2(−/−) cells showed higher molecular weights than those in the wt cells, indicating

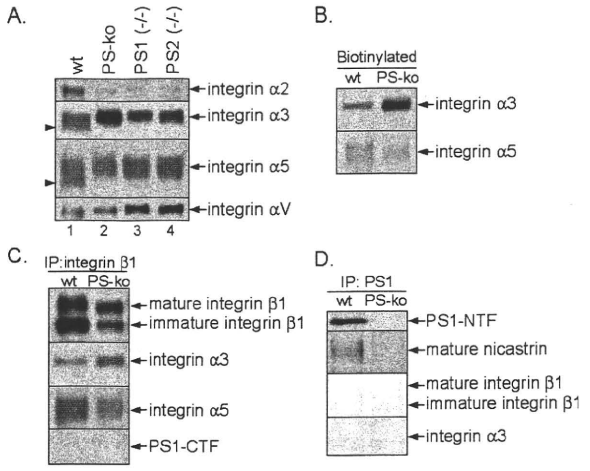

FIGURE 7: Presenilin regulates the expression, maturation and cell surface delivery of integrin α subunits. The wt, PS-ko, PS1(−/−), and PS2(−/−) cells were lysed in RIPA buffer after reaching confluence. Western blots of 50 μg of total protein from the cells were probed with anti-α2, anti-α3, anti-α5, and anti-αV integrin antibodies. Arrowheads indicate immature integrins α3 and α5; arrows indicate mature integrins α2, α3, α5, and αV (A). Biotinylated cell surface proteins of the wt and PS-ko cells were probed with anti-integrin α3 and α5 antibodies. Arrows indicate cell surface mature integrins α3 and α5 (B). Total cell lysates of wt and PS-ko cells were immunoprecipitated with a polyclonal anti-integrin β1 antibody and probed with monoclonal antibodies to integrin β1, α3, and PS1 and with a polyclonal antibody to integrin α5. Mature integrins α3 and α5 were coimmunoprecipitated with integrin β1 (C). Total cell lysates of wt and PS-ko cells were immunoprecipitated with a polyclonal anti-PS1 antibody and probed with polyclonal antibodies to PS1 and nicastrin and with monoclonal antibodies to integrin β1 and α3 (D). Mature nicastrin was coimmunoprecipitated with PS1, whereas integrins β1 and α3 were not.

that their maturation was enhanced (Figure 7A). We used cell-surface biotinylation to examine the cell-surface delivery of integrins α3 and α5. As expected, only mature integrins α3 and α5 with high molecular weights were delivered to the cell surface; moreover, the expression level of integrin α3 on the cell surface increased in the PS-ko cells, whereas that of integrin α5 remained unchanged (Figure 7B). We determined whether integrin β1 forms heterodimers with integrins α3 and α5 using immunoprecipitation analysis. Integrins α3 and α5 were coimmunoprecipitated with integrin β1, indicating the formation of functional heterodimers of integrins α3β1 and α5β1 (Figure 7C). We also found that the level of coimmunoprecipitated integrin α3 in the PS-ko cells increased compared with that in the wt cells, suggesting the enhanced maturation and cell-surface delivery, and the heterodimer formation of integrins β1 and α3 in PS-ko cells (Figure 7C). PS1 was not coimmunoprecipitated by the anti-integrin β1 antibody, indicating that PS1 was not associated with integrin β1 (Figure 7C). This was also confirmed by immunoprecipitation using the anti-PS1 antibody; mature nicastrin was coimmunoprecipitated with PS1, whereas neither integrin β1 nor integrin α3 was coimmunoprecipitated with PS1 (Figure 7D). These results suggest that PS may exert an inhibitory effect on the maturation of integrins β1 and α3 via a less direct pathway.

DISCUSSION

In this study, we demonstrated that PS deficiency leads to the enhanced maturation and cell-surface delivery of integrin β1. A marked decrease in the expression level of immature integrin β1, which is partially glycosylated and localized in ER, and an increase in that of mature integrin β1 in the PS-ko cells indicate the accelerated trafficking of integrin β1 from the ER to the Golgi apparatus. These data suggest that PS proteins also exert an inhibitory effect on the trafficking of a membrane protein from the ER to the Golgi apparatus in addition to serving as an ER-resident chaperone, which was consistent with the results of previous studies showing that the loss of function of PS results in a disrupted maturation or an enhanced intracellular retardation of nicastrin, TrkB, N-cadherin, caveolin 1, and telencephalin/ICAM (*12, 16, 17, 20, 21*). Therefore, we conclude that PS regulates the trafficking of membrane proteins from the ER to the Golgi apparatus in opposite directions, that is, PS promotes or inhibits the trafficking of membrane proteins in a protein-specific manner. In agreement with our results, results of previous studies showed that APP maturation is enhanced in PS-ko cells (*15*) and that PS1 deficiency or the loss of the PS function enhances APP maturation or causes cell-surface APP accumulation (*13, 34*). Moreover, a PS1 deletion mutant (ΔM1,2) exhibits an increased cell-surface expression level of nicotinic acetylcholine receptors (AChRs) (*15*). The post-translational maturation of integrin β1 is strictly regulated by the expression of PS. The PS-ko cells showed a marked increase in the expression level of mature integrin β1, whereas the PS1(−/−) or PS2(−/−) cells exhibited an intermediate increase in that of mature integrin β1 (Figure 1).

It is reasonable to speculate that the cell surface accumulation of integrin β1 is a result of attenuated internalization or an increased expression level of integrin β1 because a previous study showed that the loss of PS1 or PS2 function induced by the mutation of one of the critical aspartate residues or by γ-secretase inhibitors results in delayed APP reinternalization and APP accumulation on the cell surface (*14*). Another study showed that an enhanced cell-surface expression level of mature APP is not accompanied by a decrease in the expression level of immature APP in PS-ko or ΔM1,2 cells, suggesting that the total expression level of APP increases in these cells (*15*). We also found that the expression levels of both mature and immature APPs are higher in PS-ko cells than in wt cells (data not shown), in agreement with the finding of a previous study. This is also the case of acetylcholine receptors (AChRs), that is, the cell-surface and total expression levels of AChRs increase in PS1-ΔM1,2 cells (*15*). In our study, the total expression level of integrin β1 in the PS-ko cells was not altered compared with that in the wt cells because the increase in the expression level of mature integrin β1 was always accompanied by a decrease in that of immature integrin β1. The localization or trafficking of integrin β1 strictly depends on the glycosylation state of integrin β1, and partially glycosylated immature integrin β1 forms a stable pool within the ER (*26, 35, 36*). Subcellular fractionation studies showed an enhanced maturation of integrin β1 in Golgi apparatus fractions (Figure 3). Moreover, mature integrin β1 was modified by sialic acid (Figure 2). Note that the modification of complex N-linked oligosaccharides by sialic acid occurs within the Golgi apparatus (*37*). Finally, we demonstrated that the internalization of mature integrin β1 in PS-ko cells is unchanged compared with that in wt cells. Thus, these

lines of evidence suggest that an increase in the expression level of mature integrin β1 in PS-ko cells is specifically caused by the accelerated trafficking of integrin β1 from ER to the Golgi apparatus, not by an increase in integrin β1 total expression level or the delayed internalization of mature integrin β1.

PS can regulate cell adhesion via its γ-secretase substrates, such as E-cadherin, N-cadherin, CD44, and the voltage-gated sodium channel β2-subunit, and via the regulation of β-catenin and telencephalin turnover (*9, 29, 38*). Here, we demonstrated that PS regulates cell adhesion to integrin β1 ligands by modulating integrin β1 maturation and cell-surface delivery, although whether integrin β1 is a substrate of PS remains to be elucidated. We also provided evidence that the inhibition of γ-secretase activity does not enhance the expression level of mature integrin β1 or the adhesion of wt cells to fibronectin or laminin. The results of our study and previous studies show that PS1 D257A or PS1 D385A lacking γ-secretase activity expressed in a PS-null background restores nicastrin maturation; however, these mutants did not restore the expression of mature integrin β1. Because PS1 aspartate mutants do not form the mature, high molecular weight PS complexes (*30*), these results suggest that the formation of a high molecular weight PS complex is necessary for its inhibitory effect on integrin β1 maturation. Another possibility is that the loss of both the γ-secretase activity and the chaperone protein function of PS may be required to facilitate integrin β1 maturation because enhanced integrin β1 maturation was only found in PS-deficient cells.

Integrin β1 associates with multiple α-subunits to form integrin heterodimers that show cell adhesion activity (*31–33*). Our results suggest that an increase in the expression level of integrin β1 results in enhanced cell adhesion to integrin β1 ligands, fibronectin and laminin. We postulated that some integrin α-subunits are also regulated by PS deficiency. We estimated the maturation of some integrin α-subunits and observed increases in the cell-surface expression level of mature α3 subunits in PS-ko cells. Mature integrin α3 coimmunoprecipitated with integrin β1 also showed an increase in expression level in the PS-ko cells. However, previous studies have consistently shown that integrin $\alpha3\beta1$ is a strong receptor for laminin-5, laminin-10, and laminin-11 but that it mediates cell adhesion to fibronectin or laminin-1 either very poorly or not at all (*39, 40*). Some studies showed that integrin $\alpha3\beta1$ inhibits the activities of receptors for these ligands through transdominant inhibition (*41, 42*). Therefore, although the integrin $\alpha3\beta1$ heterodimer showed an increase in expression level in the PS-ko cells, the stronger adhesion of the PS-ko cells may have been due to changes in the expression levels of other integrin heterodimers. In agreement with the results of previous studies, it was found that mature nicastrin associates with PS1 and that immature nicastrin is impeded in the ER of PS-ko cells, suggesting that nicastrin maturation is directly mediated by PS. We did not find a physical interaction between PS1 and integrins β1 or α3. These results suggest that the maturation of integrins β1 and α3 is inhibited by PS via a less direct pathway, which may be activated by the absence of PS. The maturation of integrins β1 and α3 is likely regulated via the same pathway through their interaction.

PS mutants play an important role in the neurodegeneration of familial Alzheimer's disease; however, the mechanism of such neurodegeneration is not yet fully understood. Although the overexpression of human FAD PS1 mutants did not affect integrin β1 maturation in the wt cells, the chronic effects of PS1 mutants on the maturation of integrins need to be investigated in vivo. Recently, some roles of integrin β1 in the nervous system have been identified. Conditional integrin β1 gene deletion in neural crest cells leads to a delayed migration of Schwann cells and induces multiple defects in spinal nerve arborization and morphology (*43*). Interestingly, a postnatal forebrain and excitatory neuron-specific knockout of the integrin β1 mouse model shows impaired hippocampal AMPA receptor-dependent synaptic transmission, synaptic plasticity, and working memory (*44*). These studies suggest that integrin β1 is important for nervous system development and serves as a regulator of synaptic glutamate receptor functions and working memory. Thus, our results suggest that modulation of integrin β1 maturation by PS plays a role in nervous system development and memory.

ACKNOWLEDGMENT

We thank Dr. Bart De Strooper for providing the wt, PS-ko, PS1(−/−), and PS2(−/−) mouse embryonic fibroblast (MEF) cell lines.

REFERENCES

1. Hardy, J., and Selkoe, D. J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science 297*, 353–356.
2. St George-Hyslop, P. H. (2000) Genetic factors in the genesis of Alzheimer's disease. *Ann. N.Y. Acad. Sci. 924*, 1–7.
3. De Strooper, B. (2003) Aph-1, Pen-2, and nicastrin with presenilin generate an active gamma-secretase complex. *Neuron 38*, 9–12.
4. Lemere, C. A., Lopera, F., Kosik, K. S., Lendon, C. L., Ossa, J., Saido, T. C., Yamaguchi, H., Ruiz, A., Martinez, A., Madrigal, L., Hincapie, L., Arango, J. C., Anthony, D. C., Koo, E. H., Goate, A. M., Selkoe, D. J., and Arango, J. C. (1996) The E280a presenilin 1 Alzheimer mutation produces increased a beta 42 deposition and severe cerebellar pathology. *Nat. Med. 2*, 1146–1150.
5. Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and app mutations linked to familial Alzheimer's disease. *Nat. Med. 2*, 864–870.
6. Sisodia, S. S., Kim, S. H., and Thinakaran, G. (1999) Function and dysfunction of the presenilins. *Am. J. Hum. Genet. 65*, 7–12.
7. Kopan, R., and Ilagan, M. X. (2004) Gamma-secretase: proteasome of the membrane? *Nat. Rev. Mol. Cell Biol. 5*, 499–504.
8. Marjaux, E., Hartmann, D., and De Strooper, B. (2004) Presenilins in memory, Alzheimer's disease, and therapy. *Neuron 42*, 189–192.
9. Thinakaran, G., and Parent, A. T. (2004) Identification of the role of presenilins beyond Alzheimer's disease. *Pharmacol. Res. 50*, 411–418.
10. Edbauer, D., Winkler, E., Haass, C., and Steiner, H. (2002) Presenilin and nicastrin regulate each other and determine amyloid beta-peptide production via complex formation. *Proc. Natl. Acad. Sci. U.S.A. 99*, 8666–8671.
11. Herreman, A., Van Gassen, G., Bentahir, M., Nyabi, O., Craessaerts, K., Mueller, U., Annaert, W., and De Strooper, B. (2003) Gamma-secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. *J. Cell Sci. 116*, 1127–1136.
12. Leem, J. Y., Vijayan, S., Han, P., Cai, D., Machura, M., Lopes, K. O., Veselits, M. L., Xu, H., and Thinakaran, G. (2002) Presenilin 1 is required for maturation and cell surface accumulation of nicastrin. *J. Biol. Chem. 277*, 19236–19240.

13. Cai, D., Leem, J. Y., Greenfield, J. P., Wang, P., Kim, B. S., Wang, R., Lopes, K. O., Kim, S. H., Zheng, H., Greengard, P., Sisodia, S. S., Thinakaran, G., and Xu, H. (2003) Presenilin-1 regulates intracellular trafficking and cell surface delivery of beta-amyloid precursor protein. *J. Biol. Chem. 278*, 3446–3454.
14. Kaether, C., Lammich, S., Edbauer, D., Ertl, M., Rietdorf, J., Capell, A., Steiner, H., and Haass, C. (2002) Presenilin-1 affects trafficking and processing of betaapp and is targeted in a complex with nicastrin to the plasma membrane. *J. Cell Biol. 158*, 551–561.
15. Leem, J. Y., Saura, C. A., Pietrzik, C., Christianson, J., Wanamaker, C., King, L. T., Veselits, M. L., Tomita, T., Gasparini, L., Iwatsubo, T., Xu, H., Green, W. N., Koo, E. H., and Thinakaran, G. (2002) A role for presenilin 1 in regulating the delivery of amyloid precursor protein to the cell surface. *Neurobiol. Dis. 11*, 64–82.
16. Naruse, S., Thinakaran, G., Luo, J. J., Kusiak, J. W., Tomita, T., Iwatsubo, T., Qian, X., Ginty, D. D., Price, D. L., Borchelt, D. R., Wong, P. C., and Sisodia, S. S. (1998) Effects of Psl deficiency on membrane protein trafficking in neurons. *Neuron 21*, 1213–1221.
17. Uemura, K., Kitagawa, N., Kohno, R., Kuzuya, A., Kageyama, T., Chonabayashi, K., Shibasaki, H., and Shimohama, S. (2003) Presenilin 1 is involved in maturation and trafficking of N-cadherin to the plasma membrane. *J. Neurosci. Res. 74*, 184–191.
18. Gowrishankar, K., Zeidler, M. G., and Vincenz, C. (2004) Release of a membrane-bound death domain by gamma-secretase processing of the P75ntr homolog Nradd. *J. Cell Sci. 117*, 4099–4111.
19. Nyabi, O., Bentahir, M., Horre, K., Herreman, A., Gottardi-Littell, N., Van Broeckhoven, C., Merchiers, P., Spittaels, K., Annaert, W., and De Strooper, B. (2003) Presenilins mutated at Asp-257 or Asp-385 restore Pen-2 expression and nicastrin glycosylation but remain catalytically inactive in the absence of wild type presenilin. *J. Biol. Chem. 278*, 43430–43436.
20. Wood, D. R., Nye, J. S., Lamb, N. J., Fernandez, A., and Kitzmann, M. (2005) Intracellular retention of caveolin 1 in presenilin-deficient cells. *J. Biol. Chem. 280*, 6663–6668.
21. Annaert, W. G., Esselens, C., Baert, V., Boeve, C., Snellings, G., Cupers, P., Craessaerts, K., and De Strooper, B. (2001) Interaction with telencephalin and the amyloid precursor protein predicts a ring structure for presenilins. *Neuron 32*, 579–589.
22. Herreman, A., Hartmann, D., Annaert, W., Saftig, P., Craessaerts, K., Serneels, L., Umans, L., Schrijvers, V., Checler, F., Vanderstichele, H., Baekelandt, V., Dressel, R., Cupers, P., Huylebroeck, D., Zwijsen, A., Van Leuven, F., and De Strooper, B. (1999) Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. *Proc. Natl. Acad. Sci. U.S.A. 96*, 11872–11877.
23. Xia, W., Zhang, J., Ostaszewski, B. L., Kimberly, W. T., Seubert, P., Koo, E. H., Shen, J., and Selkoe, D. J. (1998) Presenilin 1 regulates the processing of beta-amyloid precursor protein c-terminal fragments and the generation of amyloid beta-protein in endoplasmic reticulum and Golgi. *Biochemistry 37*, 16465–16471.
24. Shiraishi, H., Sai, X., Wang, H. Q., Maeda, Y., Kurono, Y., Nishimura, M., Yanagisawa, K., and Komano, H. (2004) Pen-2 enhances gamma-cleavage after presenilin heterodimer formation. *J. Neurochem. 90*, 1402–1413.
25. Humphries, J. D., Schofield, N. R., Mostafavi-Pour, Z., Green, L. J., Garratt, A. N., Mould, A. P., and Humphries, M. J. (2005) Dual functionality of the anti-beta1 integrin antibody, 12g10, exemplifies agonistic signalling from the ligand binding pocket of integrin adhesion receptors. *J. Biol. Chem. 280*, 10234–10243.
26. Akiyama, S. K., and Yamada, K. M. (1987) Biosynthesis and acquisition of biological activity of the fibronectin receptor. *J. Biol. Chem. 262*, 17536–17542.
27. Argraves, W. S., Suzuki, S., Arai, H., Thompson, K., Pierschbacher, M. D., and Ruoslahti, E. (1987) Amino acid sequence of the human fibronectin receptor. *J. Cell Biol. 105*, 1183–1190.
28. De Strooper, B., Van Leuven, F., Carmeliet, G., Van Den Berghe, H., and Cassiman, J. J. (1991) Cultured human fibroblasts contain a large pool of precursor beta 1-integrin but lack an intracellular pool of mature subunit. *Eur. J. Biochem. 199*, 25–33.
29. Koo, E. H., and Kopan, R. (2004) Potential role of presenilin-regulated signaling pathways in sporadic neurodegeneration. *Nat. Med. 10*, S26–S33.
30. Yu, G., Chen, F., Nishimura, M., Steiner, H., Tandon, A., Kawarai, T., Arawaka, S., Supala, A., Song, Y. Q., Rogaeva, E., Holmes, E., Zhang, D. M., Milman, P., Fraser, P. E., Haass, C., and George-Hyslop, P. S. (2000) Mutation of conserved aspartates affects maturation of both aspartate mutant and endogenous presenilin 1 and presenilin 2 complexes. *J. Biol. Chem. 275*, 27348–27353.
31. Hynes, R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell 69*, 11–25.
32. Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell 110*, 673–687.
33. Yamada, K. M., and Even-Ram, S. (2002) Integrin regulation of growth factor receptors. *Nat. Cell Biol. 4*, E75–76.
34. Kim, S. H., Leem, J. Y., Lah, J. J., Slunt, H. H., Levey, A. I., Thinakaran, G., and Sisodia, S. S. (2001) Multiple effects of aspartate mutant presenilin 1 on the processing and trafficking of amyloid precursor protein. *J. Biol. Chem. 276*, 43343–43350.
35. Jaspers, M., de Strooper, B., Spaepen, M., van Leuven, F., David, G., van den Berghe, H., and Cassiman, J. J. (1988) Post-translational modification of the beta-subunit of the human fibronectin receptor. *FEBS Lett. 231*, 402–406.
36. Lenter, M., and Vestweber, D. (1994) The integrin chains beta 1 and alpha 6 associate with the chaperone calnexin prior to integrin assembly. *J. Biol. Chem. 269*, 12263–12268.
37. Bellis, S. L. (2004) Variant glycosylation: an underappreciated regulatory mechanism for beta1 integrins. *Biochim. Biophys. Acta 1663*, 52–60.
38. Kim, D. Y., Ingano, L. A., Carey, B. W., Pettingell, W. H., and Kovacs, D. M. (2005) Presenilin/gamma-secretase-mediated cleavage of the voltage-gated sodium channel beta2-subunit regulates cell adhesion and migration. *J. Biol. Chem. 280*, 23251–23261.
39. Delwel, G. O., de Melker, A. A., Hogervorst, F., Jaspars, L. H., Fles, D. L., Kuikman, I., Lindblom, A., Paulsson, M., Timpl, R., and Sonnenberg, A. (1994) Distinct and overlapping ligand specificities of the alpha 3a beta 1 and alpha 6a beta 1 integrins: recognition of laminin isoforms. *Mol. Biol. Cell 5*, 203–215.
40. Kreidberg, J. A. (2000) Functions of alpha3beta1 integrin. *Curr. Opin. Cell Biol. 12*, 548–553.
41. Borza, C. M., Pozzi, A., Borza, D. B., Pedchenko, V., Hellmark, T., Hudson, B. G., and Zent, R. (2006) Integrin alpha3beta1, a novel receptor for alpha3. (iv) noncollagenous domain and a trans-dominant inhibitor for integrin alphavbeta3. *J. Biol. Chem. 281*, 20932–20939.
42. Hodivala-Dilke, K. M., DiPersio, C. M., Kreidberg, J. A., and Hynes, R. O. (1998) Novel roles for alpha3beta1 integrin as a regulator of cytoskeletal assembly and as a trans-dominant inhibitor of integrin receptor function in mouse keratinocytes. *J. Cell Biol. 142*, 1357–1369.
43. Pietri, T., Eder, O., Breau, M. A., Topilko, P., Blanche, M., Brakebusch, C., Fassler, R., Thiery, J. P., and Dufour, S. (2004) Conditional beta1-integrin gene deletion in neural crest cells causes severe developmental alterations of the peripheral nervous system. *Development 131*, 3871–3883.
44. Chan, C. S., Weeber, E. J., Zong, L., Fuchs, E., Sweatt, J. D., and Davis, R. L. (2006) Beta 1-integrins are required for hippocampal ampa receptor-dependent synaptic transmission, synaptic plasticity, and working memory. *J. Neurosci. 26*, 223–232.

BI7014508

Reviews in the Neurosciences, 19, 203-212 (2008)

Angiotensin-Converting Enzyme as a Potential Target for Treatment of Alzheimer's Disease: Inhibition or Activation?

Kun Zou[1] and Makoto Michikawa[2]

[1]*Department of Neuroscience, Faculty of Pharmaceutical Sciences, Iwate Medical University, Iwate and* [2]*Department of Alzheimer's Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi, Japan*

SYNOPSIS

The accumulation of the amyloid β-protein (Aβ), the main constituent of the 'amyloid plaque', is widely considered to be the key pathological event in Alzheimer's disease (AD). In particular, the accumulation of $Aβ_{42}$ is the central event triggering neurodegeneration. Reduction of Aβ is now a major therapeutic strategy. However, only a few patients show evidence of increased Aβ production. Thus, defects in proteases that degrade Aβ could underlie some or many cases of familial and sporadic AD. Among the Aβ degrading enzymes, namely, neprilysin (NEP), insulin-degrading enzyme (IDE), endothelin-converting enzyme (ECE) and angiotensin-converting enzyme (ACE), ACE is the most commonly targeted enzyme by inhibitors in elderly populations because it plays a central role in the regulation of blood pressure and hypertension. Genetic, pathological and biochemical studies have associated ACE with AD. This review discusses genetic, molecular and clinical studies that might help explain the relationship between ACE, hypertension, Aβ degradation and AD.

KEY WORDS

angiotensin-converting enzyme (ACE), Alzheimer's disease, amyloid β-protein (Aβ), Aβ deposition, Aβ degradation, ACE inhibitor

Accepted: 13 February, 2008

Reprint address:
Kun Zou, M.D., Ph.D.
Department of Neuroscience
Faculty of Pharmaceutical Sciences
Iwate Medical University
Yahaba, Iwate 028-3694, Japan
e-mail: kunzou@iwate-med.ac.jp

ANGIOTENSIN-CONVERTING ENZYME POLYMORPHISM AND ALZHEIMER'S DISEASE

More than half a century ago, ACE was first isolated as a 'hypertensin-converting enzyme' /83/. It plays an important role in the renin-angiotensin system (RAS), which is involved in the long-term regulation of blood pressure and blood volume in the human body. The classic view of RAS is that renin acts on angiotensinogen to yield angiotensin I, which in turn is cleaved by ACE to form the active angiotensin II. Angiotensin II is a potent vasoconstrictor and exerts its hypertensive effects by its action on two receptors /12,89/. However, ACE also degrades other functional peptides, such as the vasodilator and inflammatory peptide bradykinin, substance P, cholecystokinin, and luteinizing hormone-releasing hormone (LHRH) /86,90/. ACE is both a membrane-bound zinc metallopeptidase and a dipeptidyl carboxypeptidase, and is expressed on the surface of endothelial cells and several types of epithelial and neuroepithelial cells. The active site of ACE is found in the extracellular space, and the unbound form of ACE circulates in biological fluids, such as plasma and cerebrospinal fluid (CSF), and both types of ACE have enzymatic activity /68,82,95/. It is now well established that the brain has its own intrinsic RAS with all its components present in the central nervous system /71,87/.

Plasma ACE levels are stable when measured repeatedly in the same individual, whereas large inter-individual differences are observed /1/. In 1990, Rigat and coworkers /68/ found a polymorphism involving the insertion (I) or deletion (D)

of a 287 base-pair sequence of DNA in intron 16 of the *ACE* gene. The mean serum ACE levels in DD carriers were approximately twice those found in II genotype individuals. The I/D polymorphism accounted for approximately half (47%) of the observed variance in ACE levels in this group. Later studies showed that the involvement of the I/D polymorphism is not only limited to ACE levels in the plasma, but also to tissue ACE levels /13,16/. Despite the important role played by ACE in RAS, there is no evidence to support a link between the ACE locus and essential hypertension /34,77/. This is also confirmed in mice having 1, 2, or 3 functional copies of the gene at its normal chromosomal location. Although serum ACE activity increases progressively from the 1-copy animals to the 3-copy animals, the blood pressure of the mice did not differ significantly regardless of copy number /42/. D allele carriers were found to be associated with a higher risk of diabetic nephropathy and less physical performance than II carriers /32,53,59,73/. Some studies also suggested that the D allele is a risk factor for atherosclerosis /72/, coronary heart disease and stroke /10,47,81/, whereas it may be positively associated with longevity and affect survival into old age /22,75/.

In 1999, the I allele of the *ACE* gene was demonstrated to be associated with AD in the Cardiff, London and Belfast populations by Kehoe *et al.* /37/. In their case-control studies, positive associations were found between the presence of the I allele and AD in all the three independent case-control samples (Cardiff: OR = 2.43, 95% CI = 1.4-4.4; London: OR = 2.71, 95% CI = 1.5-4.9; Belfast: OR = 1.82, 95% CI = 1.1-3; for II/ID versus DD genotypes). Later, this association was confirmed in the Japanese population by Hu *et al.* (OR = 2.7, 95% CI = 1.3-5.6) /31/ and in the Spanish population by Alvarez *et al.* (OR = 1.28, 95% CI = 1.04-1.58) /2/. Although many studies could not confirm the findings of Kehoe *et al.* /11,20,52,74/, increasing data suggested the association of the I allele with increased susceptibility to AD /49,57,84,88,91/. These conflicting results prompted the meta-analyses of ACE studies. Elkins *et al.* conducted a meta-analysis of the associations between variants of the ACE gene and late-onset AD. They analyzed 23 independent studies, consisting of 28 independent sets of cases and controls, and the I allele was found to be associated with an increased risk of late-onset AD /18/. This was subsequently confirmed by Lehmann *et al.* in a larger meta-analysis, which included 39 samples, comprising 6,037 patients with AD and 12,099 controls, from three ethnic groups (North Europeans, South Caucasians and East Asians); D homozygotes were found to have a lower risk of AD /44/. Thus, the risk of developing AD might be related to the reduced availability of ACE, which could increase the concentrations of amyloid β-protein (Aβ). This notion is further supported by studies of haplotypes of the *ACE* gene. One synonymous-coding SNP (rs4343) proximate to I/D has been associated with AD in a combined sample of four case-control samples. Individuals with a combination of the SNPs rs4343 and rs4351 had a 45-fold higher risk of developing AD /36,51/. In contrast to this finding, the D allele of the *ACE* gene was shown to be associated with cognitive decline in the elderly /3,5,67/. Because these studies included all types of dementia and the D allele is associated with atherosclerosis and stroke, the cognitive impairment observed in the ACE D homozygotes may be related to stroke dementia /47,72,81/.

ACE AND Aβ DEGRADATION

A mechanistic link between ACE and AD was first established by Hu *et al.* /30/. Affinity-purified ACE was shown to degrade synthetic $A\beta_{1-40}$ between the Asp7-Ser8 bond *in vitro*, producing a truncated 33-residue peptide that exhibited decreased aggregation and cytotoxic potential. Later, the same group further showed that the N-domain of ACE, but not its C-domain, is responsible for the degradation of synthetic $A\beta_{1-40}$ using purified recombinant truncated proteins bearing one ACE active site /62/. In contrast, Hemming and Selkoe /28/ showed that both the N- and C-domains of ACE can degrade $A\beta_{40}$ and $A\beta_{42}$ secreted by amyloid precursor protein (APP)-transfected cells using conservative mutations to inactivate each catalytic domain in the same molecule. It is possible that the C-domain can only degrade Aβ in intact cells, but not *in vitro*. There is evidence that

each catalytic domain of ACE regulates the activity of the other /7/, suggesting that the full-length protein is required for normal substrate recognition and degradation. Furthermore, recent studies suggested that ACE can cleave at multiple sites of $A\beta_{1-42}$. MALDI-TOF-MS of the reaction mixture of ACE and $A\beta_{1-42}$ revealed several peaks with masses corresponding to those of $A\beta_{1-40}$, $A\beta_{1-35}$, $A\beta_{1-34}$, $A\beta 1_{-22}$, $A\beta_{1-20}$ and $A\beta_{1-19}$ /94/. The sites of Aβ cleaved by ACE are shown in Figure 1. Interestingly, the generation of $A\beta_{1-40}$ from $A\beta_{1-42}$ by ACE suggested the existence of a novel catalytic pathway of Aβ degradation and generation, that is, a certain portion of $A\beta_{1-40}$ may be generated from secreted $A\beta_{1-42}$ in addition to that generated by γ-secretase cleavage.

Several recent studies have shown the effects of ACE inhibitors on Aβ levels or deposition in APP transgenic mouse brain. An acute, one-shot oral administration of ACE inhibitors or an injection into the intracerebroventricle did not significantly alter brain soluble Aβ levels in wild-type and young APP transgenic mice /17,94/, indicating ACE is not involved in Aβ degradation in the brain in an acute manner. Hemming *et al.* /29/ and Zou *et al.* /94/ further showed that short-term treatment of APP transgenic mice with ACE inhibitors did not cause increased brain Aβ deposition, whereas long-term treatment induced a significant enhancement of Aβ deposition, specifically, predominant $A\beta_{1-42}$ deposition, in the aged mouse brain /94/. This suggests that a slight, non-significant increase in the levels of soluble Aβ in the brain or in the $A\beta_{1-42}/A\beta_{1-40}$ ratio as a result of ACE inhibitor treatment may lead to increased brain Aβ deposition when the mice age.

Given that $A\beta_{1-40}$ can be converted from $A\beta_{1-42}$ by ACE, it is possible that $A\beta_{40}$ deposition in the AD brain is the result of $A\beta_{42}$ conversion by ACE and other $A\beta_{1-42}$ to $A\beta_{1-40}$ converting enzymes. An early immunohistological study of sporadic and familial AD brains undertaken by Iwatsubo *et al.* /33/ showed that diffuse plaques, representing the earliest stage of Aβ deposition, were exclusively positive for $A\beta_{42}$, but completely negative for $A\beta_{40}$. Interestingly, $A\beta_{40}$ positivity appeared at the core portion of mature plaques, which are considered to be the most aged portion of Aβ plaques. This may imply the conversion of $A\beta_{40}$-positive plaques from $A\beta_{42}$-positive diffused plaques by ACE or other $A\beta_{1-42}$ to $A\beta_{1-40}$ converting enzymes. It is unlikely that $A\beta_{40}$ is selectively deposited only at the center of $A\beta_{42}$-positive diffuse plaques. This notion is supported by the findings that $A\beta_{1-40}$ can inhibit $A\beta_{1-42}$ aggregation and $A\beta_{1-42}$-induced neurotoxicity *in vitro* and inhibits Aβ deposition *in vivo* /40,93/. Furthermore, McGowan *et al.* developed transgenic models that express $A\beta_{1-40}$ or $A\beta_{1-42}$ in the absence of human APP and demonstrated that mice expressing high levels of $A\beta_{1-40}$ do not develop overt amyloid pathology /50/. Although both $A\beta_{1-42}$ and $A\beta_{1-40}$ levels were elevated coordinately in late-onset sporadic AD brains /80/, the evidence from transgenic mice did not support that high levels of $A\beta_{1-40}$ can form $A\beta_{40}$-positive plaques *in vivo* /50,55/ (Fig. 2).

AMYLOID HYPOTHESIS AND AD: DIFFERENT ROLES PLAYED BY $A\beta_{1-40}$ AND $A\beta_{1-42}$

AD is a progressive neurodegenerative disorder and the most common cause of dementia, affecting millions of men and women worldwide. It is characterized by cerebral extracellular Aβ deposits /80/. Important components of the Aβ deposits are $A\beta_{1-40}$ and $A\beta_{1-42}$, derived from APP /79/. Most AD investigators consider that the abnormal deposition of Aβ or the Aβ oligomer in the brain is crucial to AD pathogenesis, which is called the 'amyloid hypothesis'. The strongest evidence for the crucial role played by Aβ in AD pathogenesis has been the mutations that underlie familial early onset cases of the disease. All of these inherited mutations affect the processing and accumulation of Aβ. Familial Alzheimer's disease (FAD) is associated with point mutations in APP in regions that are involved in the proteolytic processing of Aβ /26,45/ and with point mutations in presenilins (PSs) 1 and 2. Mutations in either PS or APP consistently increase the relative ratio between the long ($A\beta_{1-42}$) and short ($A\beta_{1-40}$) Aβ peptides /9,76/. In addition to cases of FAD, it is suggested that trisomy of chromosome 21, where the APP gene is localized, causes the overexpression of APP and the formation of Aβ deposits in Down's syndrome /65/. In epidemiological studies,

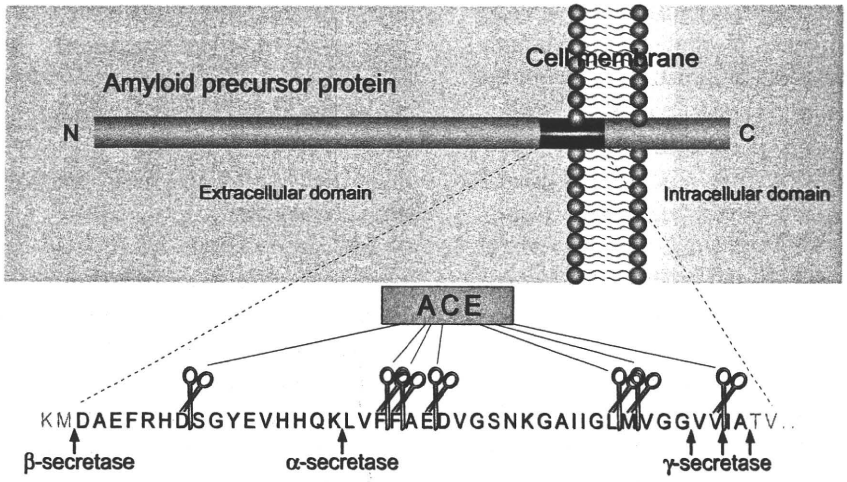

Fig. 1: Generation of amyloid β-protein (Aβ) and cleavage sites of angiotensin-converting enzyme (ACE). Aβ peptides are generated in a variety of sizes, of which the 42 amino acid form ($Aβ_{1-42}$) is thought to contribute significantly to the development of Alzheimer's disease. Aβ peptides are produced from the amyloid precursor protein (APP) by enzymatic activities known as α-, β- and γ-secretases. γ-Secretase can cleave APP at several positions, generating Aβ peptides of different lengths (38, 40 or 42 amino acids). ACE cleaves $Aβ_{1-42}$ at multiple sites. One of those cleavage sites is between 40-Val and 41-Ile, which generates $Aβ_{1-40}$ from $Aβ_{1-42}$. N = N-terminal; C = C-terminal.

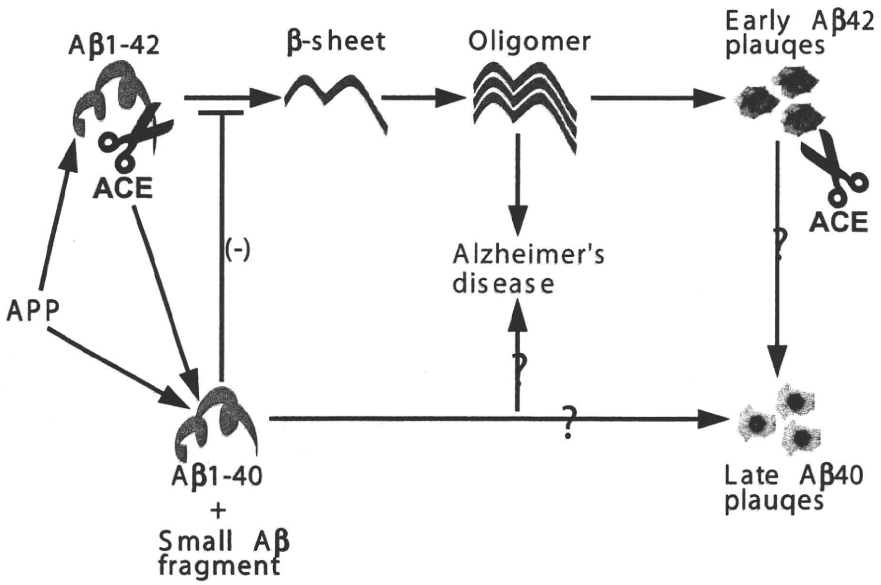

Fig. 2: Amyloidogenic processing of amyloid precursor protein (APP), formation of amyloid β-protein (Aβ) oligomers and plaques and proposed role of angiotensin-converting enzyme (ACE) in Aβ plaque formation and Alzheimer's disease (AD). Two major Aβ peptides ($Aβ_{1-40}$ and $Aβ_{1-42}$) are generated from APP. $Aβ_{1-42}$ is transformed to β-sheet structure and forms oligomers rapidly and the early stage $Aβ_{42}$-positive plaques. The oligomers of Aβ or the process of Aβ aggregation are thought to be the causes of AD. $Aβ_{1-40}$ inhibits the β-sheet transformation of $Aβ_{1-42}$ and thereby inhibits Aβ deposition *in vivo*. ACE may exert a protective effect via converting $Aβ_{1-42}$ to $Aβ_{1-40}$ or Aβ degradation. There is no evidence from *in vivo* studies showing that high levels of $Aβ_{1-40}$ can form $Aβ_{40}$-positive plaques in the brain and cause AD. Thus, in brain, the late-appearing $Aβ_{40}$-positive plaques are likely converted from the early-appearing $Aβ_{42}$-positive plaques by ACE or other $Aβ_{1-42}$ to $Aβ_{1-40}$ converting enzymes.

statistically significant relationships have been established between the Aβ plaque burden and the degree of cognitive impairment /15/, and such data have been complemented by even stronger correlations with cortical $A\beta_{42}$ levels measured biochemically /58/. Moreover, the results that the reduction in Aβ plaque burden induced by Aβ vaccination correlated with the amelioration of cognitive impairments in transgenic mice strengthened the 'amyloid hypothesis' /24/.

However, some investigators consider Aβ as a neuroprotective molecule /43/. This point of view is also supported by a number of studies. The most frequent criticism of the 'amyloid hypothesis' is that Aβ deposits are not associated with cognitive status and neurodegeneration in aging, mild cognitive impairment and AD /25,27/. Although Aβ accumulation and senile/neuritic plaque formation are striking morphological hallmarks of AD and widely used in its neuropathological diagnosis, it is clearly recognized that amyloid depositions in the brain parenchyma and blood vessels are common in non-demented individuals of advanced age /48,78/. Two recent studies showed that the biochemical alteration in cortical Aβ does not reflect the clinical diagnosis of mild cognitive impairment and AD and that the Aβ burden is not associated with brain atrophy /21,35/. In contrast to the 'amyloid hypothesis', it has been found that neuronal oxidative stress precedes Aβ deposition in Down's syndrome and AD, and the Aβ burden of the brain is negatively correlated with oxidative stress markers, suggesting an antioxidant effect of Aβ /14,60,61/. The protective effect of Aβ against lipoprotein oxidation in the CSF and plasma was reported by Kontush *et al.* /41/.

The 'amyloid hypothesis' and the 'protective amyloid hypothesis' have been proposed for nearly 20 years, and both phenomena are likely to take place simultaneously in the AD brain. $A\beta_{1-40}$ is the major soluble Aβ species in biological fluids, which represents approximately 90% of the total secreted Aβ. Finally, $A\beta_{1-42}$, although generated by neurons at a tenfold lower rate than $A\beta_{1-40}$, is the main component of amyloid plaques in the brains of patients with AD. The insoluble $A\beta_{1-42}$ level increases exponentially and steeply in an age-dependent manner, accompanied by much smaller increases in the $A\beta_{1-40}$ level /54/. Furthermore, $A\beta_{1-42}$ is deposited early and selectively in senile plaques, and this deposition is an invariant feature of all forms of AD /33/. Opposite effects of $A\beta_{1-40}$ and $A\beta_{1-42}$ on the survival of neurons were found. Monomeric $A\beta_{1-40}$ protects neurons from metal- and $A\beta_{1-42}$-induced neuronal death, whereas $A\beta_{1-42}$ exhibits neurotoxicity under physiological conditions /92,93/. $A\beta_{1-40}$, but not $A\beta_{1-42}$, rescues neurons from β- or γ-secretase inhibitor-induced cell death /66/. Moreover, some FAD presenilin (PS) mutants showed loss of function of PS, which causes a reduction in $A\beta_{1-40}$ production /6/. Two recent studies demonstrated that $A\beta_{1-42}$ is essential for parenchymal and vascular amyloid deposition in mice, and that a high $A\beta_{1-40}$ expression level inhibits Aβ deposition *in vivo* /40,50/. Thus, the 'amyloid hypothesis' and the 'protective amyloid hypothesis' might be integrated by the different roles played by $A\beta_{1-40}$ and $A\beta_{1-42}$; that is, $A\beta_{1-40}$ is neuroprotective and $A\beta_{1-42}$ is neurotoxic. In light of the neuroprotective and Aβ deposition inhibitory effects of $A\beta_{1-40}$, the inhibition of $A\beta_{1-40}$ generation or the removal/degradation of $A\beta_{1-40}$ may not be appropriate for treatment of AD. In addition to modulating the shift of γ-secretase from generating $A\beta_{1-42}$ to $A\beta_{1-40}$, the enhancement of the activities of ACE and cathepsin B, which converts $A\beta_{1-42}$ to $A\beta_{1-40}$, may be a novel treatment strategy for AD /56,94/ (Fig. 2).

ACE INHIBITORS AND AD

ACE inhibitors are widely used for the treatment of hypertension, which is a risk factor for AD. There have been very few clinical studies on the effects of ACE inhibitors on AD development and cognitive decline in patients with AD, and the results obtained to date are inconclusive /8,23,38, 39,64/. A recent study undertaken by Khachaturian *et al.* /39/, which involved more than 3,000 participants, showed the relationship of antihypertensive medication use with the incidence of AD among the elderly population. It revealed that the use of both β-blockers and diuretics is associated with the lowest risk of developing AD based on hazard ratios adjusted for age, sex, education, number of ApoE4 alleles, cholesterol, diabetes mellitus, myo-

cardial infarction, and stroke. In contrast, ACE inhibitors were shown to be the only drug class to potentially be associated with a slight increased incidence of AD (adjusted hazard ratio of 1.13) //39/. An early report by Sudilovsky *et al.* /85/ showed that ACE inhibitors appear to be ineffective in improving cognitive function in patients with AD. Louis *et al.* determined the acute effects of perindopril (an 18-week, crossover study) on cognition in elderly hypertensives, and no treatment-related changes in cognitive ability were observed /46/.

In contrast with these findings, Ohrui *et al.* showed that treatment with brain-distributed ACE inhibitors is beneficial for maintaining the cognitive ability of patients with AD with hypertension compared with non-brain-distributed ACE inhibitors or calcium channel blockers /64/. The beneficial effect of ACE seems to be limited to brain-distributed ACE inhibitors; in the analysis of individual groups (n = 4,124) of antihypertensive drugs, the incidences of AD were similar in patients administered total ACE inhibitors (2.1%), calcium-channel blockers (2.1%), β-blockers (2.6%), and diuretics (2.6%) /63/. These data are also consistent with the results of a year-long follow-up study on antihypertensive medication (ACE inhibitors in particular) undertaken by Rozzini *et al.* /70/. Individuals (n = 74) with mild cognitive impairment (MCI) who were taking ACE inhibitors were shown to have significantly more stable cognitive ability, suggesting that ACE inhibitors protect against cognitive deterioration. Although the mechanism remains unclarified, the protective effect of ACE inhibitors is likely to be attributable to their antioxidant effects /69/, vascular protective effects /19/ and direct effects on the brain RAS. Angiotensin II was shown to decrease potassium-stimulated acetylcholine (a primary neurotransmitter involved in memory) release from slices of rat entorhinal and human temporal cortex; thus, by preventing angiotensin II formation, ACE inhibitors may enhance the potassium-mediated release of acetylcholine /4/. These findings seem to disagree with studies suggesting that ACE degrades Aβ and converts $A\beta_{1-42}$ to $A\beta_{1-40}$ and that a chronic inhibition of ACE enhances $A\beta_{1-42}$ deposition in the $hAPP^{swe}$ transgenic mouse brain /28,30,62,93,94/. In addition, ACE inhibitors create an environment of reduced ACE activity that is similar to that resulting from I polymorphism, which is considered to be associated with an increased risk of AD. Taken together, there is conflicting evidence concerning the potential benefits of ACE inhibitors. Because ACE inhibitors are used globally for the treatment of hypertensive patients, who are also usually elderly people with increased risk of developing AD, further studies are required to determine the effects of long-term use of ACE inhibitors on Aβ deposition in the brain.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan (Research on Human Genome and Tissue Engineering [H17-004]), the Program for Promotion of Fundamental Studies on Health of the National Institute of Biomedical Innovation (NIBIO), Japan Society for the Promotion of Science (JSPS P04578), and Grant-in-Aid for Young Scientists (Start-up), Scientific Research (B), and Scientific Research on Priority Areas - Research on Pathomechanisms of Brain Disorders - from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18023046 and 19800040).

REFERENCES

1. Alhenc-Gelas F, Richard J, Courbon D, Warnet JM, Corvol P. Distribution of plasma angiotensin I-converting enzyme levels in healthy men: relationship to environmental and hormonal parameters. J Lab Clin Med 1991; 117: 33-39.
2. Alvarez R, Alvarez V, Lahoz CH, Martinez C, Pena J, Sanchez JM, Guisasola LM, Salas-Puig J, Moris G, Vidal JA, Ribacoba R, Menes BB, Uria D, Coto E. Angiotensin converting enzyme and endothelial nitric oxide synthase DNA polymorphisms and late onset Alzheimer's disease. J Neurol Neurosurg Psychiatry 1999; 67: 733-736.
3. Amouyel P, Richard F, Cottel D, Amant C, Codron V, Helbecque N. The deletion allele of the angiotensin I converting enzyme gene as a genetic susceptibility factor for cognitive impairment. Neurosci Lett 1996; 217: 203-205.

4. Barnes JM, Barnes NM, Costall B, Coughlan J, Kelly ME, Naylor RJ, Tomkins DM, Williams TJ. Angiotensin-converting enzyme inhibition, angiotensin, and cognition. J Cardiovasc Pharmacol 1992; 19 (Suppl 6): S63-71.
5. Bartres-Faz D, Junque C, Clemente IC, Lopez-Alomar A, Valveny N, Lopez-Guillen A, Lopez T, Cubells MJ, Moral P. Angiotensin I converting enzyme polymorphism in humans with age-associated memory impairment: relationship with cognitive performance. Neurosci Lett 2000; 290: 177-180.
6. Bentahir M, Nyabi O, Verhamme J, Tolia A, Horre K, Wiltfang J, Esselmann H, De Strooper B. Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. J Neurochem 2006; 96: 732-742.
7. Binevski PV, Sizova EA, Pozdnev VF, Kost OA. Evidence for the negative cooperativity of the two active sites within bovine somatic angiotensin-converting enzyme. FEBS Lett 2003; 550: 84-88.
8. Birkenhager WH, Forette F, Staessen JA. Dementia and antihypertensive treatment. Curr Opin Nephrol Hypertens 2004; 13: 225-230.
9. Borchelt DR, Thinakaran G, Eckman CB, Lee MK, Davenport F, Ratovitsky T, Prada CM, Kim G, Seekins S, Yager D, Slunt HH, Wang R, Seeger M, Levey AI, Gandy SE, Copeland NG, Jenkins NA, Price DL, Younkin SG, Sisodia SS. Familial Alzheimer's disease-linked presenilin 1 variants elevate Aβ1-42/1-40 ratio in vitro and in vivo. Neuron 1996; 17: 1005-1013.
10. Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D, Luc G, Bard JM, Bara L, Ricard S, et al. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. Nature 1992; 359: 641-644.
11. Chapman J, Wang N, Treves TA, Korczyn AD, Bornstein NM. ACE, MTHFR, factor V Leiden, and APOE polymorphisms in patients with vascular and Alzheimer's dementia. Stroke 1998; 29: 1401-1404.
12. Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL, et al. Identification of angiotensin II receptor subtypes. Biochem Biophys Res Commun 1989; 165: 196-203.
13. Costerousse O, Allegrini J, Lopez M, Alhenc-Gelas F. Angiotensin I-converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T-lymphocytes. Biochem J 1993; 290: 33-40.
14. Cuajungco MP, Goldstein LE, Nunomura A, Smith MA, Lim JT, Atwood CS, Huang X, Farrag YW, Perry G, Bush AI. Evidence that the beta-amyloid plaques of Alzheimer's disease represent the redox-silencing and entombment of Aβ by zinc. J Biol Chem 2000; 275: 19439-19442.
15. Cummings BJ, Pike CJ, Shankle R, Cotman CW. Beta-amyloid deposition and other measures of neuropathology predict cognitive status in Alzheimer's disease. Neurobiol Aging 1996; 17: 921-933.
16. Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. Circulation 1995; 92: 1387-1388.
17. Eckman EA, Adams SK, Troendle FJ, Stodola BA, Kahn MA, Fauq AH, Xiao HD, Bernstein KE, Eckman CB. Regulation of steady-state beta-amyloid levels in the brain by neprilysin and endothelin-converting Enzyme but not angiotensin-converting enzyme. J Biol Chem 2006; 281: 30471-30478.
18. Elkins JS, Douglas VC, Johnston SC. Alzheimer disease risk and genetic variation in ACE: a meta-analysis. Neurology 2004; 62: 363-368.
19. Enseleit F, Hurlimann D, Luscher TF. Vascular protective effects of angiotensin converting enzyme inhibitors and their relation to clinical events. J Cardiovasc Pharmacol 2001; 37 (Suppl 1): S21-30.
20. Farrer LA, Sherbatich T, Keryanov SA, Korovaitseva GI, Rogaeva EA, Petruk S, Premkumar S, Moliaka Y, Song YQ, Pei Y, Sato C, Selezneva ND, Voskresenskaya S, Golimbet V, Sorbi S, Duara R, Gavrilova S, St George-Hyslop PH, Rogaev EI. Association between angiotensin-converting enzyme and Alzheimer disease. Arch Neurol 2000; 57: 210-214.
21. Forman MS, Mufson EJ, Leurgans S, Pratico D, Joyce S, Leight S, Lee VM, Trojanowski JQ. Cortical biochemistry in MCI and Alzheimer disease: lack of correlation with clinical diagnosis. Neurology 2007; 68: 757-763.
22. Galinsky D, Tysoe C, Brayne CE, Easton DF, Huppert FA, Dening TR, Paykel ES, Rubinsztein DC. Analysis of the apo E/apo C-I, angiotensin converting enzyme and methylenetetrahydrofolate reductase genes as candidates affecting human longevity. Atherosclerosis 1997; 129: 177-183.
23. Gard PR, Rusted JM. Angiotensin and Alzheimer's disease: therapeutic prospects. Expert Rev Neurother 2004; 4: 87-96.
24. Gerlai R. Alzheimer's disease: beta-amyloid hypothesis strengthened! Trends Neurosci 2001; 24: 199.
25. Giannakopoulos P, Herrmann FR, Bussiere T, Bouras C, Kovari E, Perl DP, Morrison JH, Gold G, Hof PR. Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease. Neurology 2003; 60: 1495-1500.
26. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 1991; 349: 704-706.
27. Guillozet AL, Weintraub S, Mash DC, Mesulam MM. Neurofibrillary tangles, amyloid, and memory in aging and mild cognitive impairment. Arch Neurol 2003; 60: 729-736.
28. Hemming ML, Selkoe DJ. Amyloid beta-protein is degraded by cellular angiotensin-converting enzyme (ACE) and elevated by an ACE inhibitor. J Biol Chem

2005; 280: 37644-37650.
29. Hemming ML, Selkoe DJ, Farris W. Effects of prolonged angiotensin-converting enzyme inhibitor treatment on amyloid beta-protein metabolism in mouse models of Alzheimer disease. Neurobiol Dis 2007; 26: 273-281.
30. Hu J, Igarashi A, Kamata M, Nakagawa H. Angiotensin-converting enzyme degrades Alzheimer amyloid beta-peptide (A beta); retards A beta aggregation, deposition, fibril formation; and inhibits cytotoxicity. J Biol Chem 2001; 276: 47863-47868.
31. Hu J, Miyatake F, Aizu Y, Nakagawa H, Nakamura S, Tamaoka A, Takahash R, Urakami K, Shoji M. Angiotensin-converting enzyme genotype is associated with Alzheimer disease in the Japanese population. Neurosci Lett 1999; 277: 65-67.
32. Huang W, Gallois Y, Bouby N, Bruneval P, Heudes D, Belair MF, Krege JH, Meneton P, Marre M, Smithies O, Alhenc-Gelas F. Genetically increased angiotensin I-converting enzyme level and renal complications in the diabetic mouse. Proc Natl Acad Sci USA 2001; 98: 13330-13334.
33. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). Neuron 1994; 13: 45-53.
34. Jeunemaitre X, Lifton RP, Hunt SC, Williams RR, Lalouel JM. Absence of linkage between the angiotensin converting enzyme locus and human essential hypertension. Nat Genet 1992; 1: 72-75.
35. Josephs KA, Whitwell JL, Ahmed Z, Shiung MM, Weigand SD, Knopman DS, Boeve BF, Parisi JE, Petersen RC, Dickson DW, Jack CR Jr. Beta-amyloid burden is not associated with rates of brain atrophy. Ann Neurol 2008; 63: 204-212.
36. Kehoe PG, Katzov H, Feuk L, Bennet AM, Johansson B, Wiman B, de Faire U, Cairns NJ, Wilcock GK, Brookes AJ, Blennow K, Prince JA. Haplotypes extending across ACE are associated with Alzheimer's disease. Hum Mol Genet 2003; 12: 859-867.
37. Kehoe PG, Russ C, McIlory S, Williams H, Holmans P, Holmes C, Liolitsa D, Vahidassr D, Powell J, McGleenon B, Liddell M, Plomin R, Dynan K, Williams N, Neal J, Cairns NJ, Wilcock G, Passmore P, Lovestone S, Williams J, Owen MJ. Variation in DCP1, encoding ACE, is associated with susceptibility to Alzheimer disease. Nat Genet 1999; 21: 71-72.
38. Kehoe PG, Wilcock GK. Is inhibition of the renin-angiotensin system a new treatment option for Alzheimer's disease? Lancet Neurol 2007; 6: 373-378.
39. Khachaturian AS, Zandi PP, Lyketsos CG, Hayden KM, Skoog I, Norton MC, Tschanz JT, Mayer LS, Welsh-Bohmer KA, Breitner JC. Antihypertensive medication use and incident Alzheimer disease: the Cache County Study. Arch Neurol 2006; 63: 686-692.
40. Kim J, Onstead L, Randle S, Price R, Smithson L, Zwizinski C, Dickson DW, Golde T, McGowan E. Aβ40 inhibits amyloid deposition in vivo. J Neurosci 2007; 27: 627-633.
41. Kontush A, Berndt C, Weber W, Akopyan V, Arlt S, Schippling S, Beisiegel U. Amyloid-beta is an antioxidant for lipoproteins in cerebrospinal fluid and plasma. Free Radic Biol Med 2001; 30: 119-128.
42. Krege JH, Kim HS, Moyer JS, Jennette JC, Peng L, Hiller SK, Smithies O. Angiotensin-converting enzyme gene mutations, blood pressures, and cardiovascular homeostasis. Hypertension 1997; 29: 150-157.
43. Lee HG, Zhu X, Castellani RJ, Nunomura A, Perry G, Smith MA. Amyloid-beta in Alzheimer disease: the null versus the alternate hypotheses. J Pharmacol Exp Ther 2007; 321: 823-829.
44. Lehmann DJ, Cortina-Borja M, Warden DR, Smith AD, Sleegers K, Prince JA, van Duijn CM, Kehoe PG. Large meta-analysis establishes the ACE insertion-deletion polymorphism as a marker of Alzheimer's disease. Am J Epidemiol 2005; 162: 305-317.
45. Lendon CL, Ashall F, Goate AM. Exploring the etiology of Alzheimer disease using molecular genetics. JAMA 1997; 277: 825-831.
46. Louis WJ, Mander AG, Dawson M, O'Callaghan C, Conway EL. Use of computerized neuropsychological tests (CANTAB) to assess cognitive effects of antihypertensive drugs in the elderly. Cambridge Neuropsychological Test Automated Battery. J Hypertens 1999; 17: 1813-1819.
47. Maeda Y, Ikeda U, Ebata H, Hojo Y, Seino Y, Hayashi Y, Kuroki S, Shimada K. Angiotensin-converting enzyme gene polymorphism in hypertensive individuals with parental history of stroke. Stroke 1996; 27: 1521-1523.
48. Mann DM, Jones D, South PW, Snowden JS, Neary D. Deposition of amyloid beta protein in non-Alzheimer dementias: evidence for a neuronal origin of parenchymal deposits of beta protein in neurodegenerative disease. Acta Neuropathol (Berl) 1992; 83: 415-419.
49. Mattila KM, Rinne JO, Roytta M, Laippala P, Pietila T, Kalimo H, Koivula T, Frey H, Lehtimaki T. Dipeptidyl carboxypeptidase 1 (DCP1) and butyrylcholinesterase (BCHE) gene interactions with the apolipoprotein E ε4 allele as risk factors in Alzheimer's disease and in Parkinson's disease with coexisting Alzheimer pathology. J Med Genet 2000; 37: 766-770.
50. McGowan E, Pickford F, Kim J, Onstead L, Eriksen J, Yu C, Skipper L, Murphy MP, Beard J, Das P, Jansen K, Delucia M, Lin WL, Dolios G, Wang R, Eckman CB, Dickson DW, Hutton M, Hardy J, Golde T. Aβ42 is essential for parenchymal and vascular amyloid deposition in mice. Neuron 2005; 47: 191-199.
51. Meng Y, Baldwin CT, Bowirrat A, Waraska K, Inzelberg R, Friedland RP, Farrer LA. Association of polymorphisms in the angiotensin-converting enzyme gene with Alzheimer disease in an Israeli Arab community. Am J Hum Genet 2006; 78: 871-877.

52. Monastero R, Caldarella R, Mannino M, Cefalu AB, Lopez G, Noto D, Camarda C, Camarda LK, Notarbartolo A, Averna MR, Camarda R. Lack of association between angiotensin converting enzyme polymorphism and sporadic Alzheimer's disease. Neurosci Lett 2002; 335: 147-149.
53. Montgomery HE, Marshall R, Hemingway H, Myerson S, Clarkson P, Dollery C, Hayward M, Holliman DE, Jubb M, World M, Thomas EL, Brynes AE, Saeed N, Barnard M, Bell JD, Prasad K, Rayson M, Talmud PJ, Humphries SE. Human gene for physical performance. Nature 1998; 393: 221-222.
54. Morishima-Kawashima M, Oshima N, Ogata H, Yamaguchi H, Yoshimura M, Sugihara S, Ihara Y. Effect of apolipoprotein E allele ε4 on the initial phase of amyloid beta-protein accumulation in the human brain. Am J Pathol 2000; 157: 2093-2099.
55. Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L. High-level neuronal expression of Aβ 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. J Neurosci 2000; 20: 4050-4058.
56. Mueller-Steiner S, Zhou Y, Arai H, Roberson ED, Sun B, Chen J, Wang X, Yu G, Esposito L, Mucke L, Gan L. Antiamyloidogenic and neuroprotective functions of cathepsin B: implications for Alzheimer's disease. Neuron 2006; 51: 703-714.
57. Narain Y, Yip A, Murphy T, Brayne C, Easton D, Evans JG, Xuereb J, Cairns N, Esiri MM, Furlong RA, Rubinsztein DC. The ACE gene and Alzheimer's disease susceptibility. J Med Genet 2000; 37: 695-697.
58. Naslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, Buxbaum JD. Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. JAMA 2000; 283: 1571-1577.
59. Ng DP, Tai BC, Koh D, Tan KW, Chia KS. Angiotensin-I converting enzyme insertion/deletion polymorphism and its association with diabetic nephropathy: a meta-analysis of studies reported between 1994 and 2004 and comprising 14,727 subjects. Diabetologia 2005; 48: 1008-1016.
60. Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA. Oxidative damage is the earliest event in Alzheimer disease. J Neuropathol Exp Neurol 2001; 60: 759-767.
61. Nunomura A, Perry G, Pappolla MA, Friedland RP, Hirai K, Chiba S, Smith MA. Neuronal oxidative stress precedes amyloid-beta deposition in Down syndrome. J Neuropathol Exp Neurol 2000; 59: 1011-1017.
62. Oba R, Igarashi A, Kamata M, Nagata K, Takano S, Nakagawa H. The N-terminal active centre of human angiotensin-converting enzyme degrades Alzheimer amyloid beta-peptide. Eur J Neurosci 2005; 21: 733-740.
63. Ohrui T, Matsui T, Yamaya M, Arai H, Ebihara S, Maruyama M, Sasaki H. Angiotensin-converting enzyme inhibitors and incidence of Alzheimer's disease in Japan. J Am Geriatr Soc 2004; 52: 649-650.
64. Ohrui T, Tomita N, Sato-Nakagawa T, Matsui T, Maruyama M, Niwa K, Arai H, Sasaki H. Effects of brain-penetrating ACE inhibitors on Alzheimer disease progression. Neurology 2004; 63: 1324-1325.
65. Patterson D, Gardiner K, Kao FT, Tanzi R, Watkins P, Gusella JF. Mapping of the gene encoding the beta-amyloid precursor protein and its relationship to the Down syndrome region of chromosome 21. Proc Natl Acad Sci USA 1988; 85: 8266-8270.
66. Plant LD, Boyle JP, Smith IF, Peers C, Pearson HA. The production of amyloid beta peptide is a critical requirement for the viability of central neurons. J Neurosci 2003; 23: 5531-5535.
67. Richard F, Berr C, Amant C, Helbecque N, Amouyel P, Alperovitch A. Effect of the angiotensin I-converting enzyme I/D polymorphism on cognitive decline. The EVA Study Group. Neurobiol Aging 2000; 21: 75-80.
68. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. J Clin Invest 1990; 86: 1343-1346.
69. Rosenkranz AC, Lob H, Breitenbach T, Berkels R, Roesen R. Endothelial antioxidant actions of dihydropyridines and angiotensin converting enzyme inhibitors. Eur J Pharmacol 2006; 529: 55-62.
70. Rozzini L, Chilovi BV, Bertoletti E, Conti M, Del Rio I, Trabucchi M, Padovani A. Angiotensin converting enzyme (ACE) inhibitors modulate the rate of progression of amnestic mild cognitive impairment. Int J Geriatr Psychiatry 2006; 21: 550-555.
71. Savaskan E. The role of the brain renin-angiotensin system in neurodegenerative disorders. Curr Alzheimer Res 2005; 2: 29-35.
72. Sayed-Tabatabaei FA, Houwing-Duistermaat JJ, van Duijn CM, Witteman JC. Angiotensin-converting enzyme gene polymorphism and carotid artery wall thickness: a meta-analysis. Stroke 2003; 34: 1634-1639.
73. Sayed-Tabatabaei FA, Oostra BA, Isaacs A, van Duijn CM, Witteman JC. ACE polymorphisms. Circ Res 2006; 98: 1123-1133.
74. Scacchi R, De Bernardini L, Mantuano E, Vilardo T, Donini LM, Ruggeri M, Gemma AT, Pascone R, Corbo RM. DNA polymorphisms of apolipoprotein B and angiotensin I-converting enzyme genes and relationships with lipid levels in Italian patients with vascular dementia or Alzheimer's disease. Dement Geriatr Cogn Disord 1998; 9: 186-190.
75. Schachter F, Faure-Delanef L, Guenot F, Rouger H, Froguel P, Lesueur-Ginot L, Cohen D. Genetic associations with human longevity at the APOE and ACE loci. Nat Genet 1994; 6: 29-32.
76. Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E,

Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe D, Younkin S. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat Med 1996; 2: 864-870.

77. Schmidt S, van Hooft IM, Grobbee DE, Ganten D, Ritz E. Polymorphism of the angiotensin I converting enzyme gene is apparently not related to high blood pressure: Dutch Hypertension and Offspring Study. J Hypertens 1993; 11: 345-348.
78. Schmitt FA, Davis DG, Wekstein DR, Smith CD, Ashford JW, Markesbery WR. "Preclinical" AD revisited: neuropathology of cognitively normal older adults. Neurology 2000; 55: 370-376.
79. Selkoe DJ. Normal and abnormal biology of the beta-amyloid precursor protein. Annu Rev Neurosci 1994; 17: 489-517.
80. Selkoe DJ. Alzheimer disease: mechanistic understanding predicts novel therapies. Ann Intern Med 2004; 140: 627-638.
81. Sharma P. Meta-analysis of the ACE gene in ischaemic stroke. J Neurol Neurosurg Psychiatry 1998; 64: 227-230.
82. Sibony M, Gasc JM, Soubrier F, Alhenc-Gelas F, Corvol P. Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. Hypertension 1993; 21: 827-835.
83. Skeggs LT Jr, Kahn JR, Shumway NP. The preparation and function of the hypertensin-converting enzyme. J Exp Med 1956; 103: 295-299.
84. Sleegers K, den Heijer T, van Dijk EJ, Hofman A, Bertoli-Avella AM, Koudstaal PJ, Breteler MM, van Duijn CM. ACE gene is associated with Alzheimer's disease and atrophy of hippocampus and amygdala. Neurobiol Aging 2005; 26: 1153-1159.
85. Sudilovsky A, Cutler NR, Sramek JJ, Wardle T, Veroff AE, Mickelson W, Markowitz J, Repetti S. A pilot clinical trial of the angiotensin-converting enzyme inhibitor ceranapril in Alzheimer disease. Alzheimer Dis Assoc Disord 1993; 7: 105-111.
86. Turner AJ, Hooper NM. The angiotensin-converting enzyme gene family: genomics and pharmacology. Trends Pharmacol Sci 2002; 23: 177-183.
87. von Bohlen und Halbach O, Albrecht D. The CNS renin-angiotensin system. Cell Tissue Res 2006; 326: 599-616.
88. Wang B, Jin F, Yang Z, Lu Z, Kan R, Li S, Zheng C, Wang L. The insertion polymorphism in angiotensin-converting enzyme gene associated with the APOE epsilon 4 allele increases the risk of late-onset Alzheimer disease. J Mol Neurosci 2006; 30: 267-271.
89. Whitebread S, Mele M, Kamber B, de Gasparo M. Preliminary biochemical characterization of two angiotensin II receptor subtypes. Biochem Biophys Res Commun 1989; 163: 284-291.
90. Wright JW, Harding JW. Important role for angiotensin III and IV in the brain renin-angiotensin system. Brain Res Brain Res Rev 1997; 25: 96-124.
91. Yang JD, Feng G, Zhang J, Lin ZX, Shen T, Breen G, St Clair D, He L. Association between angiotensin-converting enzyme gene and late onset Alzheimer's disease in Han Chinese. Neurosci Lett 2000; 295: 41-44.
92. Zou K, Gong JS, Yanagisawa K, Michikawa M. A novel function of monomeric amyloid beta-protein serving as an antioxidant molecule against metal-induced oxidative damage. J Neurosci 2002; 22: 4833-4841.
93. Zou K, Kim D, Kakio A, Byun K, Gong JS, Kim J, Kim M, Sawamura N, Nishimoto S, Matsuzaki K, Lee B, Yanagisawa K, Michikawa M Amyloid beta-protein (Aβ)1-40 protects neurons from damage induced by Aβ1-42 in culture and in rat brain. J Neurochem 2003; 87: 609-619.
94. Zou K, Yamaguchi H, Akatsu H, Sakamoto T, Ko M, Mizoguchi K, Gong JS, Yu W, Yamamoto T, Kosaka K, Yanagisawa K, Michikawa M Angiotensin-converting enzyme converts amyloid beta-protein 1-42 ($A\beta_{1-42}$) to $A\beta_{1-40}$, and its inhibition enhances brain Aβ deposition. J Neurosci 2007; 27: 8628-8635.
95. Zubenko GS, Volicer L, Direnfeld LK, Freeman M, Langlais PJ, Nixon RA. Cerebrospinal fluid levels of angiotensin-converting enzyme in Alzheimer's disease, Parkinson's disease and progressive supranuclear palsy. Brain Res 1985; 328: 215-221.

FTY720 Modulates Human Oligodendrocyte Progenitor Process Extension and Survival

Veronique E. Miron, BSc,[1,2] Cha Gyun Jung, PhD,[3] Hye Jung Kim, PhD,[3] Timothy E. Kennedy, PhD,[2] Betty Soliven, MD,[3] and Jack P. Antel, MD[1]

Objective: FTY720, a sphingosine-1-phosphate (S1P) receptor agonist that crosses the blood–brain barrier, is a potential immuno-therapy for multiple sclerosis. Our objective was to assess the effect of FTY720 on process extension, differentiation, and survival of human oligodendrocyte progenitor cells (OPCs), and link the functional effects with S1P receptor expression and signaling.

Methods: Functional assays and receptor expression studies were conducted on A2B5+ OPCs derived from the human fetal central nervous system. Cells were treated with physiologically relevant concentrations of the active phosphorylated form of FTY720. S1P receptor/signaling modulators were used to elucidate the basis of the FTY720-induced functional responses.

Results: Short-term (1 day) FTY720 treatment caused initial process retraction that was reversed by uncoupling S1P3 and 5 from their G protein using suramin, and with a Rho-kinase inhibitor H1152. Retraction was associated with RhoA-mediated cytoskeletal signaling and with inhibition of OPC differentiation into more mature phenotypes. Continued FTY720 treatment (2 days) induced process extension and enhanced cell survival associated with increased extracellular signal-regulated kinases 1 and 2 phosphorylation, mimicked with the S1P1-specific agonist SEW2871, but not reversed with suramin. Quantitative real-time polymerase chain reaction showed that FTY720 induced reciprocal and cyclic modulation of S1P1 and S1P5 messenger RNA levels. The observed initial downregulation of S1P5 and subsequently of S1P1 messenger RNA supports functional responses being mediated sequentially by S1P5- and later S1P1-associated signaling.

Interpretation: FTY720 induces time-dependent modulation of S1P receptors on human OPCs with consequent functional responses that are directly relevant for the remyelination process.

Ann Neurol 2008;63:61–71

Remyelination of multiple sclerosis (MS) lesions has been recognized by histopathological and magnetic resonance–based studies, and is considered to contribute to recovery from clinical relapses.[1,2] Results from experimental models of central nervous system (CNS) demyelination indicate that remyelination is dependent on oligodendrocyte progenitor cells (OPCs) rather than on previously myelinating oligodendrocytes.[3,4] Myelin lineage progenitor cells have been identified in both the normal adult human CNS and in MS lesions.[5,6] In vitro and transplant-based studies indicate that a human white matter parenchymal cell population expressing the ganglioside A2B5 are OPCs that can evolve into myelinating cell types.[7,8] Progenitor-mediated remyelination requires survival, migration, and differentiation of these cells.[7] The emergence of potential immunomodulatory therapies that access the CNS raises the issue of their direct effects on remyelination.[9]

FTY720, a lipophilic sphingosine-1-phosphate (S1P) analogue that crosses the blood–brain barrier,[10] is being evaluated as a potential therapy for MS because of its antiinflammatory properties.[11] FTY720 is an agonist of four of five known G-protein–coupled receptors, termed S1P1, 3, 4, and 5, that are part of the endothelial differentiation gene-related (Edg) family.[12,13] Edg receptor messenger RNA (mRNA) levels are upregulated around the perimeter of MS brain lesions.[14] In lymphocytes, S1P receptor-ligand interaction leads to eventual antagonism through endocytosis of the bound receptor[15,16] and downregulation at the mRNA level.[17] This response results in blockade of S1P1-dependent T-lymphocyte efflux from secondary lymph nodes to target organs and is considered the basis for the therapeutic effect of FTY720.[18,19] In some other cell types, for example, endothelial cells, sustained treatment is suggested not to induce receptor

From the [1]Neuroimmunology Unit and [2]Center for Neuronal Survival, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada; and [3]Department of Neurology, University of Chicago, Chicago, IL.

Received Apr 6, 2007, and in revised form Jun 29. Accepted for publication Aug 3, 2007.

This article includes supplementary materials available via the Internet at http://www.interscience.wiley.com/jpages/0364-5134/suppmat

Published online Oct 4, 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.21227

Address correspondence to Dr Antel, Room 111, Neuroimmunology Unit, Montreal Neurological Institute, 3801 University, Montreal, QC, Canada H3A 2B4. E-mail: jack.antel@mcgill.ca

Published by Wiley-Liss, Inc., through Wiley Subscription Services

implications for disease pathogenesis and vaccine development. Neuromolecular Med. 3, 29–39.
Nicoll, J.A., Wilkinson, D., Holmes, C., Steart, P., Markham, H., Weller, R.O., 2003. Neuropathology of human Alzheimer disease after immunization with amyloid-β peptide: a case report. Nat. Med. 9, 448–452.
Orgogozo, J.M., Gilman, S., Dartigues, J.F., Laurent, B., Puel, M., Kirby, L.C., Jouanny, P., Dubois, B., Eisner, L., Flitman, S., Michel, B.F., Boada, M., Frank, A., Hock, C., 2003. Subacute meningoencephalitis in a subset of patients with AD after Aβ42 immunization. Neurology 61, 46–54.
Saido, T.C., Iwatsubo, T., Mann, D.M., Shimada, H., Ihara, Y., Kawashima, S., 1995. Dominant and differential deposition of distinct β-amyloid peptide species, Aβ N3(pE), in senile plaques. Neuron 14, 457–466.
Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yednock, T., Games, D., Seubert, P., 1999. Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400, 173–177.
Selkoe, D.J., 2002. Deciphering the genesis and fate of amyloid β-protein yields novel therapies for Alzheimer disease. J. Clin. Invest. 110, 1375–1381.
Shoji, M., Kanai, M., Matsubara, E., Tomidokoro, Y., Shizuka, M., Ikeda, Y., Ikeda, M., Harigaya, Y., Okamoto, K., Hirai, S., 2001. The levels of cerebrospinal fluid Aβ40 and Aβ42(43) are regulated age-dependently. Neurobiol. Aging 22, 209–215.
Shoji, M., 2002. Cerebrospinal fluid Aβ40 and Aβ42: natural course and clinical usefulness. Front. Biosci. 7, d997–d1006.
van Oijen, M., Hofman, A., Soares, H.D., Koudstaal, P.J., Breteler, M.M., 2006. Plasma Aβ1-40 and Aβ1-42 and the risk of dementia: a prospective case-cohort study. Lancet Neurol. 5, 655–660.
Weiner, H.L., Lemere, C.A., Maron, R., Spooner, E.T., Grenfell, T.J., Mori, C., Issazadeh, S., Hancock, W.W., Selkoe, D.J., 2000. Nasal administration of amyloid-β peptide decreases cerebral amyloid burden in a mouse model of Alzheimer's disease. Ann. Neurol. 48, 567–579.
Weksler, M.E., Relkin, N., Turkenich, R., LaRusse, S., Zhou, L., Szabo, P., 2002. Patients with Alzheimer disease have lower levels of serum anti-amyloid peptide antibodies than healthy elderly individuals. Exp. Gerontol. 37, 943–948.

downregulation and consequent continued signaling persists.[20]

S1P1 and S1P5 are the receptor isoforms most highly expressed on progenitor and mature rodent oligodendrocytes in vitro and in vivo.[21,22] S1P5 and S1P3 are generally linked to $G_{12/13}$, leading to activation of RhoA GTPase and process retraction[23,24]; S1P1 linkage to $G_{i/o}$ is associated with Rac1 and Ras GTPase activation, favoring subsequent process extension and survival.[15,24] Previous studies have demonstrated that S1P, the endogenous ligand for these receptors, causes process retraction in rodent O4+ preoligodendrocytes in a S1P5- and RhoA GTPase–dependent manner, a phenomenon not observed in mature rodent oligodendrocytes.[23] In contrast, S1P treatment enhances the survival of rodent mature oligodendrocytes, but not OPCs.[23] Different cellular responses to FTY720 compared with S1P could potentially exist because of differing receptor affinities and potencies.[25]

In this study, we assessed the effect of the activated phosphorylated form of FTY720 on dissociated cultures of human fetal OPCs with respect to cellular events implicated in remyelination. We specifically evaluated treatment dose- and duration-dependent effects of FTY720 on process extension, differentiation, and survival, and linked responses with relative S1P receptor levels. FTY720 concentrations used in this study are comparable with those used to modulate immune function.[26–28]

Materials and Methods

Human Fetal Oligodendrocyte Progenitor Cell Culture

Human fetal OPC cultures were derived as described elsewhere[9] from CNS tissue obtained from 19- to 23-week-old embryos, provided by the Human Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY). These studies were approved by their and our institutional review boards. In brief, diced brain tissue was incubated with 0.25% trypsin (Invitrogen, Grand Island, NY) and 25μg/ml DNase I (Roche Diagnostics, Laval, Quebec, Canada) at 37°C for 30 minutes and passed through a mesh. A2B5+ cells were isolated by immunomagnetic bead separation using an A2B5 IgM antibody (from a hybridoma), and a microbead-conjugated rat anti–mouse IgM (Miltenyi Biotech, Auburn, CA). Cells were resuspended in Dulbecco's minimum essential medium F12 supplemented with 1% penicillin-streptomycin, 1% glutamine (all from Invitrogen), N1 supplement (1X; Sigma, St. Louis, MO), thyroid hormone T3 (2ng/ml; Sigma), basic fibroblast growth factor (20ng/ml; Sigma), and platelet-derived growth factor (PDGF; 20ng/ml; Sigma), and plated on a confluent bed of lysed and washed human fetal astrocytes grown on plastic poly-lysine–coated coverslips (NunC, Rochester, NY). Purity of the cultures was verified using antibodies recognizing A2B5, the OPC marker PDGF receptor α, the neuronal markers β-tubulin III, neurofilament-M, and microtubule-associated protein 2, and the astrocytic marker glial fibrillary acidic protein.[9]

Immunocytochemistry

After fixation with 2% paraformaldehyde, cells were blocked with HHG (1nM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, 2% heat-inactivated horse serum, 10% heat-inactivated goat serum, Hanks Balanced Salt Solution [HBSS]). Primary antibodies were applied for 1 hour at 4°C. The secondary antibodies goat anti–mouse Cy3 (1:500; Jackson Immunoresearch, West Grove, PA), goat anti–rabbit Cy3 (1:250; Jackson Immunoresearch), and goat anti–mouse fluorescein isothiocyanate (1:50; Biosource, Camarillo, CA) were applied for 30 minutes at 4°C. Antibody isotype controls showed low nonspecific staining. Cell nuclei were identified using the Hoechst dye (bis-benzamide, 1:1,000; Molecular Probes, Carlsbad, CA). We were unable to demonstrate the specificity of commercially available human-reactive S1P receptor antibodies on our cells, and were thus unable to directly assess expression and modulation of S1P receptors at the protein level. This is in agreement with authors who have concluded that available S1P receptor antibodies "have not been useful for investigations of native cellular receptors"[27]; addressing this issue is not possible until better human-reactive antibodies are developed.

Pharmacological Studies

The active phosphorylated form of FTY720 (generously provided by Novartis, Basel, Switzerland) was dissolved in dimethyl sulfoxide/50mM HCl and diluted in culture media before each experiment. To identify the receptors and associated signaling pathways activated by FTY720, we treated cultures with the S1P1-specific agonist SEW2871 (10nM to 10μM; Calbiochem, San Diego, CA), or cotreated cultures with FTY720 and the S1P3/S1P5 antagonist suramin (1–100nM; EMD Bioscience, San Diego, CA) or the Rho-kinase inhibitor H1152 (10μM; Calbiochem). L-α-lysophosphatidic acid (LPA; Sigma) was used as a positive control for RhoA GTPase activation.[29] Treatments were replaced every 2 days. No effects were observed when the OPCs were treated with the appropriate vehicles used for reconstitution of these products.

Functional Assays

PROCESS EXTENSION. To quantify process outgrowth, we determined the area of A2B5 staining (1:100) or Phalloidin-Alexa 488 staining (1:400; Molecular Probes) per cell (μm^2) using a calibrated optical density image (Scion Image software, Frederick, MD) and divided it by the total number of nuclei in the image, quantified with ImageJ software using the watershed tool, to give area of staining per cell (μm^2/cell).

To assess activation of cytoskeletal modulators downstream of Rho GTPases in response to pharmacological agents, we immunostained cells with rabbit polyclonal antisera against phospho-myosin light chain (MLC)-II (1:50; Thr18/Ser19; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-cofilin (1:50; Cell Signal, Danvers, MA), total extracellular signal-regulated kinases 1 and 2 (ERK1/2; 1:100; Stressgen, Victoria, British Columbia, Canada), and mouse

antisera against phospho-ERK1/2 (1:400; Cell Signaling Technology, Danvers, MA). Phosphorylation levels were determined by assessing the immunostaining intensity in individual A2B5+ cells using the histogram function in Adobe Photoshop (Adobe Systems, San Jose, CA). Ten cells per field in each 20X objective image were quantified.

DIFFERENTIATION AND SURVIVAL. Differentiated cells were identified using an anti-galactocerebroside (GalC) antibody (1:100, supernatant from V.W. Yong, University of Calgary), and dedifferentiation was assessed using the early stem cell marker, nestin (1:50; Chemicon, Temecula, CA). The number of GalC+ or nestin+ cells was manually counted in a blinded fashion, and the percentage of positive cells was determined. For survival assays, cell death was induced by withdrawing all growth factors from the culture media. We assessed cells undergoing apoptosis using the Recombinant Terminal Deoxynucleotidyl Transferase kit (TUNEL; Promega Corporation, Madison, WI) with biotin-16-2′-deoxy-uridine-5′-triphosphate (Biotin-16-dUTP; Roche) for 1 hour at 37°C, and incubating with streptavidin-fluorescein isothiocyanate (1:1,000; Jackson Immunoresearch) for 30 minutes at 37°C. For cell survival assays, the percentage of TUNEL and A2B5 double-immunopositive cells was determined by manually counting positive cells in a blinded manner.

PROLIFERATION. Proliferation was assessed by immunostaining for a marker for cells in all phases of the active cell cycle, Ki67 (1:100; Abcam, Cambridge, MA). Cells double-immunopositive for Ki67 and A2B5 were counted in a blinded manner and normalized to total number of A2B5+ cells. Proliferation was also assessed by counting total cell numbers using the nuclear marker Hoechst and counting total A2B5+ cells.

RNA-Based Sphingosine-1-Phosphate Receptor Modulation Studies

Cells plated in poly-lysine–coated flasks were lysed with TRIzol (Invitrogen), total RNA was extracted with the Minielute Qiagen RNeasy mini kit, and samples were treated with DNase (Qiagen, Mississauga, Ontario, Canada). Reverse transcription (RT) was performed on 2μg RNA; complementary DNA was generated using random hexaprimers (Roche) and the Moloney murine leukemia virus-RT enzyme (Invitrogen) in a thermocycler at 42°C, 75°C for 60 minutes, and 4°C for 10 minutes. For real-time quantitative PCR, complementary DNA from CD8 T cells isolated from normal donors served as positive controls for S1P1, S1P4, and S1P5 mRNA levels,[30] whereas Jurkat T cells were used as a standard for S1P3.[31] These were used to create a standard curve by serial 10-fold dilutions (using primers, Taqman, and probes from Assays on Demand by Applied Biosystems, Melbourne, Australia). Transcript levels were assessed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using default temperature settings (2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C). Water control indicated undetectable levels of noise (data not shown). Actin transcript levels were used as endogenous controls for the amount of RNA transcribed, and primers/probes were designed using the PRIMER express software.[32] All S1P receptor levels were normalized to the actin levels in the corresponding sample.

Statistical Analyses

Four 20X objective images were captured for each condition for one experiment, and each experiment was repeated with three different samples. Results are presented either as percentage or fold difference over control normalized to the mean value in untreated culture conditions at the respective time point. Comparisons between conditions were made using a two-tailed Student's *t* test. Probability values less than 0.05 were considered statistically significant.

Results

Human Fetal Oligodendrocyte Progenitor Cells Express Sphingosine-1-Phosphate Receptors

As shown previously,[9] our A2B5 cultures are typically 90% positive for the OPC markers A2B5 and PDGF receptor α. Within this population, 20 to 25% of cells are O4+ pre-oligodendrocytes, 3 to 5% are GalC-positive but myelin basic protein–negative, and less than 10% are of the neuronal (β-tubulin III, neurofilament-M, microtubule-associated protein 2–positive) and astrocytic phenotypes (glial fibrillary acidic protein–positive). In our OPC cultures, we characterized the relative levels of S1P receptors that bind FTY720 by quantitative real-time PCR, and observed high levels of S1P1, low levels of S1P5, almost undetectable levels of S1P3, and no S1P4 (Fig 1). We were unable to validate the specificity of commercially available antibodies against human S1P receptors; directly assessing changes in S1P receptor protein levels will require the development of better antibodies.

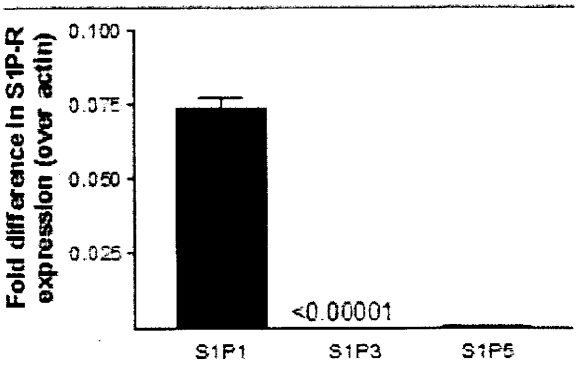

Fig 1. Human fetal oligodendrocyte progenitor cells (OPCs) have sphingosine-1-phosphate receptor (S1P-R) transcripts. Quantitative polymerase chain reaction (qPCR) results for S1P receptors were normalized to actin levels in the respective sample. Human OPCs have relative transcript abundance of S1P1>S1P5>S1P3 and undetectable levels of S1P4.

FTY720 Treatment Regulates Oligodendrocyte Progenitor Cell Process Extension

When OPC cultures were exposed to FTY720 for 1 day, we found that all concentrations were associated with reduced process extension relative to control conditions (area of A2B5 staining per cell was 61 ± 10%, 60 ± 10%, and 43 ± 6% of control for 10nM, 100nM, and 1μM, respectively; Figs 2A, B). Phalloidin staining indicated that the reduced extension was associated with the contraction of F-actin processes (area of phalloidin staining per cell 64% of control for 10nM FTY720). After 2 days of treatment, OPCs treated with the greatest dose of FTY720 (1μM) demonstrated a significant re-extension of processes relative to 1-day treated cultures (area of A2B5 staining per cell increased to 61 ± 6% of control; see Figs 2A, B). However, process area was still significantly less in FTY720-treated cultures relative to control (see Fig 2B). To determine whether the initial reduced process extension reflected slowed process outgrowth or retraction, we conducted time-course studies over the initial 24 hours of treatment. Untreated cultures demonstrated an extension and maintenance of processes over time (area of A2B5 staining per cell was 161 ± 15%, 247 ± 10%, 215 ± 14% over time 0 cultures at 2, 6, and 8 hours, respectively; see Fig 2C). Cultures treated with 1μM FTY720 for 2 hours exhibited an initial process extension comparable with untreated cultures (169 ± 31% over time 0 control); continued treatment for 6 and 8 hours induced gradual process retraction relative to time 0 cultures (114 ± 4% and 13 ± 1% of time 0 control; see Fig 2C).

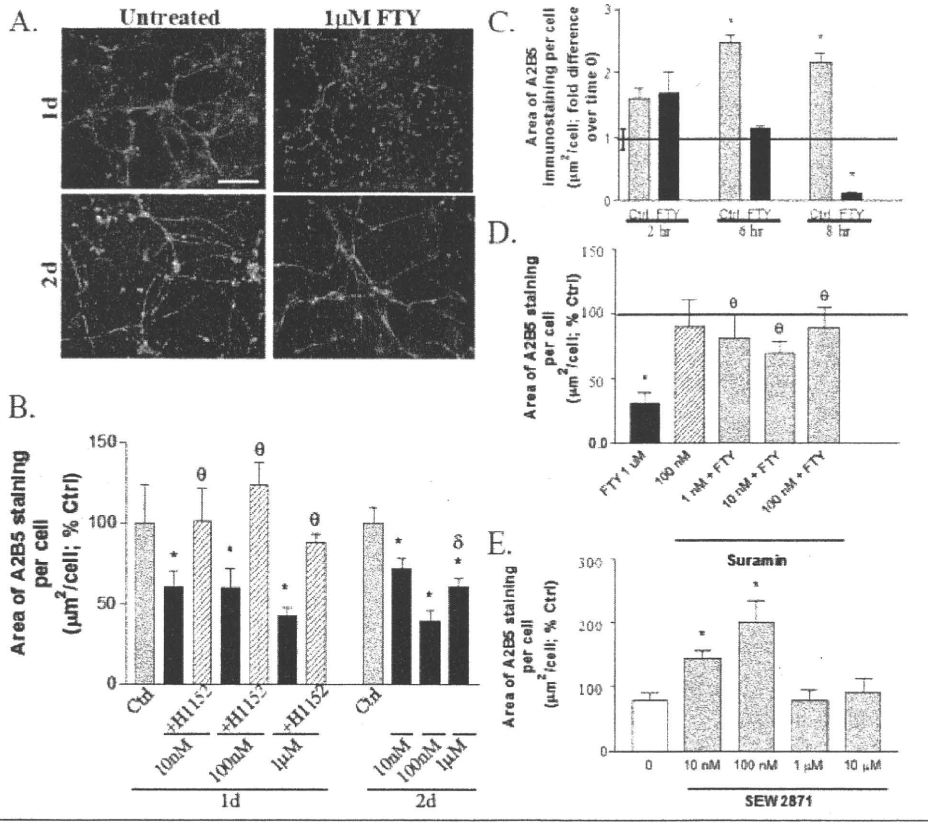

*Fig 2. FTY720 modulates oligodendrocyte progenitor cell (OPC) process extension in a time-dependent manner. (A) Representative images of human fetal OPCs immunostained against A2B5 (red) and counterstained with the nuclear marker Hoechst (blue) indicate that FTY720 (1μM) induces initial process retraction at 1 day (top) and partial recovery of retraction at 2 days (bottom). Scale bar = 50μm. (B) Quantification of process extension expressed as mean area of A2B5 staining per cell (μm²/cell) normalized to untreated control at the respective time point. FTY720-induced process retraction (10nM to 1μM) (*p < 0.05) was significantly reversed by cotreatment with the Rho kinase inhibitor H1152 (θp < 0.05). At 2 days, there was still significant process retraction compared with control (*p < 0.05), but at 1μM, significant extension compared with 1-day treated cultures was observed (δp < 0.05). (C) Quantification of area of A2B5 staining per cell (μm²/cell) normalized to time 0 cultures. Untreated cultures show progressive and maintained process extension, whereas FTY720-treated cells (1μM) demonstrate gradual process retraction. *p < 0.05. (D) Cotreatment with 1μM FTY720 and the sphingosine-1-phosphate receptor 3 (S1P3)/S1P5 antagonist suramin (1–100nM) for 1 day significantly reversed the process retraction (θp < 0.05). (E) Treatment with the S1P1-specific agonist SEW2871 for 1 day induced significant process extension at nanomolar concentrations (10–100nM) (*p < 0.05).*

We then treated OPCs with receptor- or signaling pathway–specific agonists and antagonists to elucidate the functional basis for the FTY720-induced changes in process dynamics. The FTY720-induced retraction at day 1 was significantly reversed by cotreatment with H1152 (see Fig 2B), an inhibitor of the downstream effector of RhoA GTPase, Rho kinase, and by low nanomolar concentrations of suramin (1–100nM), an agent that uncouples S1P3 and S1P5 from their G protein[33,34] (see Fig 2D). Suramin treatment alone (100nM) had no significant effect on OPC process dynamics. These results suggest that FTY720 induced initial process retraction in a RhoA and S1P3- or S1P5-dependent manner. Nanomolar concentrations of the S1P1-specific agonist SEW2871 (10, 100nM) induced significant process extension relative to control at both 1 (see Fig 2E) and 2 days (data not shown), suggesting that S1P1-associated signaling is sufficient to cause process extension in OPCs. Greater dosages of SEW2871 did not promote extension, indicating that, at these concentrations, it may be acting nonspecifically on other S1P receptors to negate the S1P1-associated effect.

We used phosphorylation of the cytoskeletal modulators MLC II and cofilin as measures of the expected activation of cytoskeletal modulators downstream of Rho GTPase. RhoA GTPase-dependent process retraction is associated with an increased MLC II phosphorylation[35] and decreased cofilin phoshorylation.[36] LPA, which binds other members of the Edg receptor family, has been shown to induce RhoA GTPase activation[29,37] and was used as a positive control. OPCs showed a significant increase in MLC II phosphorylation at 15 minutes when treated with 1μM FTY720 (4.33 ± 0.11-fold over control; Fig 3A) or with LPA (2.52 ± 0.14-fold over control; see Fig 3B). At 1 hour of treatment, MLC II phosphorylation levels in FTY720-treated cultures were significantly less than at 15 minutes (3.50 ± 0.16-fold over control; see Fig 3B), demonstrating an expected decline in activation levels over time.[35] Costaining with A2B5 indicated that the phosphorylation signal was associated with OPCs (see Fig 3C). Cofilin phosphorylation levels were significantly decreased at 1 hour of treatment with FTY720 or LPA (0.71 ± 0.02 and 0.42 ± 0.03-fold over control, respectively; see Fig 3D). H1152 was used as a positive control for increased cofilin phosphorylation,[36] which was observed at 15 minutes of treatment (1.3 ± 0.03-fold over control; see Fig 3D).

We used phosphorylation of the ERK1/2 as a measure of the expected activation of cytoskeletal modulators downstream of Rac1 and Ras GTPase. Rac1 GTPase-dependent process extension is associated with an increase in ERK1/2 phosphorylation.[38–40] We observed a significant increase in phospho-ERK1/2 both at 6 and 18 hours of FTY720 treatment relative to control (see Fig 3E; 1.64 ± 0.07 and 2.17 ± 0.08-fold over control, respectively), with a concomitant decrease in MLC II phosphorylation (see Fig 3F; 0.71 ± 0.04 and 0.71 ± 0.06, respectively). The same trend was observed when values were normalized to total ERK1/2 intensity levels (data not shown). These results correlate with FTY720 inducing an initial retraction followed by a delay in extension of processes.

FTY720 Regulates Oligodendrocyte Progenitor Cell Differentiation and Survival

As shown in Figure 4A, 2-day FTY720 treatment significantly decreased the proportion of GalC+ cells relative to untreated conditions (down to 28 ± 0.11% of control). Suramin (1, 10nM) significantly antagonized the effect of FTY720 and promoted differentiation (see Fig 4B). The ability of SEW2871 to enhance differentiation in a comparable manner (see Fig 4B) suggests that suramin cotreatment may allow FTY720 signaling through S1P1 to prevail. These findings imply that FTY720 inhibits the maturation of OPCs in an S1P3- or S1P5-dependent manner, and that S1P1-mediated signaling is sufficient in promoting OPC differentiation.

Total cell numbers were not significantly affected by FTY720 treatment (10nM to 10μM) over 1 or 2 days (see Supplementary Figs 1A, B), eliminating the possibility of a confounding increase in proliferation of other cell types. Furthermore, both short- (4 hours) and long-term (1 and 2 days) FTY720 treatment did not significantly impact the percentage of A2B5+ cells expressing the active cell cycle marker, Ki67 (see Supplementary Fig 1C).

Equal proportions of cells positive for the early stem cell marker, nestin, were observed in FTY720-treated cultures (36 and 40% with 10 and 100nM, respectively) relative to control (42%) (see Supplementary Fig 2), suggesting that FTY720 did not induce the dedifferentiation of OPCs to a less mature phenotype.

Withdrawing the growth factors PDGF, bFGF, and T3 over 2 days increased the proportion of TUNEL and A2B5 double-positive OPCs from 10 ± 1% to 89 ± 5% (Fig 5A). FTY720 treatment significantly reduced the proportion of apoptotic cells to 25 ± 3, 45 ± 12, and 50 ± 7% for 10nM, 100nM, and 1μM, respectively (see Fig 5A). SEW2871 (10nM to 10μM) also significantly reduced apoptosis (see Fig 5B), implicating that S1P1 signaling is sufficient to promote the survival of OPCs in death-inducing environments. However, cotreating cultures with FTY720 (10nM) and suramin (10, 100nM) did not significantly antagonize the survival-promoting effect of FTY720 (see Fig 5C), suggesting that S1P3 and S1P5 are not responsible for this response. These findings are consistent with FTY720 inducing the phosphorylation of the pro-survival kinases ERK1/2 (see Fig 3E).