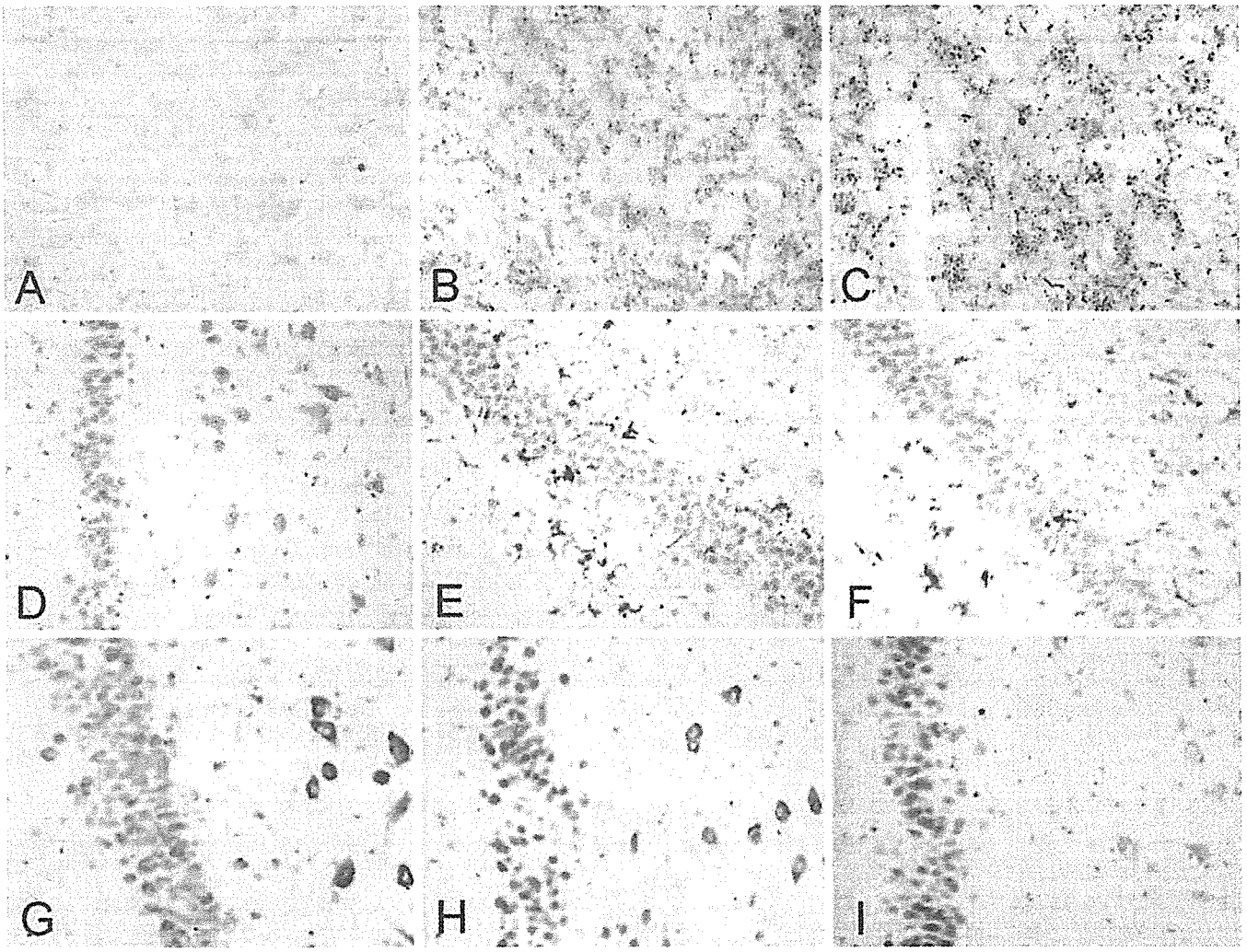

Fig. 2 Immunohistochemical studies of the dentate gyrus in NC (**A**, **D**, **G**), PD (**B**, **E**, **H**), and DLB (**C**, **F**, **I**). The first immunohistochemical study was carried out with anti-α-synuclein (**A–C**). **A** A few α-synuclein-positive presynaptic terminals are seen in NC. **B** There are many α-synuclein-positive mossy fibers in PD. **C** In DLB, more numerous mossy fibers are seen. The second immunohistochemical study was carried out with anti-HLA-DP, DQ, DR (CR3/43) (**D–F**). **D** CR3/43-positive microglia are not seen in NC. **E**, **F** In PD and DLB, many CR3/43-positive microglia were seen. The third immunohistochemical study was carried out with anti-BDNF (**G–I**). **G** Almost all neuronal cytoplasm is stained in NC. **H** Almost all pyramidal neuronal cytoplasm is stained weakly, and nuclei are stained strongly in PD. **I** In DLB, almost all pyramidal neurons cytoplasm and nuclei are stained weakly. ×380

ence in CR3/43-positive microglia counts between PD and DLB (Fig. 4).

RT-PCR method

The average ± SE ratios of the mRNA expression of neurotrophic factors and cytokines to that of GAPDH in the hippocampus, putamen and cingulate cortex of NC, PD and DLB are shown in Fig. 5. As for neurotrophic factors in the hippocampus, the mRNA expression of BDNF, GDNF and NT-3 in PD tended to decrease compared with NC. In DLB, the mRNA expression of these three neurotrophic factors showed a stronger decreasing trend than that in PD, and the mRNA expression of BDNF was significantly decreased compared with NC. As for cytokines in the hippocampus, the mRNA expression of IL-1β in PD and DLB significantly decreased compared with NC, whereas that of IL-6 in PD and DLB significantly increased compared with NC. TNF-α mRNA expression in PD and DLB tended to increase compared with NC. In the putamen, the mRNA expression of BDNF in DLB significantly decreased, and the mRNA expression of NT-3 in DLB tended to decrease compared with NC. In the putamen, the mRNA expression of IL-6 in PD and DLB significantly increased compared with NC. In the cingulate cortex, the mRNA expression of NGFβ in DLB significantly increased as did the mRNA expression of IL-1β and IL-6 in PD and DLB, and that of TNF-α in DLB when compared with NC.

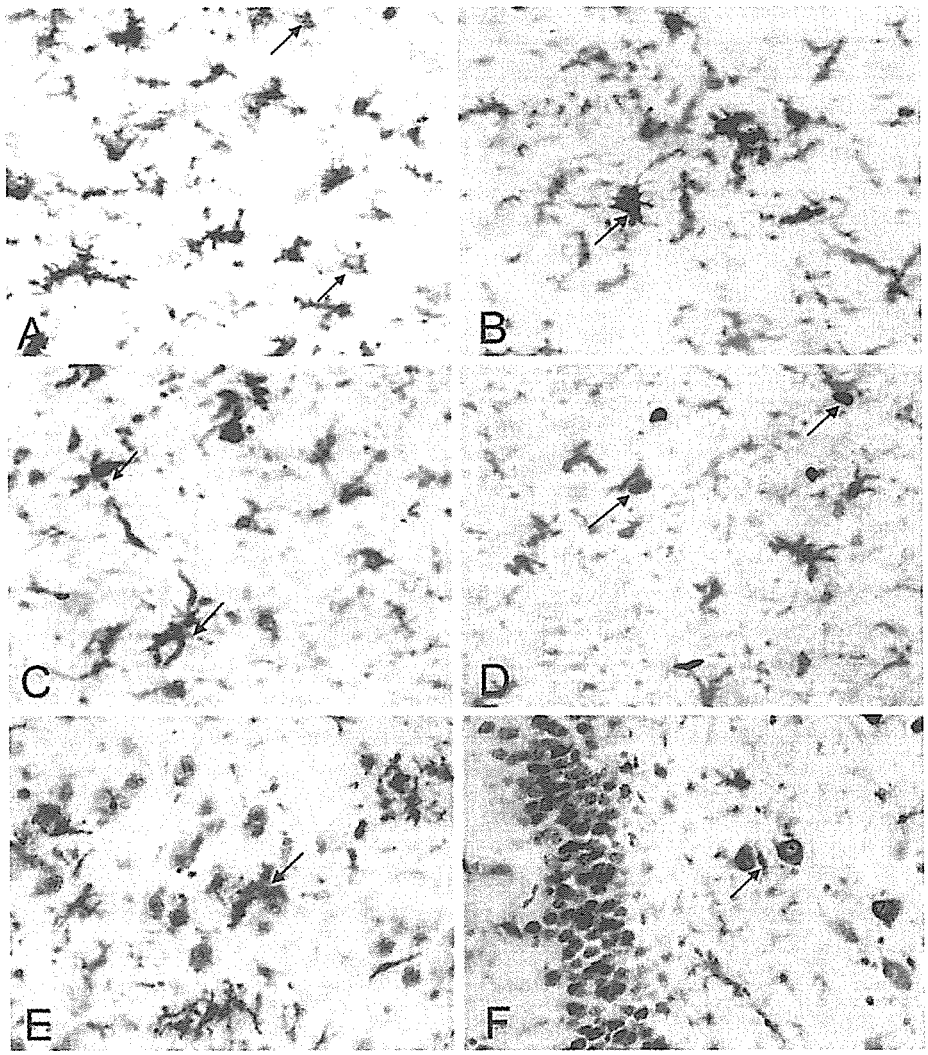

Fig. 3 Double immunohistochemical staining of the hippocampus of PD used anti-HLA-DP, DQ, DR (CR3/43) versus anti-GFAP (**A**, **B**), anti-α-synuclein (**C**, **D**), and anti-BDNF (**E**, **F**). Many CR3/43-positive microglia (*purple*) are seen in the CA2/3 region (**A**) and dentate gyrus (**B**), and a few GFAP-positive astrocytes (*brown*, *arrows*) are seen in both regions. Some CR3/43-positive microglia (*purple*, *arrows*) are associated with α-synuclein-positive Lewy neurites (*brown*) in the CA2/3 region (**C**) and Lewy body (*brown*) -containing neurons in the dentate gyrus (**D**). Some CR3/43-positive microglia (*purple*, *arrows*) are associated with weakly BDNF-positive neurons (*brown*) in the CA2/3 region (**E**) and dentate gyrus (**F**). **A**, **C**, **E**, **F** ×380; **B**, **D** ×450

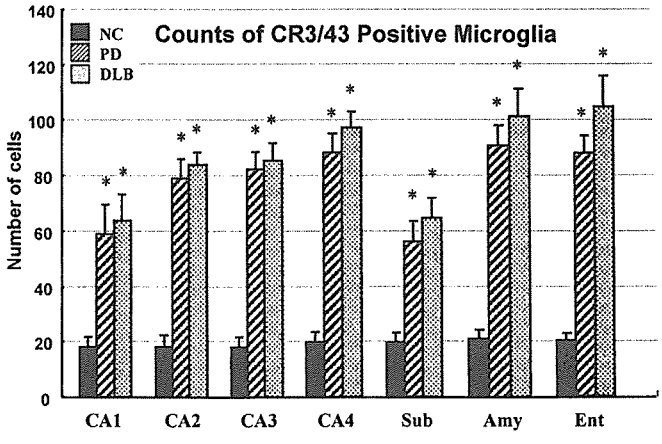

Fig. 4 Bar graphs show a comparison of the average numbers of HLA-DP, DQ, DR (CR3/43)-positive microglia in the limbic system in NC (*black bar*), PD (*striped bar*) and DLB (*dotted bar*) (number/HPF). Patients with PD and DLB have significantly higher numbers of CR3/43-positive microglia in all areas compared with NC (*$P < 0.01$), but there is no statistical difference in CR3/43-positive microglia counts between PD and DLB brains

Discussion

Comparing patients of similar age, those with PD have a higher increased risk of dementia [2, 3, 18, 43]. Mild atrophy of the hippocampus was found in both demented and non-demented cases of PD, consistent with recent postmortems and MRI findings [11, 13, 39]. Subcortical pathologies, including atrophy of the cholinergic nucleus basalis of Meynert, striatonigral degeneration, and involvement of the dopaminergic ventral tegmental area and other monoaminergic nuclei, may also contribute to dementia in PD. In the hippocampus, the decrease in choline acetyltransferase (ChAT) activity in PD with or without dementia has varied from 40% to 62% as compared with controls, and the cognitive decline in PD patients may be associated with reduced ChAT activity in the hippocampus [42].

BDNF is a neurotrophic factor that promotes cholinergic and dopaminergic neuronal differentiation [1, 28, 36, 63]. The protein and mRNA of BDNF are

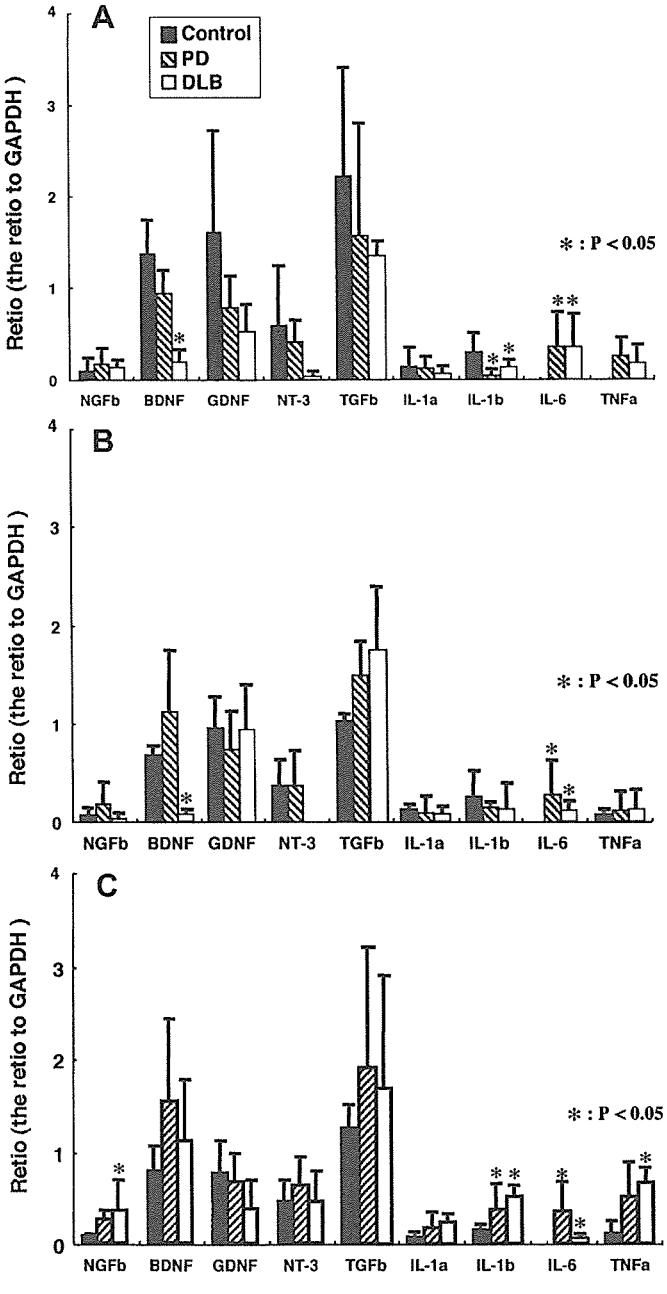

Fig. 5 Bar graphs show the average ± SE ratio of the mRNA expression of neurotrophic factors and cytokines to that of GAPDH in the hippocampus (**A**), putamen (**B**) and cingulate cortex (**C**) in NC (*black bars*), PD (*striped bars*) and DLB (*dotted bars*). **A** Hippocampus: The mRNA expression of BDNF, GDNF and NT-3 in PD tend to decrease compared with NC. In DLB, mRNA expression of these three neurotrophic factors shows a stronger decreasing trend than that in PD, and mRNA expression of BDNF is significantly decreased compared with NC ($*P < 0.05$). mRNA expression of IL-1β in PD and DLB is significantly decreased compared with that in NC ($*P < 0.05$), and mRNA expression of IL-6 in PD and DLB is significantly increased compared with that in NC ($*P < 0.05$). The TNF-α mRNA expression in PD and DLB tends to increase compared with that in NC. There is no statistical difference between PD and DLB in their mRNA expression of neurotrophic factors and cytokines. **B** Putamen: mRNA expression of BDNF in DLB is significantly decreased compared with that in NC ($*P < 0.05$), and mRNA expression of NT-3 in DLB tends to decrease compared with that in NC. mRNA expression of IL-6 in PD and DLB is significantly increased compared with that in NC ($*P < 0.05$). There is no statistical difference between PD and DLB in their mRNA expression of neurotrophic factors and cytokines. **C** Cingulate Cortex: mRNA expression of NGFβ in DLB significantly increased compared with that in NC ($*P < 0.05$). mRNA expression of IL-1β and IL-6 in PD and DLB, and that of TNF-α in DLB is significantly increased compared with that in NC ($*P < 0.05$). There is no statistical difference between PD and DLB in the mRNA expression of their neurotrophic factors and cytokines

located throughout the major target of the basal forebrain cholinergic system, i.e., the hippocampus, amygdala, and neocortex [12, 25, 33, 58, 66]. The level of BDNF protein in PD substantia nigra was reduced [51, 56, 64], and that of BDNF mRNA was decreased in the hippocampus of individuals with Alzheimer's disease [59, 64]. In the present report, we have shown that immunoreactivity and mRNA expression of BDNF were reduced in the hippocampus of PD and DLB patients. These findings may contribute to the reduced ChAT activity in the hippocampus with PD and DLB, as well as to the cognitive decline resulting from these diseases. We have already reported an increased number of activated microglia in the putamen and cingulate cortex [30]. In the putamen, the mRNA expression of BDNF in DLB was significantly decreased, but in the cingulate cortex, there was no difference compared with controls.

As for the neuropathological findings in the hippocampus of Lewy body diseases, the existence of numerous ubiquitin- or α-synuclein-positive Lewy neurites in the CA2/3 region [14, 15, 34], α-, β-synuclein-positive mossy fibers in the dentate gyrus [16], and significant neuronal loss in the presubiculum were reported [21]. In addition, atrophy of the hippocampus correlated with increasing severity in the CA2/3 Lewy neurites grade as well as with the density of Lewy bodies in the frontal lobe [20, 27]. In this report, many MHC class II-positive microglia were seen diffusely, especially in the CA2/3 region and dentate gyrus, in the hippocampus of Lewy body disease, and were associated with α-synuclein-positive Lewy neurites or Lewy body-containing neurons or neurons of reduced BDNF immunoreactivity. Neumann et al. [55] suggested that BDNF secreted by electrically active neurons inhibits the expression of MHC class II molecules on microglia. Thus, we speculate that reduced BDNF secretion of the hippocampal neurons of Lewy body disease may contribute to the up-regulation of MHC class II molecule expression on adjacent microglia.

Many studies indicate the vulnerability of neurons to the potential toxicity of microglia, which produces neurotoxic substances including superoxide anion, nitric oxide (NO), glutamate, and pro-inflammatory cytokines [8, 9, 22, 23, 26, 35, 37, 40, 47]. The presence of microglia expressing TNF-α, IL-1β, and other cytokines in the SN of PD brains has been previously reported, and Nagatsu and co-workers [50, 53] have

reported the presence using ELISA of an increased concentration of TNF-α, IL-1β and IL-6 in the striatum of PD. We have already shown [30] the presence of activated microglia and the absence of reactive astrocytosis in the putamen and hippocampus of PD, and, by immunohistochemical methods, that the activated microglia expressed TNF-α and IL-6; in the present study we have demonstrated increased expression of the mRNA of these cytokines in the putamen and hippocampus of PD and DLB. Although these data may suggest an involvement of the pro-inflammatory cytokines secreted by microglia in the degeneration of dopaminergic neurons in Lewy body disease, it is also recognized that TNF-α and IL-6 have neurotrophic mechanisms [4, 10, 19, 60, 62]. To date, glial cells are acknowledged to possess neurotrophic properties that are essential to the survival of dopaminergic and cholinergic neurons [52]. Among them, BDNF and GDNF, which can be released by activated microglia [5], seem to be the most potent factors supporting the dopaminergic neurons of the SN.

BDNF has protective effects on 6-hydroxydopamine-lesioned rat striatum and enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons [61, 65, 67]. Hippocampal neurons have endogenously abundant BDNF protein, and cultured hippocampal neurons show a response to BDNF [31]. Furthermore, the mRNA of trkB, which is the receptor of BDNF, is preserved in neurons of the putamen and hippocampus of PD [6]. These results suggest that supplements of BDNF to the hippocampus and cholinergic system may be an effective treatment for the cognitive impairment of Lewy body disease.

References

1. Aarsland D, Tandberg E, Larsen JP, Cummings JL (1996) Frequency of dementia in Parkinson's disease. Arch Neurol 53:538–542
2. Aarsland D, Andersen K, Larsen JP, Lolk A, Nielsen H, Kragh-Sørensen P (2001) Risk of dementia in Parkinson's disease. A community-based, prospective study. Neurology 56:730–736
3. Alderson RF, Alterman AL, Barde Y-A, Lindsay RM (1990) Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron 5:297–306
4. Barger SW, Horster D, Furukawa K, Goodman Y, Krieglstein J, Mattson MP (1995) Tumor necrosis factors α and β protect neurons against amyloid β-peptide toxicity: evidence for involvement of a kB-binding factor and attenuation of peroxide and Ca^{2+} accumulation. Proc Nat Acad Sci USA 92:9328–9332
5. Batchelor PE, Liberatore GT, Wong JYF, Porritt MJ, Frerichs F, Donnan GA, Howells DW (1999) Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci 19:1708–1716
6. Benisty S, Boissiere F, Faucheux B, Agid Y, Hirsch C (1998) trkB messenger RNA expression in normal human brain and in the substantia nigra of parkinsonian patients: an in situ hybridization study. Neuroscience 86:813–826
7. Braak H, Tredici KD, Rüb U, Vos RAI, Steur ENHJ, Braak E (2003) Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24:197–211
8. Cassarino DS, Fall CP, Swerdlow RH, Smith TS, Halvorsen EM, Miller SW, Parks JP, Parker WD Jr, Bennett JP Jr (1997) Elevated reactive oxygen species and antioxidant enzyme activities in animal and cellular models of Parkinson's disease. Biochim Biophys Acta 1362:77–86
9. Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK (1992) Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. J Immunol 149:2736–2741
10. Cheng B, Christakos S, Mattson MP (1994) Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. Neuron 12:139–153
11. Churchyard A, Lees AJ (1997) The relationship between dementia and direct involvement of the hippocampus and amygdala in Parkinson's disease. Neurology 49:1570–1576
12. Conner JM, Lauterborn JC, Yan Q, Gall CH, Varon S (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J Neurosci 17:2295–2313
13. Cordato NJ, Halliday GM, Harding AJ, Hely MA, Morris JGL (2000) Regional brain atrophy in progressive supranuclear palsy and Lewy body disease. Ann Neurol 47:718–728
14. Dickson DW, Ruan D, Crystal H, Mark MH, Davies P, Kress Y, Yen S-H (1991) Hippocampal degeneration differentiates diffuse Lewy body disease (DLBD) from Alzheimer's disease: light and electron microscopic immunocytochemistry of CA2–3 neurites specific to DLBD. Neurology 41:1402–1409
15. Dickson DW, Schmidt ML, Lee VM-Y, Zhao M-L, Yen S-H, Trojanowski JQ (1994) Immunoreactivity profile of hippocampal CA2/3 neurites in diffuse Lewy body disease. Acta Neuropathol 87:269–276
16. Galvin JE, Uryu K, Lee VM-Y, Trojanowski JQ (1999) Axon pathology in Parkinson's disease and Lewy body dementia hippocampus contains α-, β-, and γ-synuclein. Proc Natl Acad Sci USA 96:13450–13455
17. Gelb DJ, Oliver E, Gilman S (1999) Diagnostic criteria for Parkinson's disease. Arch Neurol 56:33–39
18. Green J, McDonald WM, Vitek JL, Evatt M, Freeman A, Haber M, Bakay RAE, Triche S, Sirockman B, DeLong MR (2002) Cognitive impairment in advanced PD without dementia. Neurology 59:1320–1324
19. Hama T, Kushima Y, Miyamoto M, Kubota M, Takei N, Hatanaka H (1991) Interleukin-6 improves the survival of mesencephalic catecholaminergic and septal cholinergic neurons from postnatal, two-week-old rats in cultures. Neuroscience 40:445–452
20. Harding AJ, Halliday GM (2001) Cortical Lewy body pathology in the diagnosis of dementia. Acta Neuropathol 102:355–363
21. Harding AJ, Lakay B, Halliday GM (2002) Selective hippocampal neuron loss in dementia with Lewy bodies. Ann Neurol 51:125–128
22. Hartmann A, Hunot S, Michel PP, Muriel MP, Vyas S, Faucheux BA, Mouatt-Prignot A, Turmel H, Srinivasan A, Ruberg M, Evan GL, Agid Y, Hirsch EC (2000) Caspase-3: a vulnerability factor and a final effector in the apoptotic cell death of dopaminergic neurons in Parkinson's disease. Proc Natl Acad Sci USA 97:2875–2880
23. Hartmann A, Troadec JD, Hunot S, Kikly K, Faucheux BA, Mouatt-Prigent A, Ruberg M, Agid Y, Hirsch EC (2001) Caspase-8 is an effector in apoptotic death of dopaminergic neurons in Parkinson's disease, but pathway inhibition results in neuronal necrosis. J Neurosci 21:2247–2255
24. Hickey WF, Kimura H (1988) Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science 239:290–292
25. Hofer M, Pagliusi SR, Hohn A, Leibrock J, Barde Y-A (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO J 9:2459–2464

26. Hunot S, Boissiere F, Faucheux B, Brugg B, Mouatt-Prigent A, Agid Y, Hirsch EC (1996) Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. Neuroscience 72:355-363
27. Hurting HI, Trojanowski JQ, Galvin J, Ewbank D, Schmidt ML, Lee VM-Y, Clark CM, Glosser G, Stern MB, Gollomp SM, Arnold SE (2000) Alpha-synuclein cortical Lewy bodies correlate with dementia in Parkinson's disease. Neurology 54:1916–1921
28. Hyman C, Hofer M, Barde Y-A, Juhasz M, Yancopoulos GD, Squinto SP, Lindsay RM (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350:230–232
29. Imamura K, Ito M, Suzumura A, Asai J, Takahashi A (1990) Generation and characterization of monoclonal antibodies against rat microglia and ontogenic distribution of positive cells. Lab Invest 63:853–861
30. Imamura K, Hishikawa N, Sawada M, Nagatsu T, Yoshida M, Hashizume Y (2003) Distribution of MHC class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta Neuropathol 106:518–526
31. Ip NY, Li Y, Yancopoulos GD, Lindsay RM (1993) Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J Neurosci 13:3394-3405
32. Jellinger KA (2000) Cell death mechanism in Parkinson's disease. J Neural Transm 107:1-29
33. Kawamoto Y, Nakamura S, Nakano S, Oka N, Akiguchi I, Kimura J (1996) Immunohistochemical localization of brain-derived neurotrophic factor in adult rat brain. Neuroscience 74:1209–1226
34. Kim H, Gearing M, Mirra SS (1995) Ubiquitin-positive CA2/3 neurites in hippocampus coexist with cortical Lewy bodies. Neurology 45:1768–1770
35. Kim WG, Mohney RP, Wilson B, Jeohn GH, Liu B, Hong JS (2000) Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. J Neurosci 20:6309–6316
36. Knüsel B, Winslow JW, Rosenthal A, Burton LE, Seid DP, Nikolics K, Hefti F (1991) Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc Natl Acad Sci USA 88:961–965
37. Koutsilieri E, Scheller C, Grünblatt E, Nara K, Li J, Pederer P (2002) Free radicals in Parkinson's disease. J Neurol 249 (Suppl 2):II/1–II/5
38. Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312–318
39. Laakso MP, Partanen K, Riekkinen P, Lehtovirta M, Helkala E-L, Hallikainen M, Hänninen T, Vainio P, Soininen H (1996) Hippocampal volumes in Alzheimer's disease, Parkinson's disease with and without dementia, and in vascular dementia: an MRI study. Neurology 46:678–681
40. Liu B, Du L, Hong JS (2000) Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. J Pharmacol Exp Ther 293:607–617
41. Mackenzie IRA (2000) Activated microglia in dementia with Lewy bodies. Neurology 55:132–134
42. Mattila PM, Röyttä M, Lönnberg P, Marjamäki P, Helenius H, Rinne JO (2001) Choline acetyltransferase activity and striatal dopamine receptors in Parkinson's disease in relation to cognitive impairment. Acta Neuropathol 102:160–166
43. Mayeux R, Chen J, Mirabello E, Marder K, Bell K, Dooneief G, Cote L, Stern Y (1990) An estimate of the incidence of dementia in idiopathic Parkinson's disease. Neurology 40:1513–1517
44. McGeer PL, Itagaki S, Boyes BE, McGeer EG (1988) Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 38:1285–1291
45. McGeer PL, Itagaki S, Tago H, McGeer EG (1988) Expression of the histocompatibility glycoprotein HLA-DR in neurological disease. Acta Neuropathol 76:550–557
46. McGeer PL, Kawamata T, Walker DG, Akiyama H, Tooyama I, McGeer EG (1993) Microglia in degenerative neurological disease. Glia 7:84–925
47. McGuire SO, Ling ZD, Lipton JW, Sortwell CE, Collier TJ, Carvey PM (2001) Tumor necrosis factor alpha is toxic to embryonic mesencephalic dopamine neurons. Exp Neurol 169:219–230
48. McKeith IG, Galasko D, Kosaka K, Perry EK, Dickson DW, Hansen LA, Salmon, DP, Lowe J, Mirra SS, Byrne EJ, Lennox G, Quinn NP, Edwardson JA, Ince PG, Bergeron C, Burns A, Miller BL, Lovestone S, Collerton D, Jansen ENH, Ballard C, Vos RA de, Wilcock GK, Jellinger KA, Perry RH (1996) Consensus guidelines for the clinical and pathological diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. Neurology 47:1113–1124
49. Mirza B, Hadberg H, Thomsen P, Moos T (2000) The absence of reactive astrocytosis is indicative of a unique inflammation process in Parkinson's disease. Neuroscience 95:425–432
50. Mogi M, Hamada M, Kondo T, Roederer P, Inagaki H, Minami M, Nagatsu T (1994) Interleukin-1β, interleukin-6, epidermal growth factor and transforming growth factor-α are elevated in the brain from parkinsonian patients. Neurosci Lett 180:147–150
51. Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, Ichinose H, Nagatsu T (1999) Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. Neurosci Lett 270:45–48
52. Nagata K, Nakajima K, Kohsaka K (1993) Plasminogen promotes the development of rat mesencephalic dopaminergic neurons in vitro. Dev Brain Res 75:31–37
53. Nagatsu T, Mogi M, Ichinose H, Togari A, Riederor P (1999) Cytokines in Parkinson's disease. NeuroSci News 2:88–90
54. Nagatsu T (2002) Parkinson's disease: changes in apoptosis-related factors suggesting possible gene therapy. J Neural Transm 109:731–745
55. Neumann H, Misgeld T, Matsumuro K, Wekerle H (1998) Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. Proc Natl Acad Sci USA 95:5779–5784
56. Parain K, Murer MG, Yan Q, Faucheux B, Agid Y, Hirsch E, Raisman-Vozari R (1999) Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra. Neuroreport 10:557–561
57. Perry VH, Hume DA, Gordon S (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. Neuroscience 15:313–326
58. Phillips HS, Hains JM, Laramee GR, Rosenthal A, Winslow JW (1990) Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. Science 250:290–294
59. Phillips HS, Hains JM, Armanini M, Laramee GR, Johonson SA, Winslow JW (1991) BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. Neuron 7:695–702
60. Satoh T, Nakamura S, Taga T, Matsuda T, Hirano T, Kishimoto T, Kaziro Y (1988) Induction of neuronal differentiation in PC12 cells by B-cell stimulatory factor 2/interleukin 6. Mol Cell Biol 8:3546–3549
61. Sauer H, Wong V, Björklund (1995) Brain-derived neurotrophic factor and neurotrophin-4/5 modify neurotransmitter-related gene expression in the 6-hydroxydopamine-lesioned rat striatum. Neuroscience 65:927–933
62. Sawada M, Suzumura A, Marunouchi T (1995) Cytokine network in the central nervous system and its roles in growth and differentiation of glial and neuronal cells. Int J Dev Neurosci 13:253–264
63. Schmidt-Kastner R, Wetmore C, Olson L (1996) Comparative study of brain-derived neurotrophic factor messenger RNA and protein at the cellular level suggests multiple roles in hippocampus, striatum and cortex. Neuroscience 74:161–183

64. Siegel GJ, Chauhan NB (2000) Neurotrophic factors in Alzheimer's and Parkinson's disease brain. Brain Res Rev 33: 199–22
65. Spina MB, Squinto SP, Miller J, Lindsay RM, Hyman C (1992) Brain-derived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and N-methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. J Neurochem 59:99–106
66. Wetmore C, Ernfors P, Persson H, Olson L (1990) Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. Exp Neurol 109:141–152
67. Yurek DM, Lu W, Hipkens S, Wiegand SJ (1996) BDNF enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons. Exp Neurol 137:105–118

Amyloid-β peptides induce cell proliferation and macrophage colony-stimulating factor expression via the PI3-kinase/Akt pathway in cultured Ra2 microglial cells

Sachiko Ito[a], Makoto Sawada[b], Masataka Haneda[a,1], Satoshi Fujii[c], Kentaro Oh-hashi[c], Kazutoshi Kiuchi[c], Masahide Takahashi[d], Ken-ichi Isobe[a,*,1]

[a] *Department of Basic Gerontology, National Institute for Longevity Sciences, 36-3 Gengo, Morioka-cho, Obu, Aichi 474-8522, Japan*
[b] *Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan*
[c] *Department of Biomolecular Science, Faculty of Engineering, Gifu University, Yanagido, Gifu 501-1193, Japan*
[d] *Department of Pathology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*

Received 28 December 2004; revised 8 February 2005; accepted 21 February 2005

Available online 5 March 2005

Edited by Jesus Avila

Abstract Alzheimer's disease is characterized by numerous amyloid-β peptide (Aβ) plaques surrounded by microglia. Here we report that Aβ induces the proliferation of the mouse microglial cell line Ra2 by increasing the expression of macrophage colony-stimulating factor (M-CSF). We examined signal cascades for Aβ-induced M-CSF mRNA expression. The induction of M-CSF was blocked by a phosphatidylinositol 3 kinase (PI3-kinase) inhibitor (LY294002), a Src family tyrosine kinase inhibitor (PP1) and an Akt inhibitor. Electrophoretic mobility shift assays showed that Aβ enhanced NF-κB binding activity to the NF-κB site of the mouse M-CSF promoter, which was blocked by LY294002. These results indicate that Aβ induces M-CSF mRNA expression via the PI3-kinase/Akt/NF-κB pathway.

Keywords: Microglia; Alzheimer's disease; Amyloid-β; Akt; NF-κB; Macrophage colony-stimulating factor

1. Introduction

Alzheimer's disease (AD) is characterized by the presence of senile plaques in the brain composed primarily of amyloid-β peptide (Aβ). Microglia have been reported to surround the Aβ plaques, which provokes a microglia-mediated inflammatory response that contributes to neuronal cell loss [1]. On the other hand, microglia play an important role in the clearance of Aβ by phagocytosis, primarily through scavenger receptor class A (SR-A, CD204), scavenger receptor-BI (SR-BI) and CD36 [2–4]. Recently, it has been reported that microglia isolated from CD36-deficient mice had marked reductions in Aβ-induced cytokine/chemokine secretion [5]. CD36 binds to Aβ in vitro [6], and is physically associated with members of the Src family tyrosine kinase [7,8], which transduce signals from this receptor [9]. Another receptors such as receptor for advanced glycosylation end-products (RAGE), integrins and heparan sulfate proteoglycans, also have been reported to bind with Aβ [10].

There are many reports that microglia are activated by Aβ, but it has been unclear whether Aβ is associated with the proliferation of microglia. Here we report that Aβ induces proliferation of the microglial cell line Ra2 by increasing macrophage colony-stimulating factor (M-CSF) expression. We also elucidated signal transduction pathways from Aβ-treatment to M-CSF mRNA expression in microglia.

2. Materials and methods

2.1. Materials

Synthetic human Aβ25–35, Aβ1–42 and Aβ1–16 were obtained from Peptide Institute Inc. Aβ35–25 was from AnaSpec Inc. Aβ25–35, Aβ1–16 and Aβ35–25 were dissolved in H_2O and Aβ 1–42 was dissolved in 0.1% NH_3 according to the manufacturer's instructions. Anti-phospho-Akt (Serine 473), anti-Akt, anti-phospho-IκBα (Serine 32), and anti-IκBα antibodies were from Cell Signaling. PP1 was from Biomol. Wortmannin, LY294002 and Akt inhibitor [1L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate] were from Calbiochem. Piceatannol was from Sigma–Aldrich. Mouse recombinant granulocyte–macrophage colony-stimulating factor (mrGM-CSF) was from Pharmingen. Mouse recombinant M-CSF (mrM-CSF) was from Techne. Aβ25-35 and Aβ1–42 were used at 50 and 10 μM, respectively, in all studies unless otherwise stated.

2.2. Cell culture

Microglial cell line Ra2 was cultured in MGI medium [Eagle's MEM supplemented with 0.2% glucose, 5 μg/ml bovine Insulin (Sigma–Aldrich), and 10% fetal bovine serum (FBS, Invitrogen)] and 0.8 ng/ml mrGM-CSF (Pharmingen) [11]. Before Aβ-treatment, Ra2 cells were cultured in MGI medium without mrGM-CSF for 16 h. Primary microglia and primary astrocytes were prepared using newborn C57BL/6 mice as described previously [12], and cultured in MGI medium containing 0.8 ng/ml mrGM-CSF. The neuroblastoma cell line Neuro2a was cultured in DMEM supplemented with 10% FBS. Primary neurons were obtained from the cortex of 14-day-old C57BL/6 mouse embryos as described previously [13] with some modifications. Neural cells cultured in DMEM supplemented with TIS (5 μg/ml

*Corresponding author. Fax: +81 52 744 2972.
E-mail address: kisobe@med.nagoya-u.ac.jp (K. Isobe).

[1] Present address. Department of Immunology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GM-CSF, granulocyte–macrophage colony stimulating factor; M-CSF, macrophage colony-stimulating factor; PBS, phosphate-buffered saline; PI3-kinase, phosphatidylinositol 3 kinase; RAGE, receptor for advanced glycation end-products

0014-5793/$30.00
doi:10.1016/j.febslet.2005.02.048

transferrin, 5 μg/ml insulin, and 5 ng/ml selenite, Sigma), 10% FBS and 5 μM cytosine arabinoside (Ara-C, Sigma). Before Aβ-treatment, primary neurons were cultured in MGI medium for 16 h.

2.3. Cell proliferation (WST-1) assay

Cell proliferation was determined by analyzing the conversion of WST-1 (light red) to its formazan derivate (dark red) using a WST-1 Cell Counting Kit (Dotite). For neutralization of M-CSF, anti-mouse M-CSF antibody (Techne) was added to the culture medium. At the end of the experiments, the media were replaced, and cells were incubated with 10 μl of the WST-1 reagent for 1 h at 37 °C in 5% CO_2. The absorbance at 450 nm was measured by using a microplate reader (Bio-Rad).

2.4. Immunoblotting

Cells were lysed in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 5% bromophenol blue). Then 50 μg of total protein was resolved by SDS–PAGE and transferred to PVDF membranes (Millipore). Immunoblotting was performed with the appropriate antibody using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia).

2.5. RT-PCR and real-time quantitative RT-PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen). For RT-PCR and real-time quantitative PCR, the primers for mouse M-CSF and β-actin genes were as follows (5′ to 3′): M-CSF sense, CCATCGAGACCCTCAGACAT; M-CSF antisense1 for RT-PCR, CCTAAGGGAAAGGGTCCTGA; M-CSF antisense2 for real-time PCR, GATGAGGACAGACAGGTGGA; β-actin sense, AGTGTGACGTTGACATCCGT; and β-actin antisense, GCAGCTCAGTAACAGTCCGC. Conventional RT-PCR was performed using 0.5 μl cDNA, and 30 cycles of amplification for M-CSF or 23 cycles for β-actin at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Quantitative real-time PCR was performed on the Smart Cycler system (Takara) using the following program: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and 8 s at 72 °C. The reactions were carried out using 0.5 μl cDNA with Smart Kit for Sybr Green I (Eurogentec). To check the specificity of reactions, a single band of the correct size was visualized by running out on 2% agarose gels. Values were expressed as relative expression of M-CSF mRNA normalized to the β-actin mRNA.

2.6. Nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts of Ra2 cells were prepared as previously described [14]. Three micrograms of nuclear extract was incubated with 5 fmol of ^{32}P end-labeled double-stranded oligonucleotides derived from M-CSF promoter in binding buffer [10 mM Tris, pH 7.5, 4% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.05 μg/μl poly(dI–dC)] for 20 min at room temperature. For competition assays, 1 pmol of unlabeled probe was incubated in the reaction mix before the addition of the ^{32}P-labeled probe. The oligonucleotides used in these experiments were as follows: NF-κB probe, 5′-GCC-TTGAGGGAAAGTCCCTAGGGGC-3′; AP1 probe, 5′-GTAGT-ATGTGTCAGTGCC-3′. For supershift assays, nuclear extracts were preincubated with anti-NF-κB p50 or p65 antibodies (Santa Cruz) for 1 h at 4 °C. The DNA–protein complex was separated on 5% native polyacrylamide gels. The dried gels were visualized using an Image Reader (Fujifilm).

2.7. Statistical analysis

Results are expressed as means ± S.D. Statistical analysis was done by a two-tailed Student's *t* test. A *P* value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Aβ promotes microglial cell proliferation

To investigate the possible role of Aβ in the activation of microglia, we examined if Aβ could sustain the cell proliferation of microglial cell line Ra2. Ra2 cells proliferate in MGI medium containing GM-CSF and stop proliferating without GM-CSF [11]. Under MGI medium without GM-CSF, the effects of M-CSF or Aβ on the proliferation of Ra2 cells were analyzed by the WST-1 assay. The addition of M-CSF induced cell proliferation dose-dependently (Fig. 1A). Aβ25–35 increased Ra2 cell proliferation dose-dependently (Fig. 1B). Aβ25–35 does not occur naturally but has shown to mimic the effects of Aβ1–42 [15–17]. Aβ1–42, which occurs in a brain affected by AD, was more effective in cell proliferation than Aβ25–35 (Fig. 1C). It has been reported that Aβ stimulates the proliferation of microglia to enclose Aβ plaque [18,19]. We examined if M-CSF provoked the cell proliferation with Aβ-treatment. The effect of Aβ on the proliferation was blocked by anti-M-CSF antibody ($P < 0.05$) (Fig. 1D). The treatment with M-CSF was performed as a control. The effect of M-CSF was blocked by anti-M-CSF antibody (Fig. 1D). We found that Aβ induces microglial cell proliferation by M-CSF production.

3.2. Aβ induces M-CSF mRNA expression in microglia

To examine whether Aβ could induce M-CSF mRNA expression in microglia, Ra2 cells were stimulated with Aβ25–35 for 16 h at various concentrations. Aβ25–35 induced M-CSF mRNA expression dose-dependently (Fig. 2A). As a result of real-time quantitative RT-PCR (Fig. 2A, right), M-CSF mRNA induction by 50 μM Aβ25–35 was about sevenfold of non-treated control. Aβ25–35 induced time-dependent increases in M-CSF mRNA expression (Fig. 2B). Aβ1–42 also induced M-CSF expression (Fig. 2A and B). GM-CSF mRNA, on the other hand, was not induced by Aβ25–35 or Aβ1–42 (data not shown). Aβ1–16 did not induce M-CSF mRNA expression (Fig. 2C), nor

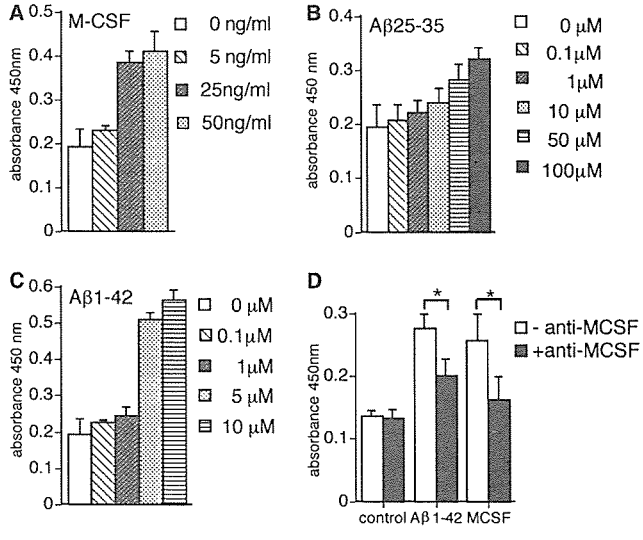

Fig. 1. Aβ promotes Ra2 cell proliferation. Cellular proliferation was measured by WST-1 assay. (A, B and C) Ra2 cells were incubated with the medium containing M-CSF, Aβ25–35 or Aβ1–42 at indicated concentrations for 48 h. (D) Ra2 cells were preincubated with 1 μg/ml anti-M-CSF antibody for 1 h before treatment with 5 μM Aβ1–42 or 25 ng/ml M-CSF for 24 h. Mean ± S.D. values from a single experiment were performed in triplicate. Similar results were obtained in each of two separate experiments (*$P < 0.05$).

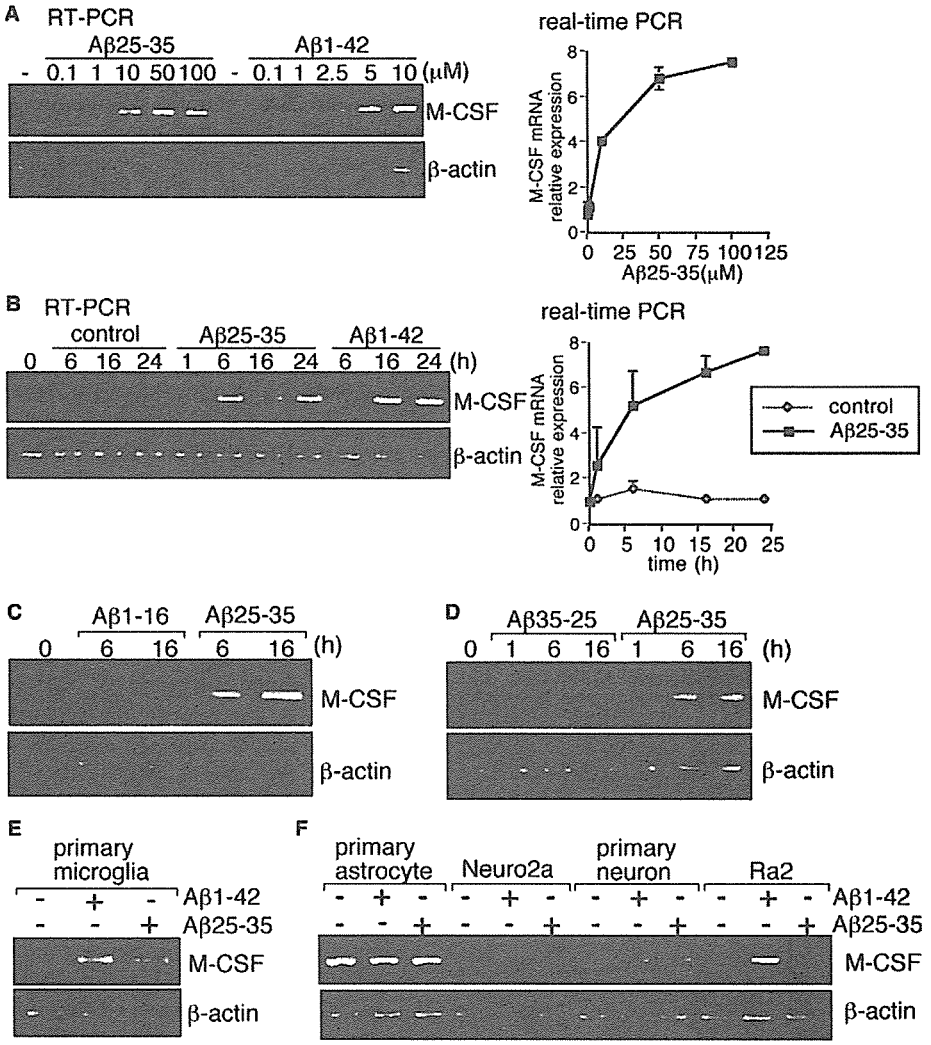

Fig. 2. Aβ stimulates M-CSF mRNA expression in microglia. (A and B) M-CSF and β-actin mRNA were determined by RT-PCR (left) and quantified by real-time PCR (right). Data represent means ± S.D. of three separate determinations. Ra2 cells were treated with Aβ25–35 or Aβ1–42 at indicated concentrations for 16 h (A). Time course of M-CSF relative expression of Ra2 cells treated with 50 μM Aβ25–35 and 10 μM Aβ1–42 (B). (C and D) Ra2 cells were treated with 50 μM Aβ1–16 (C) or 50 μM Aβ35–25 (D) for indicated times. (E and F) Primary microglia (E), primary neurons, primary astrocytes and neuroblastoma Neuro2a (F) were treated with 50 μM Aβ25–35 or 10 μM Aβ1–42 for 16 h.

did Aβ 35–25, which was a reverse sequence of Aβ25–35 (Fig. 2D). In primary microglia, as well as in Ra2 cells, Aβ25–35 and Aβ1–42 increased M-CSF mRNA expression (Fig. 2E). We also examined whether Aβ induced increases in M-CSF mRNA expression in primary astrocytes, primary neurons, and neuroblastoma cells Neuro2a. These cells constitutively expressed M-CSF mRNA, but Aβ25–35 and Aβ1–42 did not induce further expression of M-CSF mRNA (Fig. 2F). These results demonstrate that Aβ induced M-CSF mRNA expression in only microglia.

3.3. *Aβ induces M-CSF mRNA via Src family tyrosine kinase and PI3-kinase signal cascade*

Because our studies showed that Aβ25–35 had induced M-CSF expression in Ra2 cells, we examined signal cascades for Aβ-induced M-CSF mRNA expression by using several chemical inhibitors. The Src family tyrosine kinase is associated with CD36, which transduces signal cascades by Aβ [7,8,20]. In addition, Syk tyrosine kinase is activated by Aβ [21]. First, we examined if M-CSF mRNA expression was induced by Aβ via tyrosine kinase, Src family and Syk. A specific inhibitor of Src family kinase, PP1, prevented the increase in M-CSF mRNA induced by Aβ (Fig. 3A). A Syk-selective inhibitor, piceatannol, also blocked the increase in M-CSF mRNA expression (Fig. 3B). Next, to investigate whether the PI3-kinase pathway regulates Aβ-induced M-CSF expression, Ra2 cells were pretreated with the PI3-kinase inhibitors, wortmannin or LY294002. Wortmannin and LY294002 inhibited the increase in M-CSF mRNA expression dose-dependently (Fig. 3C and D). Fig. 3E shows the result of quantitative amounts of mRNA by real-time PCR. It has been reported that Aβ stimulates tyrosine kinase, PI3-kinase and Akt activation in neural and macrophage cells [21–24]. However, analysis of these signal transductions in microglia has not been reported. This is the first report that Aβ induces M-CSF expression through

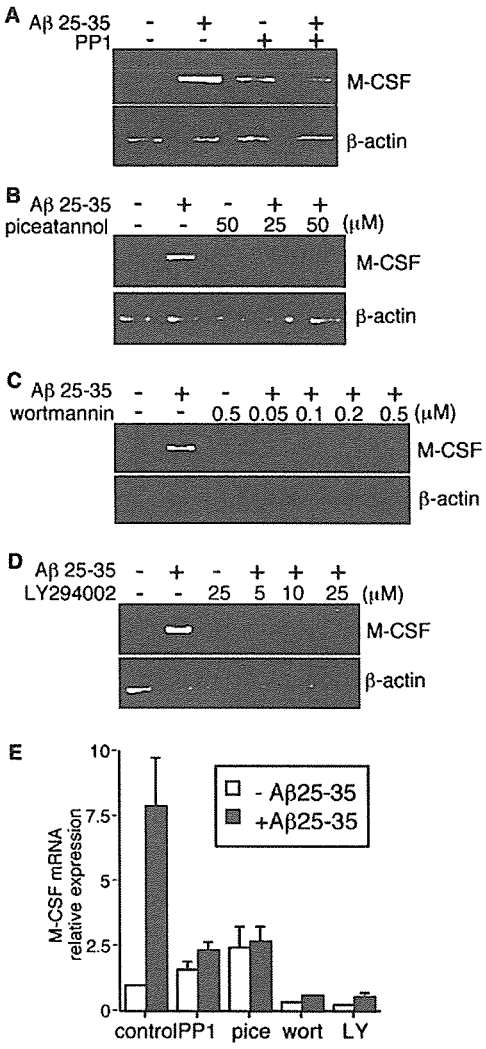

Fig. 3. Signal transduction for M-CSF mRNA expression induced by Aβ. (A–D) M-CSF and β-actin mRNA expression were determined by RT-PCR. Ra2 cells were preincubated with 10 μM PP1 (A), wortmannin (C), LY294002 (D) for 30 min or piceatannol (B) for 1 h before treatment of 50 μM Aβ25–35 for 6 h. Beacuse all inhibitors were dissolved in DMSO, control cells were treated with DMSO. (E) M-CSF mRNA expressions were measured by real-time PCR (pice, piceatannol; wort, wortmannin; LY, LY294002). Data represent means ± S.D. values of three separate determinations.

the Src family and Syk tyrosine kinases and the PI3-kinase in microglia.

3.4. Aβ activates Akt signaling pathway in microglia

We examined whether Akt was involved in the Aβ-induced M-CSF expression in Ra2 cells. Akt inhibitor blocked the increase of M-CSF mRNA expression (Fig. 4A). Immunoblotting analysis revealed that Akt was transiently phoshorylated at serine 473 by Aβ (Fig. 4B). LY294002 and PP1 suppressed the phosphorylation of Akt induced by Aβ (Fig. 4D). Piceatannol also blocked the phosphorylation of Akt (Fig. 4E). Because tyrosine kinases and PI3-kinase activate MEK/Erk/Elk [25,26], we examined whether these signal pathways were related to M-CSF mRNA expression induced by Aβ. Aβ induced MEK and Erk1/2 phosphorylation in Ra2 cells. However, specific inhibitors of MEK, U0126 and PD98059 did not inhibit Aβ-induced M-CSF mRNA expression (data not shown). These results indicate that the tyrosine kinases, Src family and Syk, and the PI3-kinase activate Akt for Aβ-induced M-CSF expression.

3.5. Aβ activates NF-κB via PI3-kinase signal cascade

Because NF-κB is a target of Akt [27], next we examined if IκBα phosphorylation was induced by Aβ. The phosphorylation of IκBα on serine 32 results in the release and nuclear translocation of active NF-κB [28]. IκBα was phosphorylated time-dependently, the phosphorylation peaked at 60 min and then declined (Fig. 4C). The phosphorylation was inhibited by LY294002 and PP1 (Fig. 4D). Piceatannol also blocked the phosphorylation of IκBα (Fig. 4E).

The M-CSF promoter region has a putative NF-κB binding site at −369–378 bp from the transcriptional start site [29]. To investigate whether this NF-κB binding site is associated with Aβ-induced M-CSF expression, EMSA was carried out with nuclear extracts prepared from untreated and Aβ-treated Ra2 cells. The amount of protein binding to the NF-κB probe was increased by Aβ-treatment (Fig. 5A, compare lanes 2 and 3). NF-κB binding activity was almost completely eliminated by adding an excess of the unlabeled NF-κB probe but not by the unlabeled AP1 probe (Fig. 5A, lanes 6 and 7). Anti-p50 antibody supershifted the complexes (Fig. 5A, lane 4) and anti-p65 antibody partially disrupted the DNA binding of the complexes (Fig. 5A, lane 5). To examine whether the Aβ-induced increase in nuclear NF-κB binding activity correlated with tyrosine kinase and PI3-kinase, Ra2 cells were preincubated with chemical inhibitors before treatment with Aβ. LY294002 reduced Aβ-induced binding to the NF-κB probe and piceatannol blocked the DNA-binding complex (Fig. 5B, lanes 4–7). These results indicate that Aβ enhances the binding of NF-κB to M-CSF promoter via the Syk tyrosine kinase and the PI3-kinase.

We have shown in the present study that Aβ proliferates microglia and induces M-CSF via the PI3-kinase/Akt/NFκB signal pathways. It has been reported that Aβ binds to CD36, which transduces signals via tyrosine kinase [6,20]. CD36 may participate in the initiation of intracellular signaling to M-CSF expression. RAGE also has been reported to induce NF-κB activation to M-CSF production [30]. Further works are needed to prove the receptors of Aβ, which induces PI3-kinase/Akt/NF-κB signal pathways to M-CSF mRNA expression. Aβ increases production of reactive oxygen species (ROS) and activates Akt in neural cells [23]. And in microglia CD36 mediates production of ROS in response to Aβ [31]. We found that antioxidants such as reduced glutathione and α-tocopherol slightly blocked M-CSF mRNA expression (data not shown). Also in microglia, ROS may partly participate in activating the signal cascade to M-CSF expression. It is important to reveal the relation among the receptors of Aβ, production of ROS and signal cascades.

Monsonego et al. [32] showed that activated microglia migrated outside the brain and could present Aβ peptide to T lymphocytes. Further analysis of microglial activation may reveal the immunological mechanism of AD, and may enhance the prospects of immune manipulation to prevent AD.

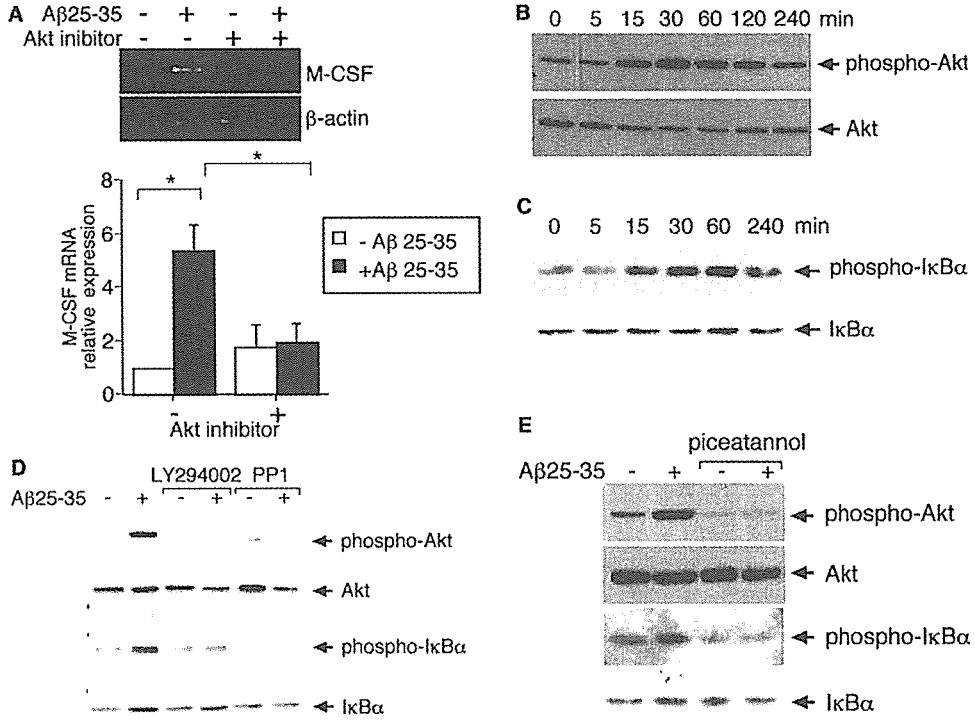

Fig. 4. Aβ-induced Akt and IκB phosphorylation through tyrosine kinase and PI3-kinase. (A) RT-PCR (top) and real-time PCR (bottom) of M-CSF mRNA. Ra2 cells were preincubated with or without 20 μM Akt inhibitor before treatment with 25 μM Aβ25–35 for 6 h. Data represent means ± S.D. values of three separate determinations. (*$P < 0.01$) (B–E) Immunoblotting analysis using anti-phospho Akt (Ser 473) or anti-phospho IκBα (Ser 32) antibody. The same blots were reprobed with anti-Akt or anti-IκB antibody. Ra2 cells were treated with Aβ25–35 for indicated times (B and C). Ra2 cells were preincubated with 25 μM LY294002 or 10 μM PP1 for 30 min or 50 μM piceatannol for 1 h before treatment with 50 μM Aβ25–35 for 30 min (D and E).

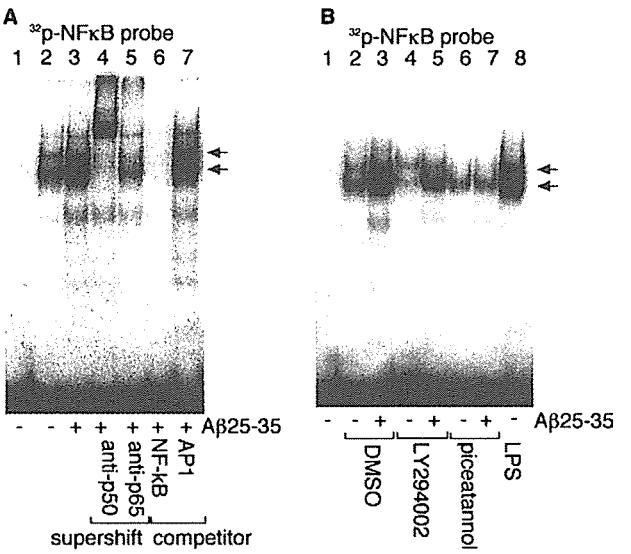

Fig. 5. NF-κB binding activity in EMSA. ^{32}P-labeled probe without nuclear extracts are showed in lane 1. (A) Ra2 cells were treated with 50 μM Aβ25–35 for 4 h (lanes 3–7). Anti-p50 or anti-p65 antibody was added to the extracts for supershift assay (lanes 4 and 5). Unlabeled competitor of NF-κB or AP1 probe was added to the extract (lanes 6 and 7). (B) Ra2 cells were preincubated with 25 μM LY294002 for 30 min (lanes 4 and 5) or 50 μM piceatannol for 1 h (lanes 6 and 7) before treatment with or without 50 μM Aβ25–35 for 4 h (lanes 2–7). Control cells were preincubated with DMSO (lanes 2 and 3). Nuclear extract of Ra2 cells treated with 1 μg/ml LPS for 4 h were used as a positive control (lane 8).

Acknowledgements: This study was supported by the Program for the Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research (of Japan), and in part by grants-in-aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

[1] Akiyama, H., et al. (2000) Inflammation and Alzheimer's disease. Neurobiol. Aging 21, 383–421.
[2] Husemann, J., Loike, J.D., Anankov, R., Febbraio, M. and Silverstein, S.C. (2002) Scavenger receptors in neurobiology and neuropathology: their role on microglia and other cells of the nervous system. Glia 40, 195–205.
[3] El Khoury, J., Hickman, S.E., Thomas, C.A., Cao, L., Silverstein, S.C. and Loike, J.D. (1996) Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. Nature 382, 716–719.
[4] Paresce, D.M., Ghosh, R.N. and Maxfield, F.R. (1996) Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. Neuron 17, 553–565.
[5] El Khoury, J.B., Moore, K.J., Means, T.K., Leung, J., Terada, K., Toft, M., Freeman, M.W. and Luster, A.D. (2003) CD36 mediates the innate host response to beta-amyloid. J. Exp. Med. 197, 1657–1666, Epub 2003 Jun 1609.
[6] Bamberger, M.E., Harris, M.E., McDonald, D.R., Husemann, J. and Landreth, G.E. (2003) A cell surface receptor complex for fibrillar beta-amyloid mediates microglial activation. J. Neurosci. 23, 2665–2674.
[7] Huang, M.M., Bolen, J.B., Barnwell, J.W., Shattil, S.J. and Brugge, J.S. (1991) Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. Proc. Natl. Acad. Sci. USA 88, 7844–7848.

[8] Bull, H.A., Brickell, P.M. and Dowd, P.M. (1994) Src-related protein tyrosine kinases are physically associated with the surface antigen CD36 in human dermal microvascular endothelial cells. FEBS Lett. 351, 41–44.
[9] Jimenez, B., Volpert, O.V., Crawford, S.E., Febbraio, M., Silverstein, R.L. and Bouck, N. (2000) Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat. Med. 6, 41–48.
[10] Verdier, Y. and Penke, B. (2004) Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. Curr. Protein Pept. Sci. 5, 19–31.
[11] Sawada, M., Imai, F., Suzuki, H., Hayakawa, M., Kanno, T. and Nagatsu, T. (1998) Brain-specific gene expression by immortalized microglial cell-mediated gene transfer in the mammalian brain. FEBS Lett. 433, 37–40.
[12] Sawada, M., Suzumura, A., Hosoya, H., Marunouchi, T. and Nagatsu, T. (1999) Interleukin-10 inhibits both production of cytokines and expression of cytokine receptors in microglia. J. Neurochem. 72, 1466–1471.
[13] Hattori, T., Takei, N., Mizuno, Y., Kato, K. and Kohsaka, S. (1995) Neurotrophic and neuroprotective effects of neuron-specific enolase on cultured neurons from embryonic rat brain. Neurosci. Res. 21, 191–198.
[14] Maehara, K., Hasegawa, T., Xiao, H., Takeuchi, A., Abe, R. and Isobe, K. (1999) Cooperative interaction of NF-kappaB and C/EBP binding sites is necessary for manganese superoxide dismutase gene transcription mediated by lipopolysaccharide and interferon-gamma. FEBS Lett. 449, 115–119.
[15] Terzi, E., Holzemann, G. and Seelig, J. (1994) Reversible random coil-beta-sheet transition of the Alzheimer beta-amyloid fragment (25–35). Biochemistry 33, 1345–1350.
[16] Shearman, M.S., Ragan, C.I. and Iversen, L.L. (1994) Inhibition of PC12 cell redox activity is a specific, early indicator of the mechanism of beta-amyloid-mediated cell death. Proc. Natl. Acad. Sci. USA 91, 1470–1474.
[17] Pike, C.J., Walencewicz-Wasserman, A.J., Kosmoski, J., Cribbs, D.H., Glabe, C.G. and Cotman, C.W. (1995) Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25–35 region to aggregation and neurotoxicity. J. Neurochem. 64, 253–265.
[18] Araujo, D.M. and Cotman, C.W. (1992) Beta-amyloid stimulates glial cells in vitro to produce growth factors that accumulate in senile plaques in Alzheimer's disease. Brain Res. 569, 141–145.
[19] Nagele, R.G., Wegiel, J., Venkataraman, V., Imaki, H. and Wang, K.C. (2004) Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. Neurobiol. Aging 25, 663–674.
[20] Moore, K.J., El Khoury, J., Medeiros, L.A., Terada, K., Geula, C., Luster, A.D. and Freeman, M.W. (2002) A CD36-initiated signaling cascade mediates inflammatory effects of beta-amyloid. J. Biol. Chem. 277, 47373–47379.
[21] Combs, C.K., Johnson, D.E., Cannady, S.B., Lehman, T.M. and Landreth, G.E. (1999) Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. J. Neurosci. 19, 928–939.
[22] Zhang, C., Qiu, H.E., Krafft, G.A. and Klein, W.L. (1996) A beta peptide enhances focal adhesion kinase/Fyn association in a rat CNS nerve cell line. Neurosci. Lett. 211, 187–190.
[23] Martin, D., Salinas, M., Lopez-Valdaliso, R., Serrano, E., Recuero, M. and Cuadrado, A. (2001) Effect of the Alzheimer amyloid fragment Abeta(25–35) on Akt/PKB kinase and survival of PC12 cells. J. Neurochem. 78, 1000–1008.
[24] Luo, Y., Sunderland, T. and Wolozin, B. (1996) Physiologic levels of beta-amyloid activate phosphatidylinositol 3-kinase with the involvement of tyrosine phosphorylation. J. Neurochem. 67, 978–987.
[25] Tang, Y., Yu, J. and Field, J. (1999) Signals from the Ras, Rac, and Rho GTPases converge on the Pak protein kinase in Rat-1 fibroblasts. Mol. Cell. Biol. 19, 1881–1891.
[26] Jiang, K., Zhong, B., Gilvary, D.L., Corliss, B.C., Hong-Geller, E., Wei, S. and Djeu, J.Y. (2000) Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. Nat. Immunol. 1, 419–425.
[27] Romashkova, J.A. and Makarov, S.S. (1999) NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 401, 86–90.
[28] Traenckner, E.B., Pahl, H.L., Henkel, T., Schmidt, K.N., Wilk, S. and Baeuerle, P.A. (1995) Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. Embo J. 14, 2876–2883.
[29] Harrington, M.A., Edenberg, H.J., Saxman, S., Pedigo, L.M., Daub, R. and Broxmeyer, H.E. (1991) Cloning and characterization of the murine promoter for the colony-stimulating factor-1-encoding gene. Gene 102, 165–170.
[30] Lue, L.F., Walker, D.G., Brachova, L., Beach, T.G., Rogers, J., Schmidt, A.M., Stern, D.M. and Yan, S.D. (2001) Involvement of microglial receptor for advanced glycation endproducts (RAGE) in Alzheimer's disease: identification of a cellular activation mechanism. Exp. Neurol. 171, 29–45.
[31] Coraci, I.S., et al. (2002) CD36, a class B scavenger receptor, is expressed on microglia in Alzheimer's disease brains and can mediate production of reactive oxygen species in response to beta-amyloid fibrils. Am. J. Pathol. 160, 101–112.
[32] Monsonego, A., Imitola, J., Zota, V., Oida, T. and Weiner, H.L. (2003) Microglia-mediated nitric oxide cytotoxicity of T cells following amyloid beta-peptide presentation to Th1 cells. J. Immunol. 171, 2216–2224.

Journal of Neurochemistry, 2005, **94**, 393–404 doi:10.1111/j.1471-4159.2005.03209.x

Peripheral injection of lipopolysaccharide enhances expression of inflammatory cytokines in murine locus coeruleus: possible role of increased norepinephrine turnover

Yoko S. Kaneko,* Keiji Mori,* Akira Nakashima,* Makoto Sawada,† Ikuko Nagatsu,‡ and Akira Ota*

**Department of Physiology, †Joint Research Division for Therapies against Intractable Diseases, Institute for Comprehensive Medical Science, and ‡Department of Anatomy, Fujita Health University School of Medicine, Toyoake, Japan*

Abstract

Cytokines and catecholamines are known to constitute a significant portion of the regulatory neuroimmune networks involved in maintaining homeostasis in the central nervous system (CNS). Although we have already reported an increase in norepinephrine (NE) turnover within the locus coeruleus (LC) at 2 and 4 h after the intraperitoneal (i.p.) injection of lipopolysaccharide (LPS), the implication of this increase remains unclear. In view of evidence that norepinephrine (NE) acts in an anti-inflammatory manner by way of negatively regulating pro-inflammatory cytokine expression, we examined the inflammatory cytokine expression levels in the LC of C3H/HeN mice (male, 8 weeks old) after an i.p. LPS injection. The mRNA expression levels of the genes encoding IL-1β and TNF-α within the LC increased during the first 2 h, and showed two peaks, the first at 4 h and the second lesser one at 15 h after the LPS injection. Microglia, which are one of the major cell types that produce pro-inflammatory cytokines in the CNS, were isolated from mouse neonate brains in order to clarify more precisely the relationship between the changes in NE content and the up-regulation of inflammatory cytokines in the LC. Simultaneous incubation of microglia with LPS and NE enhanced the expression of IL-1β at both mRNA and protein levels, but reduced the mRNA and protein levels of TNF-α. These data support the hypothesis that NE negatively regulates the expression of pro-inflammatory cytokine expression, at least in the case of TNF-α, which action could contribute to the observed anti-inflammatory properties of NE. This report, based on the results of both *in vivo* and *in vitro* experiments, is the first to suggest a relationship between NE content and cytokine expression levels in the CNS.

Keywords: inflammatory cytokine, lipopolysaccharide, locus coeruleus, norepinephrine.

J. Neurochem. (2005) **94,** 393–404.

It is well established that lipopolysaccharide (LPS), an endotoxin released from the outer membranes of gram-negative bacteria, behaves as a stimulator of the nuclear factor (NF)-κB (Müller *et al.* 1993; Fischer *et al.* 1999; Fitzgerald *et al.* 2001) and as an activator of the immune system by stimulating the secretion of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 from macrophages through mitogen-activated protein (MAP) kinase signalling pathways (Zuckerman *et al.* 1989; Quan *et al.* 1994; Poltorak *et al.* 1998). About a decade ago, the results of an *in situ* hybridization study demonstrated that systemic administration of LPS to mice induced TNF-α mRNA expression in circumventricular organs and pericircumventricular nuclei, such as the arcuate nuclei of the hypothalamus and the nucleus of the solitary tract (Breder *et al.* 1994). Therefore, TNF-α produced by a cascade of events within the central nervous system (CNS) has been expected to participate in the complex autonomic, neuroendocrine, metabolic, and behavioural responses to infection and inflammation. Following this observation, numerous groups, including ours, began to

Received November 11, 2004; revised manuscript received January 23, 2005; accepted March 31, 2005.

Address correspondence and reprint requests to Akira Ota MD PhD, Department of Physiology, Fujita Health University School of Medicine, 1–98 Dengakugakubo, Kutsukake-cho, Toyoake 470–1192, Japan. E-mail: aota@fujita-hu.ac.jp

Abbreviations used: CNS, central nervous system; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor of NF-κB; IL-1β, interleukin-1β; LC, locus coeruleus; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MEM, minimal essential medium; NE, norepinephrine; NF-κB, nuclear factor-κB; TNF-α, tumour necrosis factor-α.

use systemic LPS administration as a model for infection and inflammation in the CNS.

Systemic LPS injection has been also reported to activate the hypothalamo–pituitary–adrenal (HPA) axis by way of triggering the transcription of the genes encoding corticotropin-releasing factor (CRF) and CRF type 1 receptor (CRFR1) in the paraventricular nucleus of the hypothalamus (Rivest and Laflamme 1995; Rivest *et al.* 1995). In addition, it was also reported that systemic LPS injection induced alterations in the biosynthesis and/or projection of catecholamines in areas such as the medial prefrontal cortex, paraventricular nucleus, arcuate nucleus, locus coeruleus (LC) and hippocampus (Lavicky and Dunn 1995; Lacosta *et al.* 1999).

Then, in light of the influence of peripheral LPS on CNS function, the effects of the intraperitoneal (i.p.) administration of LPS on GTP cyclohydrolase I (GCH) mRNA and protein expression levels in the LC in the mouse brain were investigated (Kaneko *et al.* 2001, 2003), basically for the following three reasons: (i) the murine *GCH* gene up to 681 nucleotides upstream of the initiation site reveals a putative NF-κB binding site located in a GC-rich region (Shimoji *et al.* 1999); (ii) GCH is a rate-limiting enzyme participating in the *de novo* biosynthetic cascade of tetrahydrobiopterin (BH4) (Nichol *et al.* 1985), which functions as a co-factor for nitric oxide synthase (Kwon *et al.* 1989; Tayeh and Marletta 1989) and tyrosine hydroxylase (Nagatsu *et al.* 1964), the latter being the rate-limiting enzyme in the catecholamine biosynthetic pathway; and (iii) LC contains the greatest number of NE cell bodies in the brainstem and has been repeatedly implicated in stress responses. Although the above studies revealed the fact that peripherally injected LPS enhanced norepinephrine (NE) turnover within 2–4 h in the LC, accompanied by an increase in GCH expression at the site, the physiological roles assigned to such a quick elevation of NE turnover in the LC in response to peripheral LPS injection were not precisely addressed.

In contrast, it is generally recognized that pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6, as well as reactive oxygen species such as nitric oxide (NO), can provoke damage in neurological diseases (del Zoppo *et al.* 2000; Rothwell and Luheshi 2000; Asensio and Campbell 2001; Benveniste *et al.* 2001; Feinstein *et al.* 2002) including multiple sclerosis (Cannella and Raine 1995), Parkinson's disease (Nagatsu *et al.* 2000), Alzheimer's disease (Akiyama *et al.* 2000), and AIDS (Tyor *et al.* 1992). Although pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 may play an important role against infection in the CNS, they can become harmful when produced excessively over a prolonged period of time. Thus, activation of the inflammatory response in the CNS must be tightly regulated in order to prevent the potentially cytotoxic mediators, including cytokines and reactive oxygen species, from accumulating in the CNS. In addition to blood-derived cells, microglia also participate in releasing cytokines and inflammatory mediators in the CNS (Lee *et al.* 1995; Lee and Brosnan 1997; Benveniste 1998).

NE is considered to be involved in the regulation of cytokine production in microglia for the following reasons. Microglia, which are major cells that produce pro-inflammatory cytokines in the CNS, possess functional β1- and β2-adrenergic receptors (Mori *et al.* 2002; Tanaka *et al.* 2002). Stimulation of β-adrenergic receptors of microglia inhibited the release of IL-12 from the cells (Prinz *et al.* 2001). Many laboratories have reported that NE can regulate nitric oxide synthase by binding to β-adrenergic receptors to trigger a cAMP and protein kinase A-dependent pathway in microglia and astrocytes (Feinstein *et al.* 1993; Feinstein 1998; Galea and Feinstein 1999; Cho *et al.* 2001; Gavrilyuk *et al.* 2002; Dello Russo *et al.* 2004). In addition, several reports indicate that a diminished NE level or perturbation of the NE-signalling system exacerbate the neuroinflammation that occurs in patients suffering from multiple sclerosis or Alzheimer's disease (Mann *et al.* 1983; De Keyser *et al.* 1999; Heneka *et al.* 2002, 2003).

In view of all available data taken altogether, we proposed the working hypothesis that the enhanced NE turnover in the LC caused by a peripheral injection of LPS protects the area surrounding the LC, as well as the areas to which the LC-derived NE neurons project, from the propagation of a cascade of inflammatory events elicited by the expression of pro-inflammatory cytokines. In order to test our working hypothesis, we analyzed the mRNA expression levels of pro-inflammatory cytokines and IκB in the LC obtained from C3H/HeN mice intraperitoneally (i.p.)-injected with LPS and the expression level of mRNA and protein in murine microglia in primary culture incubated with LPS and/or NE. Our findings are interpreted and discussed in this paper.

Materials and methods

Chemical reagents

LPS from *Escherichia coli*, sero-type 026:B6, was purchased from Sigma Chemical Company (St Louis, MO, USA). Other reagents used in this study were of analytical grade and were purchased, unless otherwise stated, from Sigma.

Animals

Eight-week-old C3H/HeN male mice, which are LPS sensitive (Poltorak *et al.* 1998; Qureshi *et al.* 1999), were obtained from S.L.C. (Hamamatsu, Japan). The mice were kept under conditions of a 12-h light/12-h dark cycle (lights on from 08.00 to 20.00 h), constant temperature (23–25°C), and free access to food and water. The use of animals was kept to the minimum necessary to validate the data, and all animal protocols were carried out according to the National Institute of Health guidelines for the care and use of laboratory animals.

Preparation of LC samples

Brain samples containing the LC (designated as 'LC sample') were prepared chronologically after i.p. injection with 5 μg of LPS or saline as already reported (Kaneko *et al.* 2001).

Microglia from primary cultures

Microglia were harvested from mixed populations of glial cells in primary cultures prepared from neonatal C3H/HeN mouse pups as previously reported (Suzumura *et al.* 1987; Sawada *et al.* 1990). In brief, after the meninges had been carefully removed, the neonatal brain was dissociated by pipetting. The cell suspension was then plated in 75-cm^2 culture flasks (Falcon 3024; Becton-Dickinson Japan, Tokyo, Japan) at the density of one brain per flask in 10 mL of Eagle's minimum essential medium (MEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sanko Jun-yaku Co., Chiba, Japan), 5 μg/mL bovine insulin (Sigma), and 0.2% glucose. Microglia were isolated on days 14–21 by the 'shaking off' method of Suzumura *et al.* (1987). The reverse transcriptase–polymerase chain reaction (RT–PCR) was performed to detect PLP/DM-20 expression, and the results revealed that the microglia population contained neither O2-A progenitors, oligodendrocytes nor astrocytes. Then, the microglia were incubated with graded doses of LPS or NE, or with the combination of LPS and NE for the appropriate periods.

Measurement of mRNA expression levels by using quantitative real-time PCR

Total RNA was extracted from the LC samples or from 5×10^5 microglia by using RNA isolation reagent (Isogen; Nippon Gene Co.; Tokyo, Japan) according to the manufacturer's instructions. Synthesis of complementary DNA (cDNA) was performed by using 1 μg of total RNA and oligo $(dT)_{12-18}$ primer in a reverse transcription reaction (Life Technologies; Tokyo, Japan). Each primer pair used for the quantitative real-time PCR (Table 1) generated a single band with the predicted size from cDNA on a conventional PCR (data not shown). cDNA prepared from the LC sample was also used to produce the standard samples.

Quantitative real-time PCR for the sample comparable with 50 ng of cDNA was performed on an ABI 7700 PCR Instrument (Perkin Elmer Biosystems, Foster City, CA, USA) with SYBR Green (Qiagen, Hilden, Germany) according to the manufacture's instructions. Each sample was tested in triplicate by quantitative real-time PCR, and five mice and five dishes (6-cm diameter) of microglia for each group were used to calculate the means and SD. Calculated data were corrected by reference to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression level.

Measurement of cytokine protein levels in culture supernatant by using enzyme-linked immunosorbent assays (ELISA)

Prepared microglia (1×10^5 cells per well in a 24-well plate) were treated with 0.1 μg/mL LPS and/or 1 μM NE in the presence of fetal calf serum (Sanko Jun-yaku). The supernatants of the microglia cultures were collected at 6 h after LPS and/or NE stimulation, and the concentrations of IL-1β and TNF-α were measured by ELISA using monoclonal antibodies against the respective cytokines [no. MLB00 for IL-1β (BD Biosciences Pharmingen, San Diego, CA, USA); KMC0011 for IL-1β, KMC3011 for TNF-α (BioSource, Sunnyvale, CA, USA)]. ELISA was performed according to the procedure recommended by the supplier. The serum in the media neither affected the absorbance nor interfered with the assay.

Immunoprecipitation of secreted IL-1β and TNF-α protein in culture supernatant

The supernatants of the microglia cultures, which were prepared and treated as above, were collected at 6 h after LPS and/or NE stimulation. Five hundred microlitres of supernatant was incubated with the anti-IL-1β polyclonal antibody (sc-1251, 1 : 100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TNF-α polyclonal antibody (sc-1351, 1 : 100 dilution; Santa Cruz Biotechnology) or normal goat serum (Santa Cruz Biotechology). The mixtures were gently agitated for 1 h at 4°C, after which 20 μL of Protein G agarose beads (sc-2002, Santa Cruz Biotechnology) was added; and then incubation was continued for 1 h at 4°C. The beads

Table 1 Primers for real-time PCR

Gene name	Genebank accession number	Primer orientation	Nucleotide sequence (from 5′ to 3′)	Starting sequence position	Size of the PCR amplicon (bp)
Mouse IL-1β	M15131	Forward	GAGCCCATCCTCTGTGACTCAT	298	83
		Reverse	AGCCTGTAGTGCAGCTGTCTAATG	380	
Mouse TNF-α	M38296	Forward	GACCCTCACACTCAGATCATCTT	228	77
		Reverse	CCACTTGGTGGTTTGCTACGA	304	
Mouse NF-κB p65	M61909	Forward	CAGACCCAGGAGTGTTCACAGA	1370	81
		Reverse	ACATGGACACACCCTGGTTCA	1450	
Mouse IκBα	U36277	Forward	GTGATCCTGAGCTCCGAGACTT	404	93
		Reverse	TCTGCGTCAAGACTGCTACACTG	496	
Mouse I IκBβ	NM_0109081	Forward	CCCACTCCATGTAGCTGTCATC	627	93
		Reverse	CACGTAGGCTCCGGTTTATTGA	719	
Mouse caspase-1	BC008152	Forward	GAATACAACCACTCGTACACGTCTTG	465	120
		Reverse	AGATCCTCCAGCAGCAACTTCA	584	
Mouse GADPH	NM_008084	Forward	ATGTGTCCGTCGTGGATCTGA	710	81
		Reverse	ATGCCTGCTTCACCACCTTCT	790	

were washed three times with IP-kinase buffer [50 mM HEPES (pH 8.0), 150 mM NaCl, 25 mM EGTA, 1 mM EDTA, 0.1% Tween 20, and 10% glycerol], and the proteins bound to them were released and dissolved in Laemmli's sample buffer. Then, the samples were immunoblotted as described in the next chapter.

Immunoblot analysis

Detection of IL-1β and TNF-α in culture supernatant and in microglia

Microglia prepared (1×10^6 cells in a 6-cm dish) were lysed in IP-kinase buffer containing a cocktail of protease inhibitors (P8340, Sigma). The immunoprecipitated samples and the cell lysate (10 μg of proteins) were separated by sodium dodceyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) (15% acrylamide for immunoprecipitated samples, and 10% acrylamide for the cell lysate samples) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA, USA) by using the KS-8640™ blotting system (Marysol, Tokyo, Japan). The membranes were blocked with 3% skimmed milk in phosphate-buffered saline containing 0.5% Tween 20 (PBST) and then incubated with primary antibodies against mouse IL-1β (goat anti-mouse IL-1β polyclonal antibody, sc-1251, 1 : 1000 dilution; Santa Cruz Biotechnology) or those against mouse TNF-α (goat anti-mouse TNF-α polyclonal antibody sc-1351, 1 : 500 dilution; Santa Cruz Biotechnology). After the membrane had been washed thoroughly in PBST, horseradish peroxidase-conjugated secondary antibody (sc-2768, 1 : 5000 dilution; Santa Cruz Biotechnology) was applied to the membrane. Finally, the blots were developed by using an enhanced chemiluminescence detection kit (Amersham Biosciences, Uppsala, Sweden). The densitometric scanning of the corresponding band was performed and evaluated by using an Image Reader coupled with Image Gauge (Science Laboratory 98, Fujifilm, Tokyo, Japan).

Detection of caspase-1 precursor protein and its two subunits in microglia

Procedures to detect caspase-1 precursor protein and its p10 and p20 subunits in the cell lysates of microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 6 h were the same as those described in the previous chapter. The primary antibodies used were rabbit anti-mouse caspase-1 p10 polyclonal antibody (sc-514, 1 : 500 dilution; Santa Cruz Biotechnology) and goat anti-mouse caspase-1 p20 polyclonal antibody (sc-1597, 1 : 500 dilution; Santa Cruz Biotechnology), respectively. Both of them are able to recognize caspase-1 precursor protein. The blotted membranes were also developed by using an enhanced chemiluminescence detection kit (Amersham Biosciences).

Immunohistochemistry to detect IL-1β and TNF-α in microglia

Microglia prepared as described above were treated with 0.1 μg/mL LPS and/or 1 μM NE for 6 h. The cells were harvested and incubated with anti-IL-1β antibody (sc-1251, 1 : 500 dilution; Santa Cruz Biotechnology) or anti-TNF-α antibody (sc-1351, 1 : 100 dilution: Santa Cruz Biotechnology) for 30 min at 25°C and then with 3% normal goat serum (Santa Cruz Biotechnology) for 10 min at 25°C. Next, they were incubated with biotinylated anti-goat IgG antibody (E1903, Santa Cruz Biotechnology) for 30 min at 25°C, and subsequently with avidin-biotin complex for 30 min at 25°C. Finally, the specimens were stained with 3,3′-diaminobenzidine-tetrahydrochloride and hydroperoxide.

Assay of enzymatic activity of caspase-1 in microglia

The assay for the enzymatic activity of caspase-1 using the Caspase-1/ICE Colorimetric Protease Assay Kit (Medical and Biological Laboratories, Nagoya, Japan) was performed according to the supplier's instruction. All buffers, dithiothreitol (DTT), and the substrate [tyrosine-valine-alanine-aspartate coupled with chromophore-*p*-nitroanilide (YVAD-*p*NA)] used in the assay were supplied in the kit. Briefly, cell pellets (2×10^6 cells) of microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 6 h were re-suspended in 50 μL of chilled cell lysis buffer and kept on ice for 10 min. After centrifugation at 10 000 *g* for 1 min, the supernatants were adjusted to 50 μL containing 200 μg protein. They were mixed with 50 μL 2 × reaction buffer containing 10 mM dithiothreitol and incubated with 5 μL of 4 mM YVAD-*p*NA (final concentration of the substrate: 200 μM) for 2 h at 37°C. Then, the *p*NA light emission was quantified in a microtitre plate at a 405 nm wavelength.

Statistics

All numerical data were expressed as the mean ± SD. Analysis of variance (ANOVA) was used to analyze all data. If the ANOVA revealed a significant overall effect, the significance of the differences between results was determined by Sheffe's *F*-test as a post-hoc test. For all statistical analyses, $p < 0.05$ was considered to be statistically significant.

Results

Time course of mRNA expression levels of the genes encoding pro-inflammatory cytokines, IκBα and NF-κB in the LC of C3H/HeN male mice

At first, the time course of the pro-inflammatory cytokine mRNA expression level in the LC of C3H/HeN male mice after 5 μg of LPS i.p. injection was investigated by using the quantitative real-time PCR method. IL-1β and TNF-α mRNA expression levels in the LC samples were measured at times ranging from 15 min to 5 days after the LPS or vehicle i.p. injection. The mRNA expression level of the gene encoding IL-1β in LPS-injected mice increased within 2 h and reached a maximum level at 4 h (Fig. 1a). The level in the LPS-injected group was 8.3-fold higher than that of the vehicle-injected one at 2 h and 13.1-fold higher at 4 h. Then, it decreased sharply at 6 h after the injection and approached the basal level. Surprisingly, the expression was again up-regulated to become 3.5-fold higher than that of vehicle-injected mice at 15 h after the i.p. injection (Fig. 1a). The mRNA expression level of the TNF-α gene was also up-regulated within 2 h and reached a maximum level between 2 and 4 h after the i.p. injection of LPS (Fig. 1b). At that time, the expression was about 4.5-fold higher than that of the vehicle-injected mice. Then, it decreased gradually, and again increased to become 1.9-fold higher than that of vehicle-injected mice at 15 h after the injection. The

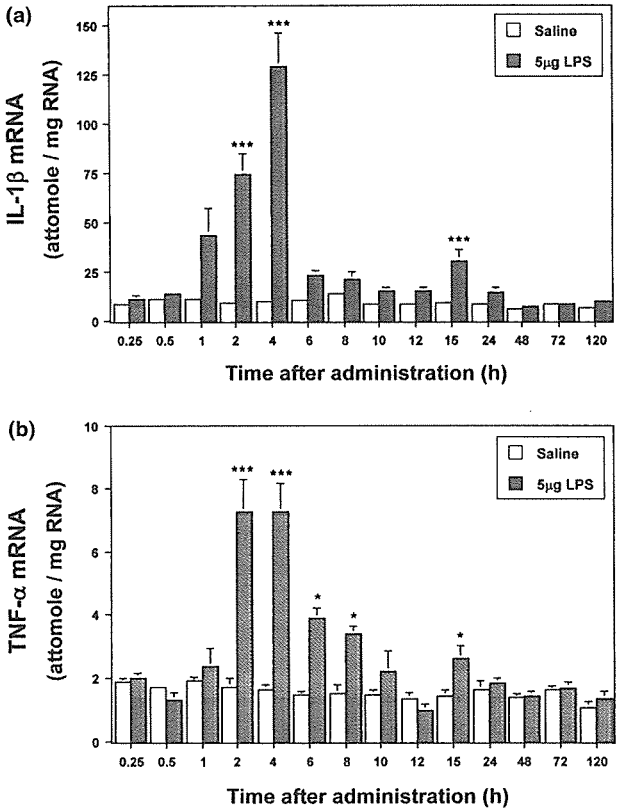

Fig. 1 Changes in mRNA expression levels of IL-1β (a) and TNF-α (b) in LC samples obtained from C3H/HeN mice injected i.p. with LPS. Mice (five mice per group) were i.p. injected with 5 μg of LPS or vehicle (saline), and decapitated at the indicated time points. Immediately after the decapitation, their brains were removed. Total RNA was extracted from LC samples and reverse transcribed to cDNA. Quantitative SYBR Green real-time PCR was performed by using the specific primers listed in Table 1 and cDNA as a template. The experiments were carried out in duplicate and repeated three times with similar results. Data are displayed as the mean (column) ± SD (bar) values. Each value marked with an asterisk was significantly greater than the corresponding one obtained from the vehicle-injected mice (control): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

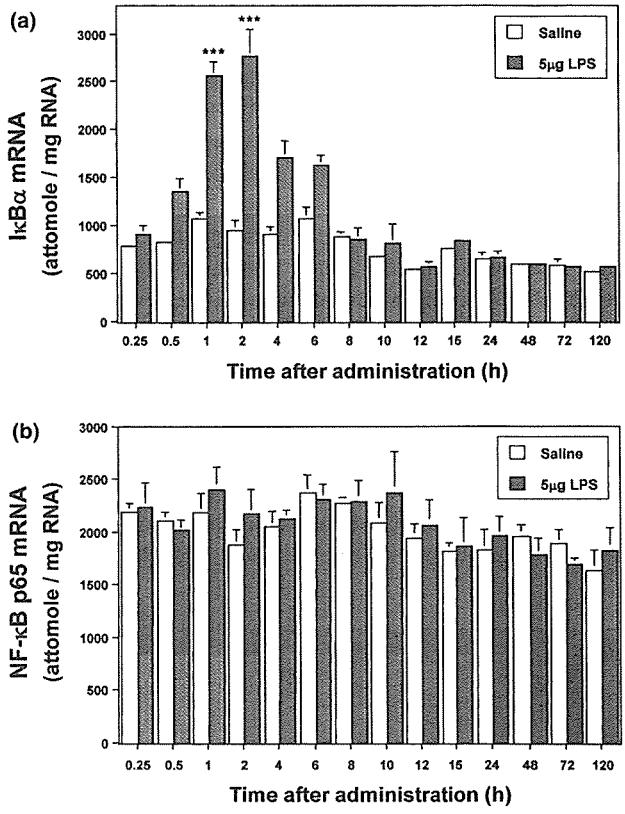

Fig. 2 Changes in mRNA expression levels of IκBα (a) and NF-κB (b) in LC samples obtained from C3H/HeN mice injected i.p. with LPS. Mice (five mice per group) were i.p. injected with 5 μg of LPS or vehicle (saline), and decapitated at the indicated time points. Immediately after the decapitation, their brains were removed. Total RNA was extracted from LC samples and reverse transcribed to cDNA. Quantitative SYBR Green real-time PCR was performed by using the specific primers listed in Table 1 and cDNA as a template. The experiments were carried out in duplicate and repeated three times with similar results. Data are displayed as the mean (column) ± SD (bar) values. Each value marked with an asterisk was significantly greater than the corresponding one obtained from the vehicle-injected mice (control): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

time-course profile of mRNA expression levels of IL-1β and TNF-α was very similar in that they responded very quickly to the i.p. injection of LPS and the second peak appeared at 15 h after the peripheral LPS injection.

As a next step, we measured the mRNA expression level of the gene encoding IκBα in the LC samples. As shown in Fig. 2(a), the IκBα mRNA expression level increased to a statistically significant level within 1 h after the LPS injection and reached a maximum level at 2 h. Its expression level in LPS-injected mice became 2.4-fold higher at 1 h and 2.9-fold higher at 2 h than that of vehicle-injected mice, at the respective times (Fig. 2a). It then decreased at 4 h post-injection and declined to almost the same level found for the vehicle-injected mice at 8 h after the injection. Contrary to the expression levels of IL-1β and TNF-α mRNAs, the IκBα mRNA expression level did not show any difference from that of the vehicle-injected mice thereafter. Next, we measured the mRNA expression level of the gene encoding NF-κB p65. However, no significant enhancement of its mRNA expression level following the peripheral LPS injection was observed (Fig. 2b).

Effects of LPS and/or NE on expression levels of IL-1β mRNA and TNF-α mRNA in microglia

It has been reported that pro-inflammatory cytokine expression is negatively regulated by NE, which could contribute to the observed anti-inflammatory properties of NE. Therefore, we conducted experiments using a cell culture system in

order to elucidate the relationship between the increase in NE content and the up-regulation of pro-inflammatory cytokines in the LC at 2 and 4 h after LPS injection.

Incubation of primary-cultured microglia with 0.1 μg/mL LPS for 1 h enhanced the expression level of IL-1β mRNA, making it about 25-fold higher, and that of TNF-α mRNA about 160-fold higher compared with those levels for the control cells (Figs 3a and b). Although the incubation of microglia with 1 μM NE for 1 h enhanced the expression levels of IL-1β mRNA 4.8-fold higher compared with that of control cells (Fig. 3a), the increase did not attain a statistically significant difference. As described above, the expression level of TNF-α mRNA was not affected by the incubation with 1 μM NE for 1 h (Fig. 3b, closed column). Incubation of cells with a combination of 0.1 μg/mL LPS and 1 μM NE for 1 h magnified the IL-1β mRNA expression, causing the level to be 2.1-fold higher than the one obtained from the incubation with LPS alone (Fig. 3a). However, TNF-α mRNA expression level enhanced by the incubation with 0.1 μg/mL LPS alone was suppressed to 40% by the addition of 1 μM NE to the incubation medium (Fig. 3b).

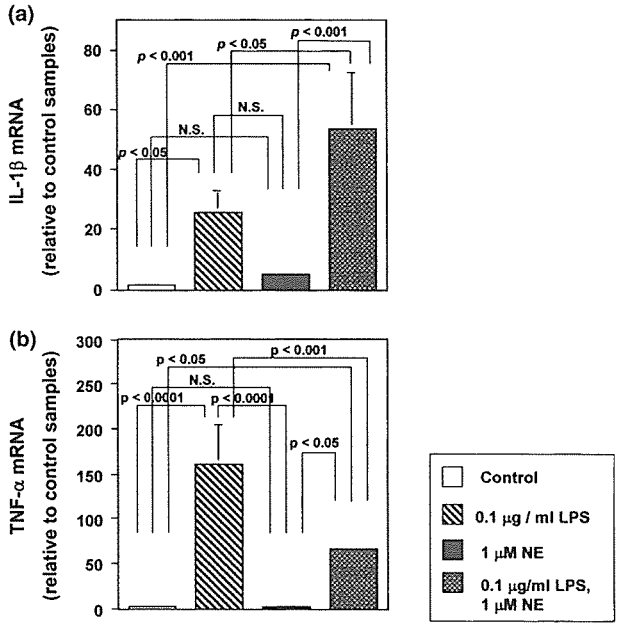

Fig. 3 The mRNA expression of IL-1β (a) and TNF-α (b) in microglia. Microglia (5×10^5 cells per 6-cm-diameter dish) prepared from primary cultures were incubated for 1 h with 0.1 μg/mL LPS and/or 1 μM NE in MEM containing 10% FBS. Total RNA was extracted from the treated microglia and reverse transcribed to cDNA. Quantitative real-time PCR was performed by using the specific primers listed in Table 1 and cDNA as a template. The amounts of IL-1β mRNA ((a) and TNF-α mRNA (b) were measured by using the SYBR Green real-time PCR method. The experiments were carried out in duplicate and repeated three times with similar results. Data shown in Fig. 3 were standardized by reference to GAPDH mRNA expression level and normalized to the standardized values obtained from control samples. They are displayed as the mean (column) ± SD (bar) values. All *p*-values for the comparisons performed are shown in the figures.

Effects of LPS and/or NE on expression levels of IL-1β and TNF-α proteins in microglia

ELISA method

The protein amounts of pro-inflammatory cytokines released into the medium of microglia cultures were measured by using the ELISA method. Although the amounts of IL-1β protein released into the culture medium were measured by using two kinds of kits (see Materials and methods), their values were at or around the detection limits of the kits (data not shown).

The incubation of the cells with 0.1 μg/mL LPS for 6 h dramatically enhanced the amount of TNF-α protein released into the medium to a value about 240-fold higher than that for the control (Fig. 4a). The incubation of the cells with 1 μM NE for 6 h did not affect the amount of secreted TNF-α protein at all (Fig. 4a). The enhanced release of TNF-α protein by the incubation of the cells with 0.1 μg/mL LPS alone was suppressed to 29% by the addition of 1 μM NE to the incubation medium (Fig. 4a)

Immunoblot analysis

Because the amount of IL-1β protein released into the culture medium could not be measured by the ELISA method, it was evaluated by a method combining immunoprecipitation and immunoblotting. Culture media of microglia that were treated with LPS and/or NE were incubated with anti-mouse IL-1β antibody or anti-mouse TNF-α antibody to immunoprecipitate secreted proteins, respectively. Immunoprecipitated proteins were analyzed by immunoblotting (Fig. 4). Secreted IL-1β protein was not detected in the supernatant of microglia stimulated with LPS or LPS/NE (Fig. 4b, left panel). By contrast, the secreted TNF-α protein was detected in the supernatant of the cultures stimulated with LPS, and the amount of the protein was reduced by the treatment with NE (Fig. 4b, right panel) whose intensity scanned by the Image Reader coupled with Image Gauge was 30% of the one of the supernatant of the cells treated with LPS alone. The molecular weight of secreted TNF-α protein was about 17 kDa, which coincides with the mature form of mouse TNF-α.

As shown by the immunoblot results in Fig. 5, incubation of the cells with 0.1 μg/mL LPS for 6 h enhanced the intracellular amounts of both IL-1β protein and TNF-α protein. Intracellular IL-1β or intracellular TNF-α protein was scarcely detected when the cells were incubated with 1 μM NE for 6 h (Fig. 5). Incubation of the cells with a combination of 0.1 μg/mL LPS and 1 μM NE for 6 h resulted in a band of IL-1β protein of greater intensity to the 1.2-fold increase than that obtained from the incubation with

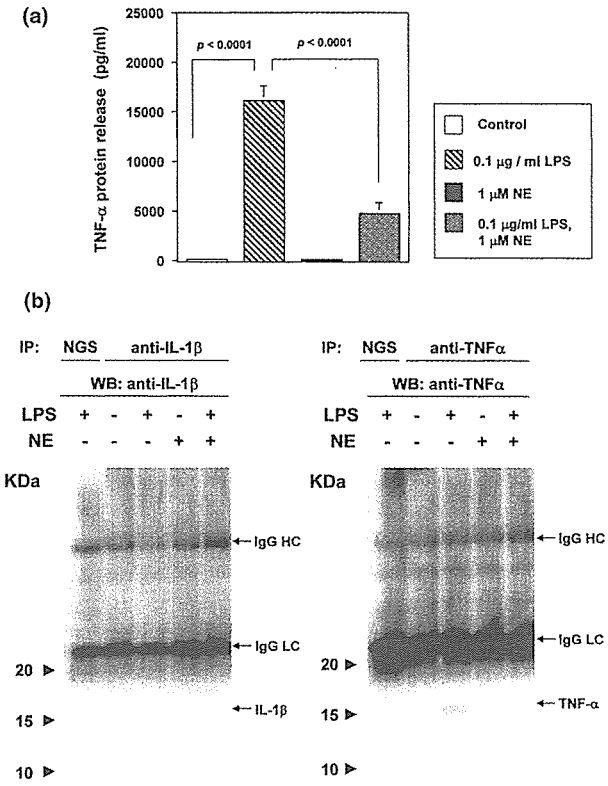

Fig. 4 The release of pro-inflammatory cytokines from microglia into culture supernatant. Microglia (1×10^5 cells per well in a 24-well plate) were incubated for 6 h with 0.1 μg/mL LPS and/or 1 μM NE in MEM containing 10% FBS. The amounts of IL-1β and TNF-α protein in the supernatants were measured by using the ELISA method described in Materials and methods. Data on the release of TNF-α into culture supernatant are displayed as the mean (column) ± SD (bar) values in (a). The serum added in the medium did not affect cytokine production. The experiments were carried out in duplicate and repeated three times with similar results. Contrary to TNF-α, IL-1β protein in culture supernatant was not detected by ELISA method. *p*-values not displayed in (a) are as follows: control versus NE-treated group, non-significant; control versus LPS/NE-treated group, $p < 0.0001$; LPS-treated group versus NE-treated group, $p < 0.0001$; NE-treated group versus LPS/NE-treated group, $p < 0.0001$. The amounts of IL-1β and TNF-α protein secreted into culture media were evaluated by the combination of immunoprecipitation and immunoblotting (b). The experiments were repeated three times with similar results, and representative data from one of them are shown. The densities of corresponding bands were scanned by Image Reader coupled with Image Gauge (see text). IgG HC, immunoglobulin heavy chain; IgG LC, immunoglobulin light chain; NGS, normal goat serum.

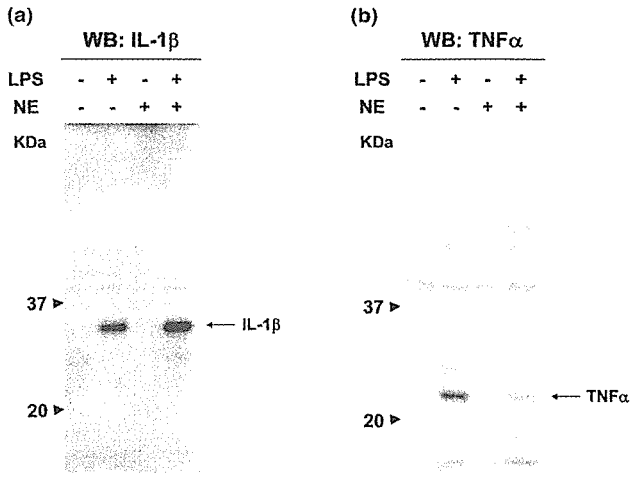

Fig. 5 Immunoblots of cell extracts prepared from microglia by using specific antibodies raised against IL-1β and TNF-α. Cell extracts prepared from microglia (1×10^6 cells) treated with or not with 0.1 μg/mL LPS and/or 1 μM NE for 6 h were subjected to immunoblot analysis as described in Materials and methods. The experiments were repeated three times with similar results. Representative data are shown in IL-1β (a) and TNF-α (b). The densities of corresponding bands were scanned by Image Reader coupled with Image Gauge (see text).

0.1 μg/mL LPS alone (Fig. 5a), whereas the band of TNF-α protein was reduced to 40% in its intensity under the same conditions (Fig. 5b). It should be noted that, in Fig. 5, the molecular weight of intracellular IL-1β protein was about 31 kDa, and that of TNF-α was about 25 kDa. These values coincide with those of the immature forms of the proteins of mouse IL-1β and mouse TNF-α. Bands corresponding to the processed forms of IL-1β (17 kDa) and TNF-α (17 kDa) proteins were not detected in the immunoblot analysis with using the cell lysates of microglia stimulated with LPS and/or NE (Fig. 5).

Immunohistochemical analysis

The data obtained by the immunoblot method were confirmed by using an immunohistochemical method (Fig. 6). The presence of both IL-1β and TNF-α proteins in microglia stimulated with 0.1 μg/mL LPS for 6 h was observed (Figs 6b and f). The cells incubated with 1 μM NE for 6 h expressed a little higher amount of IL-1β protein than the control cells (Fig. 6c), but did not produce any more TNF-α protein than the control ones (Fig. 6g). Six-hour incubation of the cells with 0.1 μg/mL LPS plus 1 μM NE increased the intracellular amount of IL-1β protein (Fig. 6d) and reduced that of TNF-α protein (Fig. 6h) compared with the levels found with LPS treatment only.

Effects of LPS and/or NE stimulation of microglia on caspase-1 in the cells

On mRNA expression level

We measured caspase-1 mRNA expression levels in microglia by using real-time PCR amplification to investigate whether caspase-1 mRNA expression levels in microglia were affected by LPS and/or NE stimulation. The pair of the primers used in the amplification is shown in Table 1. mRNA

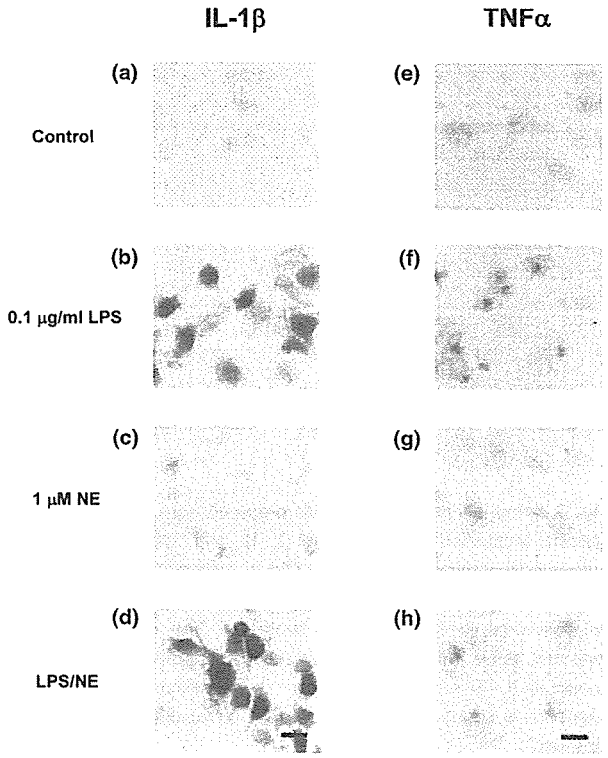

Fig. 6 Immunohistochemical staining of IL-1β and TNF-α proteins in microglia. Microglia (5×10^4 cells per cover glass in a 6-well plate) were incubated for 6 h with 0.1 μg/mL LPS and/or 1 μM NE in MEM containing 10% FBS. The cells were stained with anti-IL-1β antibody (a–d) or anti-TNF-α antibody (e–h) as described in Materials and methods. (a, e) Control cells, (b, f) cells incubated with LPS, (c, g) cells incubated with NE and (d, h) cells incubated with a combination of LPS and NE. The experiments were repeated three times with similar results and representative data are shown. Scale bar = 50 μm.

was extracted from microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 1 h. Caspase-1 mRNA was constitutively expressed in microglia and its expression level was not affected at all by the stimulation of LPS and/or NE (data not shown).

On protein expression level

On the immunoblot analysis, the expression level of 45 kDa caspase-1 precursor protein in microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 6 h was not affected by LPS and/or NE treatment. Furthermore, 45 kDa caspase-1 precursor protein in microglia under LPS and/or NE treatment was not cleaved to produce 10 kDa and 20 kDa subunits (data not shown).

On enzymatic activity

The enzymatic activity of caspase-1 in microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 6 h was below the detection limit of the assay.

Effects of LPS and/or NE on mRNA expression levels of the genes encoding IκBα, IκBβ, and NF-κB in microglia

Finally, the effects of NE on the mRNA expression levels of the genes encoding IκBα, IκBβ and NF-κB in primary-cultured microglia were investigated by using the semiquantitative real-time PCR method (Fig. 7). IκBα mRNA expression in the cells incubated with 0.1 μg/mL LPS for

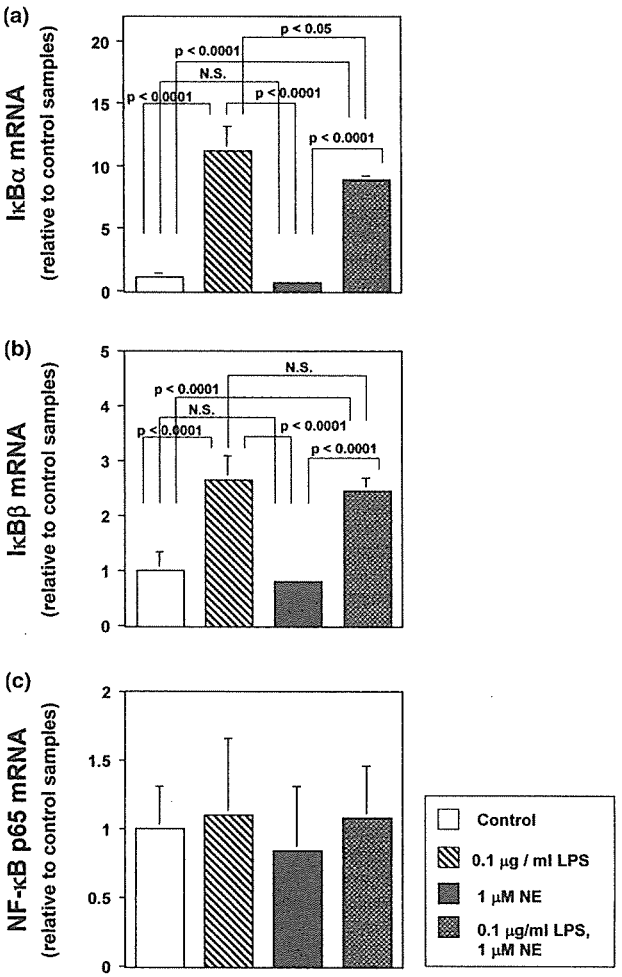

Fig. 7 The mRNA expression of transcription factors IκBα (a), IκBβ (b) and NF-κB p65 (c) in microglia. Microglia (5×10^5 cells per 6 cm-diameter dish) were incubated for 1 h with 0.1 μg/mL LPS and/or 1 μM NE in MEM containing 10% FBS. Total RNA was extracted from the treated microglia and reverse transcribed to cDNA. Quantitative real-time PCR was performed by using the specific primers listed in Table 1 and cDNA as a template. The expression levels of IκBα mRNA (a), IκBβ mRNA (b) and NF-κB p65 mRNA (c) were measured by using the SYBR Green real-time PCR method as described in Materials and methods. The experiments were carried out in duplicate and repeated three times with similar results. Data shown in the Fig. 7 were standardized by reference to GAPDH mRNA expression level and normalized to the standardized values obtained from control samples. They are displayed as the mean (column) ± SD (bar) values. All *p*-values for the comparisons performed are shown in the figures.