The role of immune activation in Alzheimer's disease

Het drukken van dit proefschrift is mede mogelijk gemaakt door financiële bijdrage van:
Internationale Stichting Alzheimer Onderzoek
Biosource BV

Cover: “Vervaging”
door mijn moeder, Wilma Smits

Gedrukt door: Ponsen & Looijen BV, Wageningen

ISBN: 90-393-2815-3

The role of immune activation in Alzheimer's disease

De rol van immuunactivatie in de ziekte van Alzheimer

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op vrijdag 5 oktober 2001 des ochtends te 10.30 uur

door

Hessel Adriaan Smits

Geboren op 23 augustus 1971 te Heerlen

Promotor: Prof. Dr. J. Verhoef

Co-promotor: Dr. H.S.L.M. Nottet

“We can’t solve problems
by using the same kind of thinking
we used when we created them.”

Albert Einstein

Voor Nathalie
en mijn ouders

Contents

1

Introduction

European Journal of Clinical Investigation (2000), 30: 526-535.
European Journal of Clinical Investigation (2001), in press.

1.1 General Introduction

During the development of the central nervous system (CNS), and in several neurological disorders, remodeling or alteration of neuronal networks occurs. These events are associated with tissue recruitment of mononuclear phagocytes and transient accumulations of brain macrophages. For instance, appearance of macrophages is an early response to experimental tissue lesions of various origins including ischemia (1), mechanical trauma (2), infections (3), autoimmune inflammation (4), or neuronal death induced by local injections of neurotoxins (5). Beyond the obvious task of cell debris removal, histological and neuropathological observations raise the issue of influence of macrophages on neuronal death and morphological remodeling of neurons. These cells have been proposed to play a pathogenic role in stroke and trauma and a variety of CNS diseases, such as multiple sclerosis (6), Parkinson's disease (7), Huntington's disease (8), human immunodeficiency virus type 1-associated dementia (HAD) (9), Alzheimer's disease (AD) (10) and recently also in Creutzfeldt-Jakob disease (11,12). Indeed, strategies to reduce the recruitment of macrophages into the CNS reduced ischaemic injury in the spinal cord (1) and suppressed experimental allergic encephalitis (4), the animal model disease for multiple sclerosis. The precise mechanisms by which macrophages mediate neuronal cell injury in these CNS disorders are still incompletely understood. Macrophages also play a role in neuroregeneration. For instance, macrophages are known to produce neurotrophic factors like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (13). Indeed, a study by Boven et al. shows an elevation of NGF and basic fibroblast growth factor (bFGF) in perivascular areas of HAD patients' brains, suggesting that these molecules may exert different actions depending on the area in which they are generated; when produced by reactive astrocytes throughout the brain parenchyma they may function as a neuronal survival factor whereas in perivascular areas they may be associated with endothelial injury (14).

1.2 Introduction to Alzheimer's disease: clinical and pathology

AD is a dementing disorder causing severe and permanent impairment of multiple cognitive faculties (15). The definition of AD may vary according to the purposes for which it is used. However, the most broadly applied criteria for the clinical definition of AD was introduced by the work group of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) in the United States (15). According to these criteria AD can be classified into definite, possible and probable levels of diagnostic certainty. Neuropsychological tests together with documented standardized mental status assessments are required and are used for establishing dementia. Supportive symptoms, although not required for diagnosis, are deficits in language, perceptual recognition and impaired activities of daily living; a familial history of a similar disorder, especially if confirmed

pathological and normal or nonspecific routine tests such as spinal fluid examination and electroencephalography (16).

Neurodegenerative disorders, including AD, produce their clinical symptoms by disrupting to various degrees the normal circuitry of the brain. The histopathology of AD includes neuritic plaques, neurofibrillary tangles, AMY plaques, loss of synapses and neurons, granulovascular degeneration and amyloid angiopathy.

Three types of amyloid-related plaques are recognized in the brain of the AD patient (17). Plaques with large or poorly circumscribed amyloid are frequently referred to as diffuse plaques. These plaques are thought to represent the earliest stages of plaque formation and are found in areas of the brain that are not symptomatic in the course of the disease, such as the cerebellum and throughout the cerebral hemispheres. The neuritic plaque is a spherical structure with a diameter of 50 to 200 μm and consist of a central core of amyloid-β proteins surrounded by dystrophic neurites (16). Reactive microglia and astrocytes are found within the plaque and at the plaque periphery (18). The third type of plaques are those that are considered to be composed exclusively of amyloid and are designated amyloid plaques. Classically they are described as 'burned out' plaque.

Another important and widely spread pathological change in AD is the neurofibrillary tangle. Neurofibrillary tangles consist of paired helical filaments that occupy the neuronal cell body and may extend into dendrites but do not occur in the axon. Paired helical filaments consist of protofilaments arranged to form a tubule and containing abnormally phosphorylated τ-protein.

Making a definitive diagnosis of AD requires a histopathological examination and until recently criteria were based on the number of neuritic plaques present in the cortex in relation to age-related standards (19). These criteria have been revised by a work group sponsored by the National Institute on Aging and the Reagan Institute of the Alzheimer's Association in the United States and now emphasize on the presence of both neuritic plaques and neurofibrillary tangles in the neurocortex (20).

Next to amyloid-containing plaques and tangle-bearing neurons, the brains of AD patients have abundant AMY plaques. They are recently described as plaques composed of a protein that is coexistent with but distinct from hyperphophorylated τ-protein. AMY plaques contain no amyloid, although they are found only in brains that also have amyloid and occur in the same brain regions as amyloid plaques (21).

1.3 Amyloid-beta

Aβ is generated by sequential endoproteolysis of the amyloid precursor protein (APP) (22) and is constitutively secreted by many cells throughout life (23). APP is a large single spanning (type 1) membrane molecule with a yet undefined cellular function. Following synthesis in the endoplasmatic reticulum (ER), the protein is transported to the cell surface via the secretory pathway. In Golgi as well as during reinternalization in endosomes, APP is cleaved by β-secretase in its N-terminal domain (24-27). This cleavage results in the release of soluble APPβ (sAPPβ) into the extracellular fluids and generates

the membrane-associated C-terminal APP fragment C99 (Figure 1) (28). C99 is subsequently cleaved by γ-secretase in the C-terminal part of its transmembrane domain, liberating Aβ into the extracellular space (29). Heterologic cleavage by γ-secretase results in the production of Aβ peptides that vary in length between 39 to 43 amino acids (23). The most abundant form of Aβ is the Aβ(1-40) peptide that accounts for approximately 90% of the secreted Aβ peptides (30,31). Although Aβ(1-40) is fibrillogenic and present in neuritic plaques, it is not as neurotoxic as Aβ(1-42) and is not able to aggregate (32-35). This final feature is important, since aggregation of Aβ fibrils is a requisite for the formation of neuritic plaques.

APP can also be processed by α-secretase that is active in late Golgi, transport vesicles and at the cell surface (24,25,36-40). α- and β-secretase compete for the APP substrate (24,25,41), although α-secretase generally cleaves APP in earlier compartments than β-secretase. α-Secretase cleavage results in the secretion of the large N-terminal fragment sAPPα and generates the membrane-bound C83 fragment (Figure 1) (42,43). α-Secretase processing of APP precludes the formation of Aβ, since it cleaves in the middle the Aβ domain (44,45). C83 is a substrate for γ-secretase that releases the p3 peptide from the membrane (46,47). The p3 peptide is probably non-pathogenic since this peptide is smaller than its longer variant Aβ and therefore not able to aggregate into plaques.

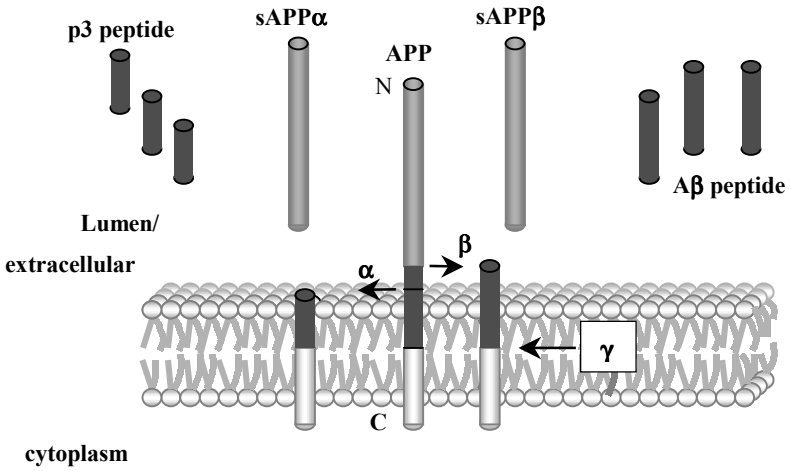

Figure 1

Generation of the amyloid-β peptide. The amyloid precursor protein (APP) is first cleaved by either α- or β-secretase, resulting in the release of soluble APPα and β (sAPPα and sAPPβ), and in the generation of membrane-associated C83 and C99. A second endoproteolytic cleavage that is carried out in the lipid bilayer, is mediated by γ-secretase. This cleavage results in the secretion of either Aβ (product of β- and γ-secretase) or p3 (product of α- and β-secretase) peptides in the extracellular fluids.

1.4 Mutations in Alzheimer's disease

AD can be divided into two genetically distinct types: familial AD (FAD) in which the disease is transmitted as an autosomal-dominant trait, and sporadic AD (SAD) which shows modest familial clustering and probably results from the synergistic action of genetic and environmental factors (48,49). There are no pathological and clinical features that distinguish FAD from SAD, except that FAD generally has an earlier age of onset. Genetic and environmental factors contribute equally to the development of both forms of AD (48,49). Most patients with AD (90%) suffer from late onset AD that occurs sporadically (50). Three modifier genes have been identified that show modest familial clustering in SAD. Inheritance of these genes results in an increased susceptibility for AD, but does not imply that the carrier will develop the disease. The best example of a modifier gene is the ε4 allele of the apoE gene on chromosome 19 that is estimated to account for approximately 50 % of the genetic component of AD (51).

FAD accounts for only a minority of all AD cases and is associated with inheritance of autosomal dominant genes. Genetic linkage studies of different FAD families have given valuable insight in the pathogenesis of AD. Analysis of the FAD-causing mutations in APP showed that all mutations resulted in an increased production of Aβ42 (52-54), the main component of neuritic plaques. This increased generation of Aβ is caused by either stimulation of the β-/γ-secretase cleavage or by inhibition of α-secretase processing. Mutations in the APP gene account only for small proportion (<3%) of all FAD-linked mutations and result in an average age of onset before 65 years (55-58). The majority of the FAD causing mutations however, are found in the two presenilin genes PS1 and PS2 (59-61). PS1 is located on chromosome 14 and is estimated to account for 70% of all FAD cases (62-64). FAD mutations in the PS1 gene are associated with the most deleterious form of AD (65), with a mean age of onset that varies between 30-60 years. In contrast to PS1, only 2 FAD-linked mutations have been found in the PS2 gene on chromosome 1. The age of onset of patients with FAD mutations in PS2 lies between 50-70 years (60). Interestingly, all FAD-linked mutations found in the PS1 and PS2 genes result in the same alternation as the mutations in APP: an increased generation of the Aβ42 (66).

1.5 Role of microglia in Alzheimer's disease

Inflammation in AD appears only to arise from within the CNS, with little or no involvement of lymphocytes and monocytes beyond their normal surveillance of the brain (67). How and when inflammation arises in the course of AD has not yet been fully resolved. Throughout the years, AD inflammation research has been focused on various subjects including cytokines, complement, chemokines, growth factors, oxidative stress, microglial activation, astrocyte reactivity. However, it is important to realize that inflammatory reactions are highly interactive and almost never occur in isolation from each

other. In the AD brain the Aβ proteins, neurofibrillary tangles and neuronal degeneration seem to be the most likely sources of inflammation. From here a bewildering number of subsystems come into play, each with multiple interactions with other subsystems. These interactions make it possible for one set of mediators to activate most others. Presently there is no reason to believe that any one mechanism is more primary than the others.

As pointed out earlier neuritic plaques are composed of a core of dense aggregated Aβ peptides and are surrounded by microglia and astrocytes, in contrast to diffuse plaques which seem not to be surrounded by those cells (68). Although Aβ can act as a potent and direct neurotoxic agent (69-72) an increasing amount of evidence show that neuritic plaques elicit a cascade of cellular events which lead to neuronal pathology (73,74). In the late nineteen eighties the group of McGeer and the group of Rozemuller and Eikelenboom showed that reactive microglia, which show increased immune-labeling for the human leukocyte antigen (HLA-DR) and for glycoproteins of the leukocyte function-associated antigens (LFA-1) family, i.e. LFA-1, P150,95 and iC3b-receptor, are closely associated with neuritic and amyloid plaques, but not with diffuse plaques (7,75-78). Using electron microscopic reconstruction Wisniewski and coworkers have also noted interactions between microglia and neuritic plaques (79). Because of their altered morphology and increased expression of MHC-II, cytokines, chemokines and complement these microglia are, like microglia in a variety of neuropathologic conditions, appropriately referred to as being activated (80-84). However, there is one stumbling block. The *in-vivo* situation and the *in-vitro* situation are not per definition the same. Products expressed by microglia or cells used as a model for microglia *in-vitro* might not be produced in the brain. For example astrocytes in culture express MHC-II but they do not so in the brain (85-87). Therefore in the conventional immunological sense astrocytes in AD cortex might not be an activated astrocyte.

Many groups have performed a lot of effort in trying to unravel the role of the microglia. Herein a lot of research have been focused on the neurotoxic properties. Microglia have been found to produce interleukin-1 (IL-1), IL-1β, IL-6, complement C3, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ) when activated (74,88-95). Next to these pro-inflammatory products microglia are able to produce neurotoxic products including reactive oxygen species, cytokines and secreted proteases (96-98). Moreover, there are also reports that microglia produce neurotrophic molecules like nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) (99).

1.6 Astrocytes

Like microglia, astrocytes are located at sites of Aβ deposition. Unlike microglia a few reactive astrocytes are present in virtually all diffuse plaques. The highest densities however are in neuritic plaques. Astrocytes are rarely associated with 'burned out' plaques (100). The positioning of astrocytes in plaques differs from that of microglia. Astrocytes somas form a corona at the edge of the neuritic halo that, in turn, may surround a dense

core Aβ deposit. Processes from the astrocytes cover and interact with the neuritic layer (100), in a manner reminiscent of glial scarring and there is, in fact, recent evidence that plaque-associated astrocytes may be creating barriers. When microglia are presented with naked Aβ depositions onto the culture substratum they can readily clear the deposit. However, when Aβ deposits are first presented to astrocytes they deposit proteoglycans that greatly inhibit the microglial attack (101). Astrocytes may therefore impair the natural ability of microglia to clear plaques.

1.7 Intracellular pathways

Research of the microglial signal transduction pathways mediating the neurotoxic response of Aβ has revealed the mitogen-activated protein kinase (MAPK) superfamily members ERK1/2 and p38 MAPK as important mediators (102,103). Besides, an increasing amount of evidence shows that the transcription factor nuclear factor-κB (NF-κB) can be stimulated in microglia by Aβ(25-35) (104,105). Upregulation of ERK1/2 and p38 MAPK can lead to the activation of numerous transcription factors in their turn leading to the activation of cytokines and neurotrophic factors. Activation of NF-κB can lead to the transcription of genes expressing TNF-α, IL-1, IL-6, monocyte chemoattractant protein-1 (MCP-1) and nitric oxide (NO) synthase (reviewed in (106)). The production of reactive oxygen species as a neurotoxic response of Aβ in microglia seems to involve the NADPH oxidase system (96,107). Protein kinase C is thought to be involved as a mediator of the NADPH oxidase system (108).

It remains unclear how above-mentioned signal transduction pathways that mediate the neurotoxic response are stimulated by Aβ. Presumably Aβ induces these cellular effects by engaging a binding protein (or proteins) presented by plasma membranes. The receptor for advanced glycation end products (RAGE) is described to be a possible candidate. It was first reported in 1996 that besides binding advanced glycation end products and amphoterin, RAGE is able to mediate Aβ-induced cellular activation in macrophages and in neurons (109,110). Another receptor capable of binding Aβ and present on macrophages is the class A scavenger receptor (111,112). Because Aβ(25-35) is capable of blocking the binding of Aβ(1-40/42) to RAGE and class A scavenger receptors on the one hand and reports of groups showing that the 1-16 domain of the Aβ protein is most likely to be involved in the binding of the protein to the macrophages (113-115) on the other hand and additional reports showing that RAGE and class A scavenger receptors are not involved in the Aβ-stimulated ERK1/2 activation (102) it remains unclear how Aβ is coupled to signal transduction pathways.

The cytokines and reactive oxygen species released by microglia / macrophages can not only be direct neurotoxic, they can also activate surrounding astrocytes. Astrocytes can be shown to be associated with many, but not all senile plaques. They are most closely associated with plaques with a dense amyloid core and an attendant microglial reaction. Stimulation of astrocytes by IL-1β, TNF-α and reactive oxygen species activates NF-κB (116,117). Moreover, *in vitro* studies show that Aβ(1-42) is capable of activating NF-κB

and stimulating production of IL-1β and NO in rat astrocytes (118,119). On the other hand, it has also been shown that activity of iNOS in microglial cells is inhibited by the presence of astroglial cells, probably involving TGF-β (120). More important, astrocytes have also been shown to inhibit phagocytic properties of microglial cells (101).

1.8 Chemokines

Since the discovery of the role of the CCR5 chemokine receptor in HIV pathogenesis (121,122) the status of chemokines and their receptors has raised considerably. To date, the known chemokine family in humans consists of approximately 50 ligands and 20 G protein-coupled receptors. Bone marrow-derived cells are the principle targets of chemokines. Because motility is an essential part of their function, chemokines play a central role in leukocyte physiology by controlling basal and inflammatory trafficking. But, chemokine receptor activation is not only responsible for motion, it can also play a role in gene transcription, infections, angiogenesis, mitogenic effects or apoptosis. Moreover, many other cell types also express chemokine receptors. These cells include smooth muscle cells, epithelial cells, neurons and stromal cells. Thus, next to localizing cells of the immune system to specific places, chemokines might be involved in aspects of tissue homeostasis.

Chemokines are small, about 8~14 kDa, secreted molecules that exhibit very specific cysteine motifs in their amino acid sequence. Almost all members have four cysteines, and depending on the motif of the first two residues they have been classified into CXC, CC, C, and CX_3C classes. The only exception is lymphotactin which has only two cysteine residues (123). Two disulfide bonds are formed between the first and the third Cys and the second and the fourth residues. In addition the CXC family has been subdivided into two groups, depending on the presence of the ELR motif preceding the first cysteine: the ELR-CXC chemokines and the non-ELR-CXC chemokines. Chemokine receptors are G protein-coupled, seven-transmembrane receptors. Based on the chemokine class they bind, the receptors have been named CXCR, CCR, XCR, and CX_3CR (124).

1.9 Scope of this thesis

Although much research has been done on the physiology of the neuritic plaque, the exact mechanisms responsible for the decay of the neurons surrounding this plaque is still not clear. Moreover, almost all research has been focused on and is still focused on interpreting the processes occurring within the neuritic plaque. The answer to the question how are these glial cells attracted towards the plaque is still a black box. Because *in-vivo* microglial cells and astrocytes are in immediate vicinity, the research presented in this thesis has not just been focused on the response of the macrophage when activated by amyloid-beta. More important the effect of the astrocyte on the response of the macrophage has therefore been investigated. Besides, the interaction of the macrophage and the

astrocyte has been a pivotal element in resolving the role of these cells in the inflammatory response. In addition the role of several signal-transduction pathways that are pivotal in the macrophage-mediated inflammatory response towards Aβ are investigated.

References

1. Giulian, D. and C. Robertson. 1990. Inhibition of mononuclear phagocytes reduces ischemic injury in the spinal cord. Ann.Neurol. 27:33.
2. Stoll, G., B. D. Trapp, and J. W. Griffin. 1989. Macrophage function during Wallerian degeneration of rat optic nerve: clearance of degenerating myelin and Ia expression. J.Neurosci. 9:2327.
3. Sharer, L. R., L. G. Epstein, E. S. Cho, V. V. Joshi, M. F. Meyenhofer, L. F. Rankin, and C. K. Petito. 1986. Pathologic features of AIDS encephalopathy in children: evidence for LAV/HTLV-III infection of brain. Hum.Pathol. 17:271.
4. Huitinga, I., N. van Rooijen, C. J. De Groot, B. M. Uitdehaag, and C. D. Dijkstra. 1990. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J.Exp.Med. 172:1025.
5. Coffey, P. J., V. H. Perry, and J. N. Rawlins. 1990. An investigation into the early stages of the inflammatory response following ibotenic acid-induced neuronal degeneration. Neuroscience 35:121.
6. Li, H., J. Newcombe, N. P. Groome, and M. L. Cuzner. 1993. Characterization and distribution of phagocytic macrophages in multiple sclerosis plaques. Neuropathol.Appl.Neurobiol. 19:214.
7. McGeer, P. L., S. Itagaki, B. E. Boyes, and E. G. McGeer. 1988. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 38:1285.
8. McGeer, P. L., S. Itagaki, and E. G. McGeer. 1988. Expression of the histocompatibility glycoprotein HLA-DR in neurological disease. Acta Neuropathol.(Berl) 76:550.
9. Price, R. W., B. Brew, J. Sidtis, M. Rosenblum, A. C. Scheck, and P. Cleary. 1988. The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. Science 239:586.
10. Meda, L., M. A. Cassatella, G. I. Szendrei, L. Otvos, Jr., P. Baron, M. Villalba, D. Ferrari, and F. Rossi. 1995. Activation of microglial cells by beta-amyloid protein and interferon-gamma. Nature 374:647.
11. Brown, D. R., B. Schmidt, and H. A. Kretzschmar. 1996. Role of microglia and host prion protein in neurotoxicity of a prion protein fragment. Nature 380:345.
12. Muhleisen, H., J. Gehrmann, and R. Meyermann. 1995. Reactive microglia in Creutzfeldt-Jakob disease. Neuropathol.Appl.Neurobiol. 21:505.
13. Barde, Y. A. 1989. Trophic factors and neuronal survival. Neuron 2:1525.
14. Boven, L. A., J. Middel, P. Portegies, J. Verhoef, G. H. Jansen, and H. S. Nottet. 1999. Overexpression of nerve growth factor and basic fibroblast growth factor in AIDS dementia complex. J.Neuroimmunol. 97:154.
15. McKhann, G., D. Drachman, M. Folstein, R. Katzman, D. Price, and E. M. Stadlan. 1984. Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group under the auspicies of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurol. 34:939.
16. Cummings, J. L., H. V. Vinters, G. M. Cole, and Z. S. Khachaturian. 1998. Alzheimer's disease: etiologies, pathophysiology, cognitive reserve, and treatment opportunities. Neurology 51:S2.
17. Dickson, D. W. 1997. The pathogenesis of senile plaques. J.Neuropathol.Exp.Neurol. 56:321.

18. Mandybur, T. I. and C. C. Chuirazzi. 1990. Astrocytes and the plaques of Alzheimer's disease. Neurology 40:635.
19. Mirra, S. S., A. Heyman, D. McKeel, S. M. Sumi, B. J. Crain, L. M. Brownlee, F. S. Vogel, J. P. Hughes, G. van Belle, and L. Berg. 1991. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41:479.
20. Anonymous. 1997. Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease. Neurobiol.Aging 18:S1.
21. Schmidt, M. L., V. M. Lee, M. Forman, T. S. Chiu, and J. Q. Trojanowski. 1997. Monoclonal antibodies to a 100-kd protein reveal abundant A beta-negative plaques throughout gray matter of Alzheimer's disease brains. Am.J.Pathol. 151:69.
22. Kang, J., H. G. Lemaire, A. Unterbeck, J. M. Salbaum, C. L. Masters, K. H. Grzeschik, G. Multhaup, K. Beyreuther, and B. Muller-Hill. 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325:733.
23. Selkoe, D. J. 1994. Normal and abnormal biology of the beta-amyloid precursor protein. Annu.Rev.Neurosci. 17:489.
24. Haass, C., C. A. Lemere, A. Capell, M. Citron, P. Seubert, D. Schenk, L. Lannfelt, and D. J. Selkoe. 1995. The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. Nat.Med. 1:1291.
25. Thinakaran, G., D. B. Teplow, R. Siman, B. Greenberg, and S. S. Sisodia. 1996. Metabolism of the "Swedish" amyloid precursor protein variant in neuro2a (N2a) cells. Evidence that cleavage at the "beta-secretase" site occurs in the golgi apparatus. J.Biol.Chem. 271:9390.
26. Perez, R. G., S. Soriano, J. D. Hayes, B. Ostaszewski, W. Xia, D. J. Selkoe, X. Chen, G. B. Stokin, and E. H. Koo. 1999. Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Abeta42. J.Biol.Chem. 274:18851.
27. Haass, C., E. H. Koo, A. Mellon, A. Y. Hung, and D. J. Selkoe. 1992. Targeting of cell-surface beta-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. Nature 357:500.
28. Vassar, R., B. D. Bennett, S. Babu-Khan, S. Kahn, E. A. Mendiaz, P. Denis, D. B. Teplow, S. Ross, P. Amarante, R. Loeloff, Y. Luo, S. Fisher, J. Fuller, S. Edenson, J. Lile, M. A. Jarosinski, A. L. Biere, E. Curran, T. Burgess, J. C. Louis, F. Collins, J. Treanor, G. Rogers, and M. Citron. 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286:735.
29. Haass, C. and D. J. Selkoe. 1993. Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. Cell 75:1039.
30. Dovey, H. F., S. Suomensaari-Chrysler, I. Lieberburg, S. Sinha, and P. S. Keim. 1993. Cells with a familial Alzheimer's disease mutation produce authentic beta-peptide. Neuroreport 4:1039.
31. Vigo-Pelfrey, C., D. Lee, P. Keim, I. Lieberburg, and D. B. Schenk. 1993. Characterization of beta-amyloid peptide from human cerebrospinal fluid. J.Neurochem. 61:1965.
32. Jarrett, J. T., E. P. Berger, and P. T. Lansbury. 1993. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry 32:4693.

33. Hilbich, C., B. Kisters-Woike, J. Reed, C. L. Masters, and K. Beyreuther. 1991. Aggregation and secondary structure of synthetic amyloid beta A4 peptides of Alzheimer's disease. J.Mol.Biol. 218:149.
34. Burdick, D., B. Soreghan, M. Kwon, J. Kosmoski, M. Knauer, A. Henschen, J. Yates, C. Cotman , and C. Glabe. 1992. Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. J.Biol.Chem. 267:546.
35. Jarrett, J. T. and P. T. Lansbury. 1993. Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell 73:1055.
36. De Strooper, B., L. Umans, F. Van Leuven, and B. H. Van Den. 1993. Study of the synthesis and secretion of normal and artificial mutants of murine amyloid precursor protein (APP): cleavage of APP occurs in a late compartment of the default secretion pathway. J.Cell Biol. 121:295.
37. Kuentzel, S. L., S. M. Ali, R. A. Altman, B. D. Greenberg, and T. J. Raub. 1993. The Alzheimer beta-amyloid protein precursor/protease nexin-II is cleaved by secretase in a trans-Golgi secretory compartment in human neuroglioma cells. Biochem.J. 295:367.
38. Sambamurti, K., J. Shioi, J. P. Anderson, M. A. Pappolla, and N. K. Robakis. 1992. Evidence for intracellular cleavage of the Alzheimer's amyloid precursor in PC12 cells. J.Neurosci.Res. 33:319.
39. Sisodia, S. S. 1992. Beta-amyloid precursor protein cleavage by a membrane-bound protease. Proc.Natl.Acad.Sci.U.S.A 89:6075.
40. Koo, E. H. and S. L. Squazzo. 1994. Evidence that production and release of amyloid beta-protein involves the endocytic pathway. J.Biol.Chem. 269:17386.
41. Skovronsky, D. M., D. B. Moore, M. E. Milla, R. W. Doms, and V. M. Lee. 2000. Protein kinase C-dependent alpha-secretase competes with beta-secretase for cleavage of amyloid-beta precursor protein in the trans-golgi network. J.Biol.Chem. 275:2568.
42. Weidemann, A., G. Konig, D. Bunke, P. Fischer, J. M. Salbaum, C. L. Masters, and K. Beyreuther. 1989. Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. Cell 57:115.
43. Oltersdorf, T., P. J. Ward, T. Henriksson, E. C. Beattie, R. Neve, I. Lieberburg, and L. C. Fritz. 1990. The Alzheimer amyloid precursor protein. Identification of a stable intermediate in the biosynthetic/degradative pathway. J.Biol.Chem. 265:4492.
44. Esch, F. S., P. S. Keim, E. C. Beattie, R. W. Blacher, A. R. Culwell, T. Oltersdorf, D. McClure, and P. J. Ward. 1990. Cleavage of amyloid beta peptide during constitutive processing of its precursor. Science 248:1122.
45. Sisodia, S. S., E. H. Koo, K. Beyreuther, A. Unterbeck, and D. L. Price. 1990. Evidence that beta-amyloid protein in Alzheimer's disease is not derived by normal processing. Science 248:492.
46. Haass, C., M. G. Schlossmacher, A. Y. Hung, C. Vigo-Pelfrey, A. Mellon, B. L. Ostaszewski, I. Lieberburg, E. H. Koo, D. Schenk, and D. B. Teplow. 1992. Amyloid beta-peptide is produced by cultured cells during normal metabolism. Nature 359:322.
47. Haass, C., A. Y. Hung, M. G. Schlossmacher, D. B. Teplow, and D. J. Selkoe. 1993. Beta-amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. J.Biol.Chem. 268:3021.
48. Lendon, C. L., F. Ashall, and A. M. Goate. 1997. Exploring the etiology of Alzheimer disease using molecular genetics. JAMA 277:825.
49. Strittmatter, W. J. and A. D. Roses. 1995. Apolipoprotein E and Alzheimer disease. Proc.Natl.Acad.Sci.U.S.A. 92:4725.

50. Fraser, P. E., D. S. Yang, G. Yu, L. Levesque, M. Nishimura, S. Arawaka, L. C. Serpell, E. Rogaeva, and P. George-Hyslop. 2000. Presenilin structure, function and role in Alzheimer disease. Biochim.Biophys.Acta 1502:1.
51. Corder, E. H., A. M. Saunders, W. J. Strittmatter, D. E. Schmechel, P. C. Gaskell, G. W. Small, A. D. Roses, J. L. Haines, and M. A. Pericak-Vance. 1993. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261:921.
52. Cai, X. D., T. E. Golde, and S. G. Younkin. 1993. Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. Science 259:514.
53. Citron, M., T. Oltersdorf, C. Haass, L. McConlogue, A. Y. Hung, P. Seubert, C. Vigo-Pelfrey, I. Lieberburg, and D. J. Selkoe. 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360:672.
54. Suzuki, N., T. T. Cheung, X. D. Cai, A. Odaka, L. Otvos, Jr., C. Eckman, T. E. Golde, and S. G. Younkin. 1994. An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264:1336.
55. Goate, A., M. C. Chartier-Harlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. Haynes, N. Irving, and L. James. 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349:704.
56. Chartier-Harlin, M. C., F. Crawford, H. Houlden, A. Warren, D. Hughes, L. Fidani, A. Goate, M. Rossor, P. Roques, and J. Hardy. 1991. Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene. Nature 353:844.
57. Murrell, J., M. Farlow, B. Ghetti, and M. D. Benson. 1991. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. Science 254:97.
58. Karlinsky, H., G. Vaula, J. L. Haines, J. Ridgley, C. Bergeron, M. Mortilla, R. G. Tupler, M. E. Percy, Y. Robitaille, and N. E. Noldy. 1992. Molecular and prospective phenotypic characterization of a pedigree with familial Alzheimer's disease and a missense mutation in codon 717 of the beta-amyloid precursor protein gene. Neurology 42:1445.
59. Sherrington, R., E. I. Rogaev, Y. Liang, E. A. Rogaeva, G. Levesque, M. Ikeda, H. Chi, C. Lin, G. Li, and K. Holman. 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375:754.
60. Rogaev, E. I., R. Sherrington, E. A. Rogaeva, G. Levesque, M. Ikeda, Y. Liang, H. Chi, C. Lin, K. Holman, and T. Tsuda. 1995. Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature 376:775.
61. Levy-Lahad, E., W. Wasco, P. Poorkaj, D. M. Romano, J. Oshima, W. H. Pettingell, C. E. Yu, P. D. Jondro, S. D. Schmidt, and K. Wang. 1995. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 269:973.
62. Schellenberg, G. D., T. D. Bird, E. M. Wijsman, H. T. Orr, L. Anderson, E. Nemens, J. A. White, L. Bonnycastle, J. L. Weber, and M. E. Alonso. 1992. Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14. Science 258:668.
63. George-Hyslop, P., J. Haines, E. Rogaev, M. Mortilla, G. Vaula, M. Pericak-Vance, J. F. Foncin, M. Montesi, A. Bruni, and S. Sorbi. 1992. Genetic evidence for a novel familial Alzheimer's disease locus on chromosome 14. Nat.Genet. 2:330.

64. Van Broeckhoven, C., H. Backhovens, M. Cruts, G. De Winter, M. Bruyland, P. Cras, and J. J. Martin. 1992. Mapping of a gene predisposing to early-onset Alzheimer's disease to chromosome 14q24.3. Nat.Genet. 2:335.
65. Wisniewski, T., W. K. Dowjat, J. D. Buxbaum, O. Khorkova, S. Efthimiopoulos, J. Kulczycki, W. Lojkowska, J. Wegiel, H. M. Wisniewski, and B. Frangione. 1998. A novel Polish presenilin-1 mutation (P117L) is associated with familial Alzheimer's disease and leads to death as early as the age of 28 years. Neuroreport 9:217.
66. Scheuner, D., C. Eckman, M. Jensen, X. Song, M. Citron, N. Suzuki, T. D. Bird, J. Hardy, M. Hutton, W. Kukull, E. Larson, E. Levy-Lahad, M. Viitanen, E. Peskind, P. Poorkaj, G. Schellenberg, R. Tanzi, W. Wasco, L. Lannfelt, D. Selkoe, and S. Younkin. 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.
67. Wekerle, H., D. Sun, R. L. Oropeza-Wekerle, and R. Meyermann. 1987. Immune reactivity in the nervous system: modulation of T-lymphocyte activation by glial cells. J.Exp.Biol. 132:43.
68. Sasaki, A., H. Yamaguchi, A. Ogawa, S. Sugihara, and Y. Nakazato. 1997. Microglial activation in early stages of amyloid beta protein deposition. Acta Neuropathol.(Berl) 94:316.
69. Pike, C. J., A. J. Walencewicz, C. G. Glabe, and C. W. Cotman. 1991. Aggregation-related toxicity of synthetic beta-amyloid protein in hippocampal cultures. Eur.J.Pharmacol. 207:367.
70. Yankner, B. A., L. K. Duffy, and D. A. Kirschner. 1990. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. Science 250 :279.
71. Behl, C., J. B. Davis, R. Lesley, and D. Schubert. 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77:817.
72. Yaar, M., S. Zhai, P. F. Pilch, S. M. Doyle , P. B. Eisenhauer, R. E. Fine, and B. A. Gilchrest. 1997. Binding of beta-amyloid to the p75 neurotrophin receptor induces apoptosis. A possible mechanism for Alzheimer's disease. J.Clin.Invest. 100:2333.
73. Davies, P. 1994. Neuronal abnormalities, not amyloid, are the cause of dementia in Alzheimer's disease. In Alzheimer's Disease. R.D.Terry, R.Katzman and K.L.Bick, eds. Raven Press, New York, p. 327.
74. Giulian, D., L. J. Haverkamp, J. Li, W. L. Karshin, J. Yu, D. Tom, X. Li, and J. B. Kirkpatrick. 1995. Senile plaques stimulate microglia to release a neurotoxin found in Alzheimer brain. Neurochem.Int. 27:119.
75. McGeer, E. G. and P. L. McGeer. 1997. The role of the immune system in neurodegenerative disorders. Mov Disord. 12:855.
76. McGeer, P. L., E. G. McGeer, T. Kawamata, T. Yamada, and H. Akiyama. 1991. Reactions of the immune system in chronic degenerative neurological diseases. Can.J.Neurol.Sci. 18:376.
77. Rozemuller, J. M., P. Eikelenboom, and F. C. Stam. 1986. Role of microglia in plaque formation in senile dementia of the Alzheimer type. An immunohistochemical study. Virchows Arch.B Cell Pathol.Incl.Mol.Pathol. 51:247.
78. Rozemuller, J. M., P. Eikelenboom, S. T. Pals, and F. C. Stam. 1989. Microglial cells around amyloid plaques in Alzheimer's disease express leucocyte adhesion molecules of the LFA-1 family. Neurosci.Lett. 101:288.
79. Wisniewski, H. M., J. Wegiel, K. C. Wang, M. Kujawa, and B. Lach. 1989. Ultrastructural studies of the cells forming amyloid fibers in classical plaques. Can.J.Neurol.Sci. 16:535.

80. Gehrmann, J., G. Mies, P. Bonnekoh, R. Banati, T. Iijima, G. W. Kreutzberg, and K. A. Hossmann. 1993. Microglial reaction in the rat cerebral cortex induced by cortical spreading depression. Brain Pathol. 3:11.
81. Morioka, T., A. N. Kalehua, and W. J. Streit. 1993. Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. J.Comp. Neurol. 327:123.
82. Morioka, T., T. Baba, K. L. Black, and W. J. Streit. 1992. Immunophenotypic analysis of infiltrating leukocytes and microglia in an experimental rat glioma. Acta Neuropathol.(Berl) 83:590.
83. Streit, W. J. and D. L. Sparks. 1997. Activation of microglia in the brains of humans with heart disease and hypercholesterolemic rabbits. J.Mol.Med. 75:130.
84. Streit, W. J., S. A. Walter, and N. A. Pennell. 1999. Reactive microgliosis. Prog.Neurobiol. 57:563.
85. Gresser, O., A. Hein, S. Riese, and A. Regnier-Vigouroux. 2000. Tumor necrosis factor alpha and interleukin-1 alpha inhibit through different pathways interferon-gamma-induced antigen presentation, processing and MHC class II surface expression on astrocytes, but not on microglia. Cell Tissue Res. 300:373.
86. Horwitz, M. S., C. F. Evans, F. G. Klier, and M. B. Oldstone. 1999. Detailed in vivo analysis of interferon-gamma induced major histocompatibility complex expression in the the central nervous system: astrocytes fail to express major histocompatibility complex class I and II molecules. Lab Invest 79:235.
87. Lee, S. C., M. Collins, P. Vanguri, and M. L. Shin. 1992. Glutamate differentially inhibits the expression of class II MHC antigens on astrocytes and microglia. J.Immunol. 148:3391.
88. Lorton, D., J. M. Kocsis, L. King, K. Madden, and K. R. Brunden. 1996. beta-Amyloid induces increased release of interleukin-1 beta from lipopolysaccharide-activated human monocytes. J.Neuroimmunol. 67:21.
89. Klegeris, A., D. G. Walker, and P. L. McGeer. 1997. Interaction of Alzheimer beta-amyloid peptide with the human monocytic cell line THP-1 results in a protein kinase C-dependent secretion of tumor necrosis factor-alpha. Brain Res. 747:114.
90. Frei, K., U. V. Malipiero, T. P. Leist, R. M. Zinkernagel, M. E. Schwab, and A. Fontana. 1989. On the cellular source and function of interleukin 6 produced in the central nervous system in viral diseases. Eur.J.Immunol. 19:689.
91. Giulian, D., T. J. Baker, L. C. Shih, and L. B. Lachman. 1986. Interleukin-1 of the central nervous system is produced by ameboid microglia. J.Exp.Med. 164:594.
92. Griffin, W. S., J. G. Sheng, G. W. Roberts, and R. E. Mrak. 1995. Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. J.Neuropathol.Exp.Neurol. 54:276.
93. Haga, S., K. Ikeda, M. Sato, and T. Ishii. 1993. Synthetic Alzheimer amyloid beta/A4 peptides enhance production of complement C3 component by cultured microglial cells. Brain Res. 601:88.
94. McGeer, P. L., H. Akiyama, S. Itagaki, and E. G. McGeer. 1989. Immune system response in Alzheimer's disease. Can.J.Neurol.Sci. 16:516.
95. Sawada, M., N. Kondo, A. Suzumura, and T. Marunouchi. 1989. Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. Brain Res. 491:394.
96. Klegeris, A. and P. L. McGeer. 1997. beta-amyloid protein enhances macrophage production of oxygen free radicals and glutamate. J.Neurosci.Res. 49:229.
97. Klegeris, A., D. G. Walker, and P. L. McGeer. 1994. Activation of macrophages by Alzheimer beta amyloid peptide. Biochem.Biophys.Res.Commun. 199:984.
98. McDonald, D. R., K. R. Brunden, and G. E. Landreth. 1997. Amyloid fibrils activate tyrosine kinase-dependent signaling and superoxide production in microglia. J.Neurosci. 17:2284.

99. Elkabes, S., E. M. DiCicco Bloom, and I. B. Black. 1996. Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. J.Neurosci. 16:2508.
100. Mrak, R. E., J. G. Sheng, and W. S. Griffin. 1996. Correlation of astrocytic S100 beta expression with dystrophic neurites in amyloid plaques of Alzheimer's disease. J.Neuropathol.Exp.Neurol. 55:273.
101. DeWitt, D. A., G. Perry, M. Cohen, C. Doller, and J. Silver. 1998. Astrocytes regulate microglial phagocytosis of senile plaque cores of Alzheimer's disease. Exp.Neurol. 149:329.
102. McDonald, D. R., M. E. Bamberger, C. K. Combs, and G. E. Landreth. 1998. beta-Amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. J.Neurosci. 18:4451.
103. Combs, C. K., D. E. Johnson, S. B. Cannady, T. M. Lehman, and G. E. Landreth. 1999. Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. J.Neurosci. 19:928.
104. Bonaiuto, C., P. P. McDonald, F. Rossi, and M. A. Cassatella. 1997. Activation of nuclear factor-kappa B by beta-amyloid peptides and interferon-gamma in murine microglia. J.Neuroimmunol. 77:51.
105. Heese, K., C. Hock, and U. Otten. 1998. Inflammatory signals induce neurotrophin expression in human microglial cells. J.Neurochem. 70:699.
106. Baeuerle, P. A. and T. Henkel. 1994. Function and activation of NF-kappa B in the immune system. Annu.Rev.Immunol. 12:141.
107. Meda, L., C. Bonaiuto, P. Baron, L. Otvos, Jr., F. Rossi, and M. A. Cassatella. 1996. Priming of monocyte respiratory burst by beta-amyloid fragment (25-35). Neurosci.Lett. 219:91.
108. Benna, J. E., P. M. Dang, M. Gaudry, M. Fay, F. Morel, J. Hakim, and M. A. Gougerot-Pocidalo. 1997. Phosphorylation of the respiratory burst oxidase subunit p67(phox) during human neutrophil activation. Regulation by protein kinase C-dependent and independent pathways. J.Biol.Chem. 272:17204.
109. Yan, S. D., X. Chen, J. Fu, M. Chen, H. Zhu, A. Roher, T. Slattery, L. Zhao, M. Nagashima, J. Morser, A. Migheli, P. Nawroth, D. Stern, and A. M. Schmidt. 1996. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature 382:685.
110. Du Yan, S., H. Zhu, J. Fu, S. F. Yan, A. Roher, W. W. Tourtellotte, T. Rajavashisth, X. Chen, G. C. Godman, D. Stern, and A. M. Schmidt. 1997. Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. Proc.Natl.Acad.Sci.U.S.A. 94:5296.
111. El Khoury, J., S. E. Hickman, C. A. Thomas, L. Cao, S. C. Silverstein, and J. D. Loike. 1996. Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. Nature 382:716.
112. Honda, M., H. Akiyama, Y. Yamada, H. Kondo , Y. Kawabe, M. Takeya, K. Takahashi, H. Suzuki, T. Doi, A. Sakamoto, S. Ookawara, M. Mato, P. J. Gough, D. R. Greaves, S. Gordon, T. Kodama, and M. Matsushita. 1998. Immunohistochemical evidence for a macrophage scavenger receptor in Mato cells and reactive microglia of ischemia and Alzheimer's disease. Biochem.Biophys.Res.Commun. 245:734.
113. Giulian, D., L. J. Haverkamp, J. H. Yu, W. Karshin, D. Tom, J. Li, J. Kirkpatrick, L. M. Kuo, and A. E. Roher. 1996. Specific domains of beta-amyloid from Alzheimer plaque elicit neuron killing in human microglia. J.Neurosci. 16:6021.
114. Van Muiswinkel, F. L., S. F. Raupp, N. M. de Vos, H. A. Smits, J. Verhoef, P. Eikelenboom, and H. S. Nottet. 1999. The amino-terminus of the amyloid-beta protein is critical for the cellular binding and consequent activation of the respiratory burst of human macrophages. J.Neuroimmunol. 96:121.

115. Smits, H. A., F. L. Van Muiswinkel, N. M. de Vos, J. Verhoef, and H. S. Nottet. 1999. The amyloid-beta (1-42)-induced respiratory burst of primary human macrophages is enhanced in the presence of amyloid-beta (25-35). In Alzheimer's Disease and Related Disorders. K.Iqbal, D.F.Swaab, B.Winblad and H.M.Wisniewski, eds. John Wiley & Sons Ltd, Sussex, p. 457.
116. Tanaka, C., H. Kamata, H. Takeshita, H. Yagisawa, and H. Hirata. 1997. Redox regulation of lipopolysaccharide (LPS)-induced interleukin-8 (IL-8) gene expression mediated by NF kappa B and AP-1 in human astrocytoma U373 cells. Biochem.Biophys.Res.Commun. 232:568.
117. Lieb, K., B. L. Fiebich, H. Schaller, M. Berger, and J. Bauer. 1996. Interleukin-1 beta and tumor necrosis factor-alpha induce expression of alpha 1-antichymotrypsin in human astrocytoma cells by activation of nuclear factor-kappa B. J.Neurochem. 67:2039.
118. Akama, K. T., C. Albanese, R. G. Pestell, and L. J. Van Eldik. 1998. Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. Proc.Natl.Acad.Sci.U.S.A. 95:5795.
119. Hu, J., K. T. Akama, G. A. Krafft, B. A. Chromy, and L. J. Van Eldik. 1998. Amyloid-beta peptide activates cultured astrocytes: morphological alterations, cytokine induction and nitric oxide release. Brain Res. 785:195.
120. Vincent, V. A., F. J. Tilders, and A. M. Van Dam. 1997. Inhibition of endotoxin-induced nitric oxide synthase production in microglial cells by the presence of astroglial cells: a role for transforming growth factor beta. Glia 19:190.
121. Littman, D. R. 1998. Chemokine receptors: keys to AIDS pathogenesis? Cell 93:677.
122. Berger, E. A., P. M. Murphy, and J. M. Farber. 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu.Rev.Immunol. 17:657-700.:657.
123. Kelner, G. S., J. Kennedy, K. B. Bacon, S. Kleyensteuber, D. A. Largaespada, N. A. Jenkins, N. G. Copeland, J. F. Bazan, K. W. Moore, and T. J. Schall. 1994. Lymphotactin: a cytokine that represents a new class of chemokine. Science 266:1395.
124. Rossi, D. and A. Zlotnik. 2000. The biology of chemokines and their receptors. Annu.Rev.Immunol. 18:217-42:217.

2

The Aβ(1-42)-induced respiratory burst of primary human macrophages is enhanced in the presence of Aβ(25-35)

Hessel A. Smits[1]
Freek L. van Muiswinkel[2]
N. Machiel de Vos[1]
Jan Verhoef[1]
Hans S.L.M. Nottet[1]

1 *Eijkman-Winkler* Institute, section Neuroimmunology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

2 Research Institute Neurosciences, Vrije Universiteit, Faculty of Medicine, Department of Psychiatry, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

Alzheimer's Disease and Related Disorders (1999) K. Iqbal, D.F. Swaab, B. Winblad and H.M. Wisniewski, eds. John Wiley & Sons Ltd, Sussex, p. 457-461.

Introduction

Previously, we and others have shown that Aβ, which is the full-length homologue to the Aβ actually deposited in the Alzheimer's disease brain, is capable of priming and/or triggering the respiratory burst of cultured rat microglial cells (1). Using primary cultures of human monocyte-derived macrophages as a model of human microglial cells, we recently discovered that the amino-terminus of Aβ is critical for the cellular binding and consequent activation of the phagocytic respiratory burst (Van Muiswinkel et al., unpublished observations). Furthermore, in line with previous studies of Giulian and colleagues (2,3), we found that Aβ(1-16) behaves like a pharmacological antagonist. Thus, using flow cytometry with Aβ(1-42)-FITC and lucigenin-enhanced chemiluminescence, it was found that Aβ(1-16), which has been described as containing a cell attachment domain (2,3), inhibited both the cellular binding and the Aβ(1-42)-induced release of superoxide anion in human macrophages. In the present study, we have used the same experimental model to investigate the effects of Aβ(25-35) on the Aβ(1-42)-induced oxidative burst.

Materials and methods

Amyloid-β peptides

The synthetic Aβ peptides Aβ(1-42) and Aβ(25-35) (Bachem AG, Switzerland), prepared as stock solutions in sterile water at a concentration of 500 μM, were stored at –20 °C. FITC-labeled Aβ(1-42) was prepared by incubating Aβ(1-42) at a concentration of 10 mg/ml with 0.1 mg/ml FITC in PBS (pH=7.4) for 1 h at 22 °C. Subsequently, using a 1 kDa Spectra/Por dialysation membrane, Aβ(1-42)-FITC was dialyzed overnight against PBS to remove unbound FITC, diluted to a concentration of 220 μM and stored at –20 °C.

Human monocyte-derived macrophages

Monocytes were derived from peripheral blood mononuclear cells by Ficoll-Paque (Pharmacia Biotech, Sweden) density gradients and purified by centrifugal elutriation as described previously (4). Cells were seeded at a concentration of $2x10^6$ cells/ml in Teflon Erlenmeyer flasks (Nalgene, USA) and grown as suspension at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Culture medium was composed of Iscove's modified Dulbecco's medium (Gibco Life technologies, The Netherlands) supplemented with 10% heat-inactivated human AB serum, 2 mM L-glutamine, 19 mM sodium bicarbonate, 10 μg/ml gentamicin and 10 μg/ml ciprofloxacin. After 7 days, monocyte-derived macrophages (MDMs) were harvested from the flasks, washed with Hanks' balanced salt solution (HBSS) and used for the experiments.

Superoxide anion production

Superoxide anion production was measured as lucigenin-enhanced chemiluminescence (4). Briefly, cells were transferred to polystyrene vials ($2x10^5$/vial), placed into a luminometer (Packard Instruments, Belgium) and incubated for 30 min at 37 °C in HBSS containing 250 nM bis-N-methylacridinum (Lucigenin; Sigma Chemical Co., The Netherlands) to assess the spontaneous release of superoxide anions. Subsequently, Aβ peptides were added and chemiluminescence (expressed as millivolts) was monitored for 30-60 min.

Flow cytometry

For Aβ(1-42)-FITC binding studies, $2x10^5$ cells were incubated at 37 °C with HBSS containing 2.5 μM Aβ(1-42)-FITC, either alone or in combination with 50 μM Aβ(25-35). After 30 min, the cells were washed with HBSS and fixed for 15 min with PBS containing 2% paraformaldehyde. Thereafter, flow cytometry analysis was performed by analyzing 10000 cells per experimental condition on a FACScan flow cytometer (Becton Dickinson and Co., USA) equipped with a computer-assisted data analysis system.

Results

Effect Aβ(25-35) on the Aβ(1-42)-induced superoxide anion release

To investigate the effect of Aβ(25-35) on the Aβ(1-42)-induced superoxide anion production, MDMs were incubated with Aβ(1-42), either alone or in combination with various amounts of Aβ(25-35). Results are shown in Figure 1. While the spontaneous burst amounted to 6.9 ± 1.3 mv, it was found that the respiratory burst was enhanced to 18.8 ± 0.4 mv by 10 μM Aβ(1-42). Moreover, while Aβ(25-35) did not trigger the respiratory burst by itself, Aβ(25-35) dramatically enhanced the Aβ(1-42) release of superoxide anion, i.e., in the presence of 200 μM Aβ(25-35) the Aβ(1-42)-induced release amounted to 67.8 ± 2.1 mv.

Effect of Aβ(25-35) on the cellular binding of Aβ(1-42)-FITC

To characterize the nature of the aforementioned effects of Aβ(25-35) on the Aβ(1-42)-stimulated superoxide release, we next investigated the effect of Aβ(25-35) on the cellular binding of Aβ(1-42) to human macrophages. Therefore, MDMs were incubated with Aβ(1-42)-FITC, either alone or in combination with Aβ(25-35). As shown in Figure 2, it was found that 2.5 μM Aβ(1-42)-FITC readily binds to MDMs. Control experiments performed at 4 °C or in the presence of 10 μg/ml cytochalasine B revealed that under our conditions Aβ(1-42)-FITC accumulates predominantly at the cell surface. Interestingly, whereas preincubation of cells with Aβ(25-35) did not have any effect, the cellular binding was markedly enhanced when 2.5 μM Aβ(1-42)-FITC was added together with 50 μM Aβ(25-35). This phenomenon was even more pronounced when Aβ(1-42)-FITC and

Aβ(25-35) were first incubated for 30 min prior to adding the peptides to the cells (Figure 2).

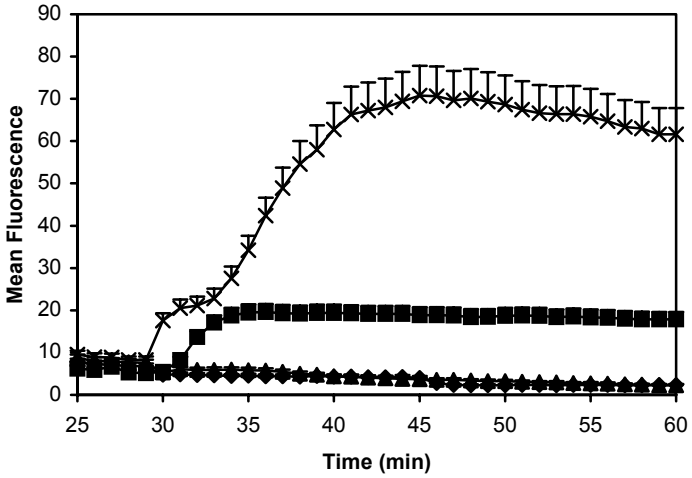

Figure 1

Effect of Aβ(25-35) on the Aβ(1-42)-induced superoxide anion release from human monocyte-derived macrophages. Superoxide anion release was measured in MDMs, either incubated with HBSS (*), 10 μM Aβ(1-42) (closed triangle), 200 μM Aβ(25-35) (closed diamond) or a combination thereof (x).

Discussion

In summary, the results of the current study demonstrate that the cellular binding of Aβ(1-42) and consequent activation of the respiratory burst of MDMs is enhanced in the presence of Aβ(25-35). Intriguingly, this effect was only observed when Aβ(1-42) and Aβ(25-35) were first preincubated in the absence of cells or when both peptides were added as a mixture. Although the nature of this effect is, as yet, largely enigmatic, various cellular mechanisms might be implicated. For example, Aβ(25-35) is known to induce changes in intracellular calcium homeostasis and/or to trigger tyrosine kinase-dependent intracellular signal-transduction pathways (5,6), phenomena which are both thought to be implicated in the activation and/or regulation of the respiratory burst (6,7). Thus, in such a way Aβ(25-35) might be able to prime MDMs for an enhanced Aβ(1-42)-induced respiratory burst. Alternatively, the effects of Aβ(25-35) might be due to its direct influence on the biophysical properties and/or the assembly state of Aβ(1-42). Particularly since preincubation of cells with Aβ(25-35) prior to the addition of Aβ(1-42) did not have any effect, in our opinion the latter explanation seems most plausible. Hence, notwithstanding the fact that Aβ(25-35) fragment as such does not exists in the AD brain, the results

obtained in our study might be relevant in that they provide new insight into the structural features required for Aβ-microglia interactions and consequent microglia-driven pathogenic mechanisms.

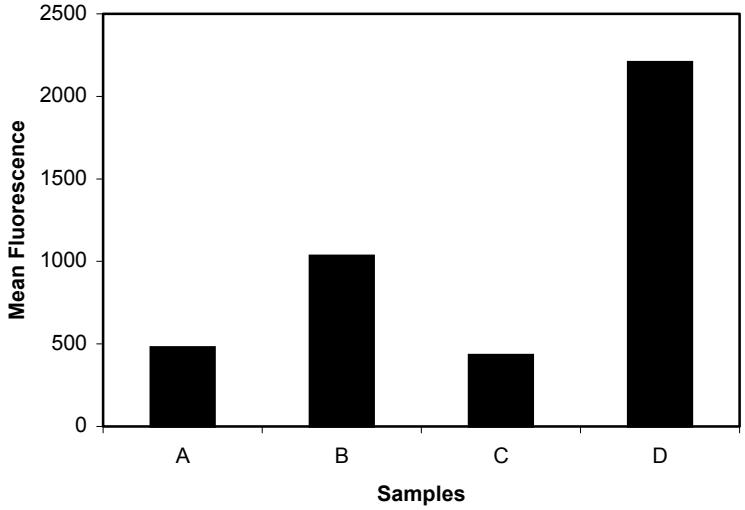

Figure 2

Effect of Aβ(25-35) on the binding of Aβ(1-42)-FITC to human monocyte-derived macrophages. Cells were incubated for 30 min with either 2.5 μM Aβ(1-42)-FITC (A), or Aβ(1-42)-FITC together with 50 μM Aβ(25-35) (B), or preincubated with Aβ(25-35) followed by Aβ(1-42)-FITC (C), or preincubation with a mixture of Aβ(25-35) and Aβ(1-42)-FITC (D).

Acknowledgements

This work was supported by Grant No. 903-51-104 from the Dutch Organisation for Scientific Research (NWO) to H.S.L.M.N.

References

1. Van Muiswinkel, F. L., R. Veerhuis and P. Eikelenboom. 1999. Amyloid beta protein primes cultured rat microglial cells for an enhanced phorbol 12-myristate 13-acetate-induced repiratory burst activity. *J.Neurochem.* 66:2468.
2. Giulian, D., L. J. Haverkamp, J. H. Yu, W. Karshin, D. Tom, J. Li, J. Kirkpatrick, Y.-M. Kuo and A.E. Roher. 1996. Specific domains of beta-amyloid from Alzheimer plaque elicit neuron killing in human microglia. *J.Neurosci.* 16:6021.

3. Giulian, D. 1997. Immune responses and dementia. Reis DJ and Posner JB eds. Ann. NY Acad. Sci., New York, p. 91.
4. Nottet, H. S. L. M., L. de Graaf, N. M. de Vos, L. Bakker, J. A. G. van Strijp, M. R. Visser and J. Verhoef. 1993. Phagocytic function of monocyte-derived macrophages is not affected by human immunodeficiency virus type 1 infection. *J.Infect.Dis.* 168:84.
5. Korotzer, A. R., E. R. Whittemore and C. W. Cotman, 1995. Differential regulation by beta-amyloid peptides of intracellular free Ca^{2+} concentration in cultured rat microglia. *Eur.J.Pharm.* 288:125.
6. McDonald, D. R., M. E. Bamberger, C. K. Combs, G. E. Landreth. 1998. Beta-amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP-1 monocytes. *J.Neurosci.* 18:4451.
7. Colton, C. A., M. Jia, M. S. Li, D. L. Gilbert. 1994. K^+ modulation of microglial superoxide production: involvement of voltage-gated Ca^{2+} channels. *Am.J.Physiol.Cell Physiol.* 266:C1650.

3

Intracellular pathways involved in TNF-α and superoxide anion release by Aβ(1-42)-stimulated primary human macrophages

Hessel A. Smits
N. Machiel de Vos
Jesse W.Y. Wat
Tjomme van der Bruggen
Jan Verhoef
Hans S.L.M. Nottet

Eijkman-Winkler Institute, section Neuroimmunology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Journal of Neuroimmunology (2001): 115, 144-151.

Abstract

In this study the intracellular signal transduction pathways leading to the production of TNF-α and superoxide anions by amyloid-β-stimulated primary human monocyte-derived macrophages was investigated. Using Western blotting and specific inhibitors it is shown that both ERK 1/2 and p38 MAPK signal transduction pathways as well as PKC are involved in the amyloid-β-stimulated superoxide anion production. In contrast, only ERK 1/2 MAPK seems to be involved in TNF-α production: questioning the connection between PKC and ERK 1/2 activation. Our results suggest the use of ERK 1/2 MAPK inhibitors in the prevention of macrophage activation in the context of Alzheimer's disease.

Introduction

One of the characteristics of Alzheimer's disease (AD) is the presence of extracellular depositions in the cortical gray matter (1). These so-called neuritic plaques are mainly composed of aggregated amyloid-β (Aβ) peptides. Studies with synthetic Aβ have shown that it can act as a potent and direct neurotoxic agent (2-4). Furthermore, neuritic plaques are reported to be surrounded by microglia (5-8). Activated microglia, or so called brain macrophages, are able to produce neurotoxic factors and a lot of research has focused on the ability of Aβ to induce these events. Studies have shown that Aβ is able to induce proinflammatory interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) but also neurotrophic molecules like nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) in THP-1 monocytes (9-13). Aβ is also reported to stimulate the generation of reactive oxygen species (ROS) in rat microglia, THP-1 monocytes (14-17) and recently also in primary human macrophages (18,19). Moreover, an excessive release of ROS not only leads to oxidative stress but may also potentiate the inflammatory response, e.g. by triggering redox-sensitive expression of various inflammatory genes (20,21).

The intracellular pathways of Aβ-stimulated microglia leading to the production of these neurotoxic products are still largely enigmatic. Research of these signal transduction pathways mediating the neurotoxic response of Aβ has revealed the mitogen-activated protein kinase (MAPK) superfamily members ERK 1/2 and p38 MAPK to be important mediators (22,23). Besides, an increasing amount of evidence shows that the transcription factor nuclear factor-κB (NF-κB) can be stimulated by Aβ in neurons (3,21,24) and by Aβ(25-35) in microglia (12,12,25). Upregulation of ERK 1/2 and p38 MAPK can lead to the activation of numerous transcription factors in their turn leading to the activation of cytokines and neurotrophic factors. Activation of NF-κB can lead to the transcription of genes expressing TNF-α, IL-1, IL-6, monocyte chemoattractant protein-1 (MCP-1) and nitric oxide (NO) synthase (reviewed in (20)).

In this report the intracellular pathways of Aβ-stimulated monocyte-derived macrophages leading to the production of TNF-α and superoxide anions were investigated.

Using Westernblot analysis the activation of ERK 1/2, p38 MAPK and protein kinase C (PKC) by Aβ was studied. More specific the role of ERK 1/2, p38 MAPK and protein kinase C (PKC) in the production of TNF-α and superoxide anions was studied using PD098059 (26,27), SB202190 (28,29) and bisindolylmaleimide (30) as specific inhibitors. In addition, staurosporine was used to study the role of PKC and protein kinase A (PKA), protein kinase G (PKG) and calmodulin-dependant protein kinase (CaM) (31).

Materials and methods

Materials

The synthetic Aβ peptide Aβ(1-42) was obtained from Genosphere Biotechnologies (Paris, France). PKC mouse monoclonal antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). ERK 1/2 phospho-specific rabbit polyclonal antibody and p38 MAPK phospho-specific rabbit polyclonal antibody were bought from New England BioLabs (Frankfurt am Main, Germany). Goat Anti-Rabbit IgG-HRP secondary antibody was obtained from Southern Biotechnology Associates (Birmingham, AL, USA). PD098059, SB202190 and staurosporine were obtained from Calbiochem (La Jolla, CA, USA). Bisindolylmaleimide, gentamycin sulfate and bis-*N*-methylacridinum were purchased from Sigma (Zwijndrecht, The Netherlands). Iscove's modified Dulbecco's medium was obtained from Gibco Life Technologies (Breda, the Netherlands). Ciprofloxacin was bought from Bayer Pharmaceuticals (Mijdrecht, The Netherlands) and Ficoll-paque from Pharmacia Biotech (Uppsala, Sweden).

Amyloid-β

Lyophilized synthetic Aβ(1-42) peptide, initially dissolved at a concentration of 1 mM in sterile double-distilled pyrogen-free water, was aliquoted and stored at –20 °C.

Cells

Monocytes were derived from peripheral blood mononuclear cells by Ficoll-paque density gradients and purified by centrifugal elutriation as described previously (32). Cells were seeded at a concentration of 2 x 10^6 cells / ml in Teflon Erlenmeyer flasks (Nalgene, USA) and grown as suspension at 37 °C in a humidified atmosphere of 5% CO^2 / 95% air. Culture medium was composed of Iscove's modified Dulbecco's medium supplemented with 10% (v/v) heat-inactivated human AB serum, 2 mM L-glutamine, 19 mM sodium bicarbonate, 10 μg/ml gentamicin and 0.5 μg/ml ciprofloxacin. After 7 days, monocyte-derived macrophages (MDMs) were harvested from the flasks, washed with Hanks' balanced salt solution (HBSS) and used for experiments.

Westernblot analysis of Aβ-stimulated ERK 1/2, p38 MAPK and PKC activation

To measure ERK 1/2, p38 MAPK and PKC activation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed, followed by Western blotting. Blots then were stained with a purified rabbit antiserum containing antibodies directed against Thr202- and Tyr204-phosphorylated (i.e., active) ERK 1 (p44) and ERK 2 (p42) or Thr180- and Tyr182-phosphorylated p38 MAPK or with a purified mouse antiserum containing antibodies against a highly conserved epitope within the hinge region of PKC. First macrophages were stimulated with 10 μM Aβ(1-42) for the indicated times (Fig. 1) at 37 °C. After incubation, the cells were washed with ice-cold phosphate-buffered saline and resuspended in 80 μl lysis buffer (1% Triton X-100, 50 mM Tris-HCl [pH 8.0], 100 mM NaCl) containing several inhibitors (1 μg of antipain per ml, 2 μg of benzamidine per ml, 1 μg of leupeptin per ml, 1 μg of chymostatin per ml, 1 μg of pepstatin A per ml, 1 mM phenylmethylsulfonyl fluoride). After addition of 80 ml 2x sample buffer (final concentrations of 2% SDS, 2% β-mercaptoethanol, and 10% glycerol in 300 mM Tris-Cl pH 6.8), the lysates were boiled for 2 min. Samples were run on a 10% polyacrylamide minigel for 1 h at 150 V and blotted for 2 h (50 V) on a 0.2-μm-pore-size polyvinylidene difluoride (PVDF) membrane at 4 °C. The PVDF membrane was blocked with 0.5% Tween-20 in PBS for 1 h at room temperature and subsequently incubated for 1 h with the ERK 1/2 or p38 MAPK phosphospecific rabbit antibodies diluted 1:2,000 in blocking buffer or with the PKC mouse antibody diluted 1:2,000. After being washed six times with PBS/0.5% Tween-20 for 3 min, the PVDF membrane was incubated at room temperature for 2 h with goat anti-rabbit-peroxidase conjugate (1:8,000 in blocking buffer) or with goat anti-mouse-peroxidase conjugate. The PVDF filter was again washed six times with PBS/0.5% Tween-20 and then twice with PBS for 5 min. Thereafter, proteins were visualized with enhanced chemiluminescence on film (Kodak X-OMAT LS).

TNF-α ELISA

Macrophages were grown in teflon flasks for six days after which they were seeded in a 48-well plate (4 x 10^5 / well). Next day cells were preincubated with specific enzyme inhibitors for 30 min after which they were incubated with the same inhibitors together with 10 μM Aβ(1-42) at 37 °C in a humidified atmosphere of 5% CO^2 / 95% air. After 5 hours a sample of the supernatant was taken and the TNF-α concentration was quantified with an ELISA. In short, TNF-α was captured by monoclonal anti-human TNF-α antibody and detected with a biotinylated anti-human TNF-α antibody according to the manufacturer's instructions (Pelikine compact human TNF-α ELISA kit, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Superoxide anion production

Superoxide production was measured as lucigenin-enhanced chemiluminescence (33). Briefly, cells were transferred to polystyrene vials (2 x 10^5 / vial), placed into a luminometer (Packard Instruments, Belgium) and incubated for 30 min at 37 °C in HBSS

containing 250 nM bis-*N*-methylacridinum (Lucigenin) to assess the spontaneous release of superoxide. Subsequently, cells were preincubated with specific enzyme inhibitors for 30 min after which 10 µM Aβ(1-42) was added and chemiluminescence (expressed as millivolts, mV) was monitored for 30 min.

Results

To resolve the intracellular pathways in Aβ-stimulated MDMs leading to the production of TNF-α and superoxide anions, the stimulation of ERK 1/2, p38 MAPK and PKC in macrophages stimulated with Aβ(1-42) was investigated. First primary human macrophages stimulated for 2, 5, 10, 20 and 40 min with 10 µM Aβ(1-42) were stained on Western blot for phosphorylated ERK 1/2. Results showed an increased ERK 1/2 activity after 2 min (Fig. 1A). The decline of ERK 1/2 activity started 5 min after stimulation.

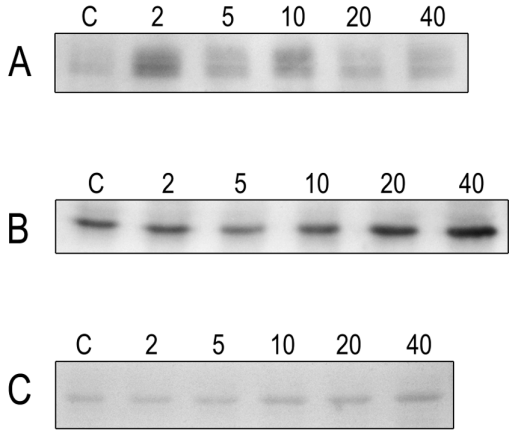

Figure 1

Aβ(1-42) specifically activates ERK 1/2 in primary human macrophages. Cells were incubated with 10 µM Aβ(1-42) for 2, 5, 10, 20 and 40 min. Aliquotes of the cell lysates were resolved by SDS-PAGE, Western blotted and visualized by chemiluminescence. (A) anti-phospho-ERK rabbit antibodies show an increase in activated ERK 1/2 after 2 min incubation with Aβ(1-42) compared to control cells. (B) anti-phospho-p38 rabbit antibodies show no stimulation of p38 MAPK activity after incubation with Aβ(1-42). (C) Mouse antiserum containing antibodies against PKC show no increase in PKC after stimulation with Aβ(1-42). We isolated monocytes from 4 different donors and consequently performed a whole cell extraction on Aβ-stimulated macrophages after 7 days. Each whole cell extract was blotted and stained at least two times.

Figure 1B indicates that Aβ(1-42) could not activate p38 MAPK beyond the background activity in control cells (Fig 1B, lane 1). There seems to be a slight increase in p38 activity after 40 min. However, this effect was not reproducible. All extracts showed a constant p38 activity. Aβ(1-42)-stimulated macrophages showed no PKC activity on

Western blots (Fig 1C). We isolated monocytes from 4 different donors and consequently performed a whole cell extraction on Aβ-stimulated macrophages after 7 days. Each whole cell extract was blotted and stained at least two times.

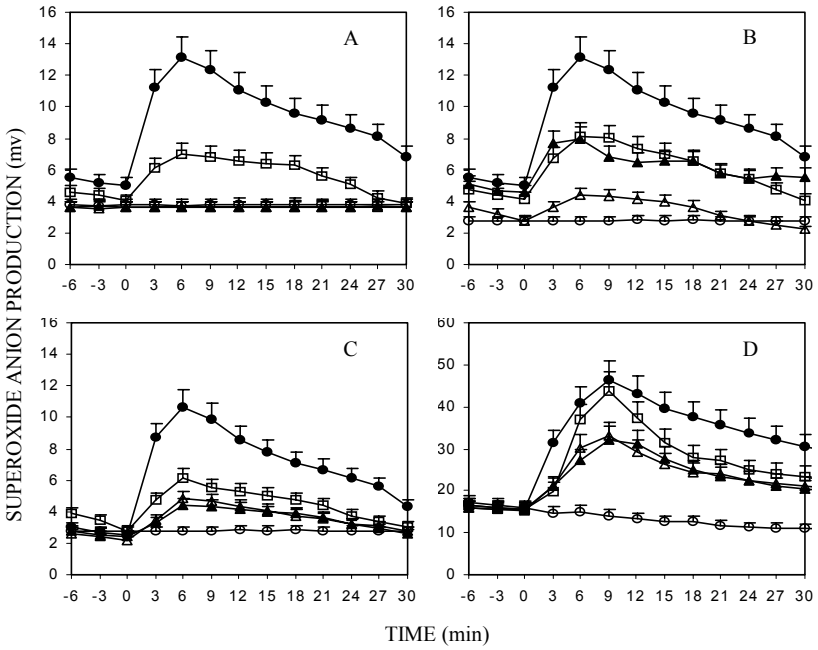

Figure 2

Inhibition of the Aβ(1-42)-induced superoxide anion production in primary human macrophages. Cells were preincubated with inhibitors for 30 min after which they were exposed to buffer (open circle) or to 10 μM Aβ(1-42) (closed circle). Superoxide anion production was inhibited with 10 (open square), 1 (open triangle) and 0.1 (closed triangle) μM staurosporine (**A**), 100 (open square), 33 (open triangle) and 10 (closed triangle) nM bisindolylmaleide (**B**), 50 (open square), 25 (open triangle) and 12.5 (closed triangle) μM PD098059 (**C**) and 50 (open square), 25 (open triangle) and 12.5 (closed triangle) μM SB202190 (**D**).

Macrophages, isolated from several donors, showed a consequent increase (~ 3.4 fold) of superoxide anion production within the first 6 min after exposure to 10 μM Aβ(1-42) (Fig. 2A-D). To determine whether ERK 1/2, p38 MAPK and PKC are involved in the activation of the NADPH-complex and consequent release of superoxide anions, macrophages were incubated with specific enzyme inhibitors. Incubation with staurosporine revealed a partial inhibition of superoxide anion production at a concentration of 100 nM and complete inhibition at concentrations of 1 μM and higher (Fig. 2A). Incubation with bisindolylmaleimide, a specific PKC inhibitor, showed a partial inhibition at 10 and 33 nM and a nearly complete inhibition at 100 nM (Fig. 2B). Complete inhibition was seen at 500 nM and higher concentrations (data not shown). To determine whether

ERK 1/2 and the p38 MAPK signal transduction pathways are involved in the superoxide anion production, macrophages were incubated with PD098059 as a specific inhibitor for ERK 1/2 and SB202190 as a specific inhibitor for p38 MAPK. Incubation with 12.5, 25 and 50 μM PD098059 revealed a partial inhibition of the superoxide anion production (Fig. 2C). Treatment with SB202190 at 12.5, 25 and 50 μM also resulted in a partially inhibited superoxide anion production (Fig. 2D). These results suggest that the PKC, ERK 1/2 and p38 MAPK signal transduction pathways are involved in superoxide anion production of Aβ(1-42)-induced macrophages.

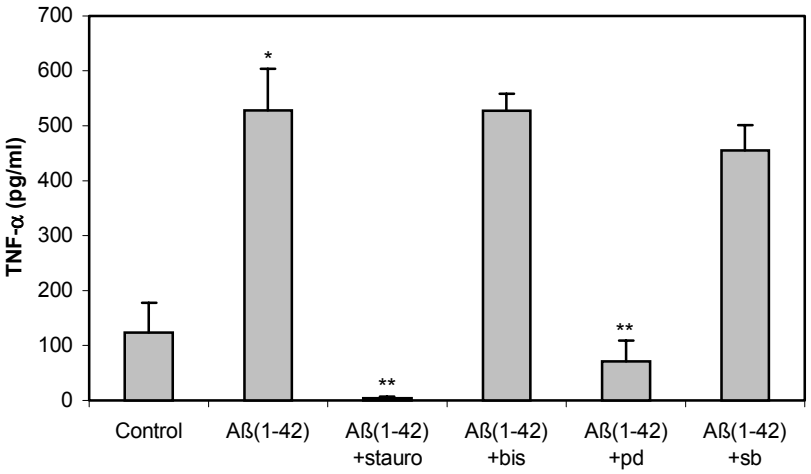

Figure 3

Inhibition of the Aβ(1-42)-induced TNF-α production in primary human macrophages. Cells were preincubated with 10 μM staurosporine (**stauro**), 500 nM bisindolylmaleimide (**bis**), 50 μM pd098059 (**pd**) and 50 μM sb202190 (**sb**) after which they were incubated with 10 μM Aβ(1-42) together with these inhibitors for 5 hours. Supernatant samples were taken and TNF-α levels were quantified. Results are representative of three independent experiments (* $p<0.05$ compared to unstimulated cells, ** $p<0.05$ compared to Aβ(1-42)-stimulated cells).

Macrophages stimulated with 10 μM Aβ(1-42) also revealed a 4.3 fold ($p<0.05$) increase in TNF-α production (Fig. 3). To check whether PKC is involved in Aβ-induced TNF-α production, cells were incubated with staurosporine as well as bisindolylmaleimide. Incubating macrophages with 50 μM staurosporine revealed a complete block of the TNF-α production ($p<0.01$), whereas treatment with 500 nM bisindolylmaleimide showed no significant decrease in TNF-α production. To investigate the involvement of ERK 1/2 in the Aβ-stimulated TNF-α production cells were incubated with 50 μM PD098059. As shown in Fig. 3, PD098059 resulted in a complete inhibition of TNF-α production ($p<0.02$).

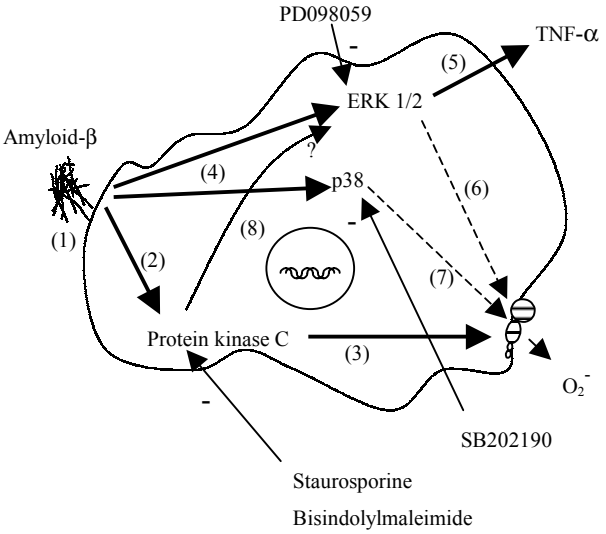

Figure 4

Mechanism of macrophage activation by Aβ leading to the release of pro-inflammatory superoxide anions and TNF-α. A schematic is shown indicating the relationships between Aβ on one hand and superoxide anion and TNF-α on the other hand. Aβ can activate macrophages (1) which triggers Protein Kinase C (2) and subsequently stimulates NADPH (3) to release superoxide anions. Aβ stimulation also induces ERK 1/2 and p38 MAPK (4). ERK 1/2 activation induces TNF-α production (5) and partially induces NADPH activity (6). P38 activation only induces NADPH activation (7). Protein kinase C is suggested to activate ERK 1/2 as outlined in the discussion (8).

Macrophages were incubated with 50 μM SB202190 to check whether p38 MAPK is involved in TNF-α production. The results clearly indicate that there is no significant decrease of TNF-α (Fig 2). All together, these results indicate that although staurosporine inhibits TNF-α production PKC seems not involved in the Aβ stimulated TNF-α production due to the fact that bisindolylmaleimide does not show any inhibition. The ERK 1/2 pathway seems involved in the Aβ-stimulated TNF-α production in contrast to p38 MAPK pathway (Fig. 4).

Discussion

Neuritic plaques in brains of people suffering from AD are found to be surrounded by microglia. These microglia are normally in a resting state but they can be activated by numerous signals from their surroundings. Depending on the signal, these microglia can then produce a number of pro-inflammatory products. In AD these microglial cells are activated and are thought to produce a number of pro-inflammatory products. By doing so these microglia are thought to stimulate and enhance the neuronal damage and by activating

surrounding microglia are thought to continue the inflammatory process. In the study presented in this paper we have tried to partially unravel the intracellular pathways activated in microglia as a response to Aβ, leading to the production of the pro-inflammatory products TNF-α and superoxide anions. Our results show that upon Aβ(1-42) stimulation macrophages produce TNF-α. Using specific inhibitors it was shown results show that ERK 1/2, and not p38 MAPK and PKC, plays an important role in the production of TNF-α. Western blot analysis confirms the ability of Aβ to stimulate the activation of ERK 1/2. Regarding the production of superoxide anions, the situation is different. Bisindolylmaleimide can completely inhibit the superoxide anion production indicating an important role for PKC. We could however not detect an activation of PKC on Western blot. Furthermore, inhibiting properties of PD098059 and SB202190 indicate participation of ERK 1/2 and p38 MAPK in the signal transduction pathway leading to superoxide anion production. However, using these inhibitors the superoxide anion production could not be blocked completely, indicating a possible existence of multiple pathways.

Despite extensive research over the last years, the precise pathways following Aβ stimulation are not yet fully understood. Differences in the source and in the use of Aβ and differences in the cells used as a model for human microglia often results in different outcomes. Studies performed with rat microglia and THP-1 monocytes stimulated with Aβ(1-40) showed an activation of ERK 1/2 as well as p38 MAPK (22). Studies by Pyo et al. using Aβ(25-35)-stimulated rat microglia showed a correlation between ERK 1/2 and p38 MAPK and TNF-α production (34), in contrast to results presented in this paper. In our study Aβ(1-42) and human macrophages were used in contrast to Aβ(25-35) and rat microglia that were used by Pyo et al.

Although the signal transduction pathways of the Aβ-induced TNF-α production in mononuclear cells have been studied by several investigators, to our knowledge no studies have been performed on the Aβ-induced signal transduction pathways that lead to superoxide anion production. A study by Yagisawa et al. on fMLP-stimulated human monocytes showed a partial inhibition of the superoxide release by PD098059 or SB202190 (35). In neutrophils stimulated by fMLP or PMA a relation between NADPH oxidase and p38 MAPK has also been demonstrated (36). Using SB202190 as a specific inhibitor, our results show p38 MAPK as an intermediate for superoxide anion production and show p38 MAPK not to be involved in TNF-α production. Looking at the Western blots we could not detect Aβ(1-42) to be able to activate p38 MAPK beyond the level found in control cells. However, because of the use of a specific inhibitor we hypothesize p38 MAPK to be an intermediate in the signal transduction pathway leading to the production of superoxide anions, independent of stimulation with Aβ. Because the pathway between Aβ located near the cell membrane and the activation of p38 MAPK is not yet understood, further investigation is necessary to define the role of p38 MAPK.

This paper shows results indicating that PKC is not involved in the TNF-α production but seems to be important in the superoxide anion production. Using a monoclonal antibody to stain Western blots we could not detect an activation of PKC. Because of the many function of PKC in the cell it is possible that the amount of PKC activated in the cell is very low. Moreover our results show that the superoxide anion

production could nearly completely be inhibited using only 100 nM bisindolylmaleimide. It is possible that because of this low amount of PKC activated we could not detect its activity on Western blot. Several other studies have indicated that PKC might be involved in the pathways following Aβ treatment. A minor induction of TNF-α, which could be inhibited by PKC inhibitor H-7, was found in Aβ(1-40)-induced THP-1 monocytes (11). Also Siedlar et al. found that upon stimulation of monocytes with colorectal cancer cells, PKC activation is involved (37). It has also been shown that stimulation with Aβ(25-35) and the consequent chemotaxis of microglial cells also required PKC as an intracellular intermediate (38). Recently Combs et al. showed that upon stimulation with fibrillar Aβ PKC activity is required for protein tyrosine phosphorylation (23). Whether there is a link between PKC and ERK 1/2 is still not clear, as shown in Fig. 4. Combs et al. found ERK 1/2 being a downstream event of PKC activation (23). Haring et al. however found ERK 1/2 and PKC to be operating in parallel in the secretion of the soluble form of amyloid precursor protein in PC12 cells (39). Because of the complete inhibition of PKC and the partial inhibition of ERK 1/2 in the superoxide anion production, our results suggest a link (Fig. 4). It seems however be possible to activate ERK 1/2 independent of PKC since our results indicate a difference between bisindolylmaleimide and staurosporine in Aβ-stimulated TNF-α production. Moreover, staurosporine not only inhibits PKC but also inhibits PKA, PKG and CaM.

The capabilities of staurosporine to inhibit various kinases together with the different results of bisindolylmaleimide and staurosporine suggest that other enzymes are involved in the Aβ-stimulated production of TNF-α. Other studies showed that inhibition of PKA did not change TNF-α levels in monocytes (37,40). Also Wood et al. showed that in microglia PKG seems not to be involved in LPS-stimulated TNF-α release (41). Furthermore, Mattsson et al. found CaM kinase to be involved in the LPS-stimulated induction of TNF-α in human monocytes (42). All together these data suggest that nor PKC, nor PKA or PKG are likely to be involved in the Aβ(1-42)-induced TNF-α production and that CaM might be responsible.

Acknowledgments

This work was supported by the Dutch Organization for Scientific Research (NWO) grant 903-51-141 to H.S.L.M. Nottet. H.S.L.M. Nottet is a fellow of the Royal Netherlands Academy of Sciences and Art.

References

1. Dickson, D. W. 1997. Neuropathological diagnosis of Alzheimer's disease: a perspective from longitudinal clinicopathological studies. *Neurobiol.Aging 18*:S21.
2. Yankner, B. A., L. K. Duffy, and D. A. Kirschner. 1990. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science 250*:279.
3. Behl, C., J. B. Davis , R. Lesley, and D. Schubert. 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell 77*:817.
4. Pike, C. J., N. Ramezan Arab, and C. W. Cotman. 1997. Beta-amyloid neurotoxicity in vitro: evidence of oxidative stress but not protection by antioxidants. *J.Neurochem. 69*:1601.
5. McGeer, P. L., S. Itagaki, H. Tago, and E. G. McGeer. 1987. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. *Neurosci.Lett. 79*:195.
6. Wisniewski, H. M., J. Wegiel, K. C. Wang, M. Kujawa, and B. Lach. 1989. Ultrastructural studies of the cells forming amyloid fibers in classical plaques. *Can.J.Neurol.Sci. 16*:535.
7. Rozemuller, J. M., P. Eikelenboom, S. T. Pals, and F. C. Stam. 1989. Microglial cells around amyloid plaques in Alzheimer's disease express leucocyte adhesion molecules of the LFA-1 family. *Neurosci.Lett. 101*:288.
8. Sasaki, A., H. Yamaguchi, A. Ogawa, S. Sugihara, and Y. Nakazato. 1997. Microglial activation in early stages of amyloid beta protein deposition. *Acta Neuropathol.(Berl) 94*:316.
9. Giulian, D., L. J. Haverkamp, J. Li, W. L. Karshin, J. Yu, D. Tom, X. Li, and J. B. Kirkpatrick. 1995. Senile plaques stimulate microglia to release a neurotoxin found in Alzheimer brain. *Neurochem.Int. 27*:119.
10. Lorton, D., J. M. Kocsis, L. King, K. Madden, and K. R. Brunden. 1996. beta-Amyloid induces increased release of interleukin-1 beta from lipopolysaccharide-activated human monocytes. *J.Neuroimmunol. 67*:21.
11. Klegeris, A., D. G. Walker, and P. L. McGeer. 1997. Interaction of Alzheimer beta-amyloid peptide with the human monocytic cell line THP-1 results in a protein kinase C-dependent secretion of tumor necrosis factor-alpha. *Brain Res. 747*:114.
12. Heese, K., C. Hock, and U. Otten. 1998. Inflammatory signals induce neurotrophin expression in human microglial cells. *J.Neurochem. 70*:699.
13. Ariga, T. and R. K. Yu. 1999. GM1 inhibits amyloid beta-protein-induced cytokine release. *Neurochem.Res. 24*:219.
14. Klegeris, A., D. G. Walker, and P. L. McGeer. 1994. Activation of macrophages by Alzheimer beta amyloid peptide. *Biochem.Biophys.Res.Commun. 199*:984.
15. Meda, L., C. Bonaiuto, P. Baron, L. Otvos, Jr., F. Rossi, and M. A. Cassatella. 1996. Priming of monocyte respiratory burst by beta-amyloid fragment (25-35). *Neurosci.Lett. 219*:91.
16. Klegeris, A. and P. L. McGeer. 1997. beta-amyloid protein enhances macrophage production of oxygen free radicals and glutamate. *J.Neurosci.Res. 49*:229.
17. Tjernberg, L. O., D. J. Callaway, A. Tjernberg, S. Hahne, C. Lilliehook, L. Terenius, J. Thyberg, and C. Nordstedt. 1999. A molecular model of Alzheimer amyloid beta-peptide fibril formation. *J.Biol.Chem. 274*:12619.

18. Smits, H. A., F. L. Van Muiswinkel, N. M. de Vos, J. Verhoef, and H. S. Nottet. 1999. The amyloid-beta (1-42)-induced respiratory burst of primary human macrophages is enhanced in the presence of amyloid-beta (25- 35). In *Alzheimer's Disease and Related Disorders*. K.Iqbal, D.F.Swaab, B.Winblad and H.M.Wisniewski, eds. John Wiley & Sons Ltd, Sussex, p. 457.
19. Van Muiswinkel, F. L., S. F. Raupp, N. M. de Vos, H. A. Smits, J. Verhoef, P. Eikelenboom, and H. S. Nottet. 1999. The amino-terminus of the amyloid-beta protein is critical for the cellular binding and consequent activation of the respiratory burst of human macrophages. *J.Neuroimmunol. 96*:121.
20. Baeuerle, P. A. and T. Henkel. 1994. Function and activation of NF-kappa B in the immune system. *Annu.Rev.Immunol. 12*:141.
21. Kaltschmidt, B., M. Uherek, B. Volk, P. A. Baeuerle , and C. Kaltschmidt. 1997. Transcription factor NF-kappaB is activated in primary neurons by amyloid beta peptides and in neurons surrounding early plaques from patients with Alzheimer disease. *Proc.Natl.Acad.Sci.U.S.A. 94*:2642.
22. McDonald, D. R., M. E. Bamberger, C. K. Combs, and G. E. Landreth. 1998. beta-Amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. *J.Neurosci. 18*:4451.
23. Combs, C. K., D. E. Johnson, S. B. Cannady, T. M. Lehman, and G. E. Landreth. 1999. Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. *J.Neurosci. 19*:928.
24. Bales, K. R., Y. Du, R. C. Dodel, G. M. Yan, E. Hamilton Byrd, and S. M. Paul. 1998. The NF-kappaB/Rel family of proteins mediates Abeta-induced neurotoxicity and glial activation. *Brain Res.Mol.Brain Res. 57*:63.
25. Bonaiuto, C., P. P. McDonald, F. Rossi, and M. A. Cassatella. 1997. Activation of nuclear factor-kappa B by beta-amyloid peptides and interferon-gamma in murine microglia. *J.Neuroimmunol. 77*:51.
26. Cescato, R., E. Dumermuth, M. Spiess, and P. A. Paganetti. 2000. Increased generation of alternatively cleaved beta-amyloid peptides in cells expressing mutants of the amyloid precursor protein defective in endocytosis. *J.Neurochem. 74*:1131.
27. Postuma, R. B., W. He, J. Nunan, K. Beyreuther, C. L. Masters, C. J. Barrow, and D. H. Small. 2000. Substrate-bound beta-amyloid peptides inhibit cell adhesion and neurite outgrowth in primary neuronal cultures. *J.Neurochem. 74*:1122.
28. Goodman, Y. and M. P. Mattson. 1996. Ceramide protects hippocampal neurons against excitotoxic and oxidative insults, and amyloid beta-peptide toxicity. *J.Neurochem. 66*:869.
29. Yates, S. L., L. H. Burgess, J. Kocsis-Angle, J. M. Antal, M. D. Dority, P. B. Embury, A. M. Piotrkowski, and K. R. Brunden. 2000. Amyloid beta and amylin fibrils induce increases in proinflammatory cytokine and chemokine production by THP-1 cells and murine microglia. *J.Neurochem. 74*:1017.
30. Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, L. Duhamel, D. Charon, and J. Kirilovsky. 1991. The Bisindolylmaleimide GF 109203X Is a Potent and Selective Inhibitor of Protein Kinase C. *J.Biol.Chem. 266*:15771.
31. Barger, S. W., D. Horster, K. Furukawa, Y. Goodman , J. Krieglstein, and M. P. Mattson. 1995. Tumor necrosis factors alpha and beta protect neurons against amyloid beta-peptide toxicity: evidence for involvement of a kappa B-binding factor and attenuation of peroxide and Ca2+ accumulation. *Proc.Natl.Acad.Sci.U.S.A 92*:9328.

32. Boven, L. A., L. Gomes, C. Hery, F. Gray, J. Verhoef, P. Portegies, M. Tardieu, and H. S. Nottet. 1999. Increased peroxynitrite activity in AIDS Dementia Complex: implications for neuropathogenesis of HIV-1 infection. *J.Immunol. 162*:4319.
33. Nottet, H. S., L. de Graaf, N. M. de Vos, L. J. Bakker, J. A. van Strijp, M. R. Visser, and J. Verhoef. 1993. Phagocytic function of monocyte-derived macrophages is not affected by human immunodeficiency virus type 1 infection. *J.Infect.Dis. 168*:84.
34. Pyo, H., I. Jou, S. Jung, S. Hong, and E. H. Joe. 1998. Mitogen-activated protein kinases activated by lipopolysaccharide and beta-amyloid in cultured rat microglia. *Neuroreport. 9*:871.
35. Yagisawa, M., K. Saeki, E. Okuma, T. Kitamura, S. Kitagawa, H. Hirai, Y. Yazaki, F. Takaku, and A. Yuo. 1999. Signal transduction pathways in normal human monocytes stimulated by cytokines and mediators: comparative study with normal human neutrophils or transformed cells and the putative roles in functionality and cell biology. *Exp.Hematol. 27*:1063.
36. Lal, A. S., A. D. Clifton, J. Rouse, A. W. Segal, and P. Cohen. 1999. Activation of the neutrophil NADPH oxidase is inhibited by SB 203580, a specific inhibitor of SAPK2/p38. *Biochem.Biophys.Res.Commun. 259*:465.
37. Siedlar, M., B. Mytar, M. Hyszko, and M. Zembala. 1999. Involvement of protein kinases in signalling for nitric oxide (NO) and tumour necrosis factor alpha (TNF) production by monocytes stimulated with colorectal DeTa cancer cells: the lack of evidence for the role of TNF in the regulation of NO production. *Immunol.Lett. 65*:189.
38. Nakai, M., K. Hojo, T. Taniguchi, A. Terashima, T. Kawamata, T. Hashimoto, K. Maeda, and C. Tanaka. 1998. PKC and tyrosine kinase involvement in amyloid beta (25-35)-induced chemotaxis of microglia. *Neuroreport 9*:3467.
39. Haring, R., A. Fisher, D. Marciano, Z. Pittel, Y. Kloog, A. Zuckerman, N. Eshhar, and E. Heldman. 1998. Mitogen-activated protein kinase-dependent and protein kinase C- dependent pathways link the m1 muscarinic receptor to beta-amyloid precursor protein secretion. *J.Neurochem. 71*:2094.
40. Geng, Y., B. Zhang, and M. Lotz. 1993. Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. *J.Immunol. 151*:6692.
41. Wood, P. L. 1994. Differential regulation of IL-1 alpha and TNF alpha release from immortalized murine microglia (BV-2). *Life Sci. 55*:661.
42. Mattsson, E., H. Van Dijk, K. Van Kessel, J. Verhoef, A. Fleer, and J. Rollof. 1996. Intracellular pathways involved in tumor necrosis factor-alpha release by human monocytes on stimulation with lipopolysaccharide or staphylococcal peptidoglycan are partly similar. *J.Infect.Dis. 173*:212.

4

Activation of human macrophages by amyloid-β is attenuated by astrocytes

Hessel A. Smits[1]
Astrid J. van Beelen[1]
N. Machiel de Vos[1]
Annemarie Rijsmus[1]
Tjomme van der Bruggen[1]
Jan Verhoef[1]
Freek L. van Muiswinkel[2]
Hans S.L.M. Nottet[1]

1 *Eijkman-Winkler* Institute, section Neuroimmunology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
2 Research Institute Neurosciences, Vrije Universiteit, Faculty of Medicine, Department of Psychiatry, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

The Journal of Immunology (2001), 166: 6869-6876.

Abstract

In Alzheimer's disease neuritic amyloid-β plaques together with surrounding activated microglia and astrocytes are thought to play an important role in the inflammatory events leading to neurodegeneration. Studies have indicated that amyloid-β can be direct neurotoxic and, by activating these glial cells to produce oxygen radicals and pro-inflammatory cytokines. This report shows that, using primary human monocyte-derived macrophages as model cells for microglia, amyloid-β(1-42) stimulate these macrophages to the production of superoxide anions and TNF-α. In contrast, astrocytes do not produce both inflammatory mediators when stimulated with amyloid-β(1-42). In cocultures with astrocytes and amyloid-β(1-42)-stimulated macrophages decreased levels of both superoxide anion and TNF-α were detected. These decreased levels of potential neurotoxins were due to binding of amyloid-β(1-42) to astrocytes since FACSscan analysis demonstrated binding of FITC-labeled amyloid-β(1-42) to astrocytoma cells and pretreatment of astrocytes with amyloid-β(1-16) prevented the decrease of superoxide anion in cocultures of human astrocytes and amyloid-β(1-42)-stimulated macrophages. In order to elucidate an intracellular pathway involved in TNF-α secretion, the activation state of NF-κB was investigated in macrophages and astrocytoma cells after amyloid-β(1-42) treatment. Interestingly, while activation of NF-κB could not be detected in amyloid-β-stimulated macrophages it was readily detected in astrocytoma cells. These results not only demonstrate that amyloid-β stimulation of astrocytes and macrophages result in different intracellular pathway activation but also indicate that astrocytes attenuate the immune response of macrophages to amyloid-β(1-42) by interfering with amyloid-β(1-42) binding to macrophages.

Introduction

Alzheimer's disease (AD) is the most common cause of progressive dementia and a neurodegenerative disorder, which despite scientific progress in recent years is still a disease of unknown etiology. One pathological hallmark of AD is the presence of neuritic plaques in the cortical gray matter (1). These extracellular depositions are mainly composed of aggregated amyloid-β (Aβ) peptides and are surrounded by dystrophic neurites and glial cells. The Aβ peptide is derived through changes in the processing of the amyloid-β precursor protein (APP) and according to the 'amyloid cascade hypothesis' these changes are central to the disease process (2). Aβ is a 4 kDa peptide of 39-43 amino acids (3,4) and studies with synthetic Aβ show that it can act as a potent and direct neurotoxic agent (5-7).

There is however an increasing amount of evidence that Aβ can be indirect neurotoxic by activating surrounding glial cells. Neuritic plaques, but not diffuse plaques, are reported to be surrounded by microglia (8-11). Since microglia are able to produce cytokines as well as chemotactic and neurotoxic factors a lot of research has focused on the ability of Aβ to induce these events in microglia. Recent studies show that Aβ is able to

trigger the generation of reactive oxygen species (ROS) in rat microglia and THP-1 monocytes (12-14). Excessive release of ROS not only leads to oxidative stress but may also potentiate the inflammatory response, e.g. by triggering redox-sensitive expression of various inflammatory genes (5,15,16). Aβ has also been shown to induce interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and neurotrophic molecules like nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) in THP-1 monocytes (17-19).

Aβ, cytokines and ROS released by microglia cannot only be direct neurotoxic, they may also activate surrounding astrocytes. Astrocytes are associated with many, but not all senile plaques. They are most closely associated with plaques with a dense amyloid core and an attendant microglial reaction (20-22). *In vitro* studies show that Aβ can stimulate the production of IL-1β and NO in rat astrocytes (23,24). It has also been shown that activity of iNOS in microglial cells is inhibited by the presence of astroglial cells, probably involving TGF-β (25). In addition, astrocytes have been shown to inhibit phagocytic properties of microglial cells (26).

Although extensive research has shown that microglia as well as astrocytes are involved in the inflammatory process occurring around neuritic plaques it remains unclear how Aβ activates these microglia and astrocytes. Research of the microglial signal transduction pathways mediating the neurotoxic response of Aβ has revealed the mitogen-activated protein kinase (MAPK) superfamily members ERK1/2 and p38 MAPK as important mediators (27,28). Besides, an increasing amount of evidence shows that the transcription factor nuclear factor-κB (NF-κB) can be stimulated by Aβ in neurons (5,16,29) and by Aβ(25-35) in microglia (30,31). Upregulation of ERK1/2 and p38 MAPK can lead to the activation of numerous transcription factors in their turn leading to the activation of cytokines and neurotrophic factors. Activation of NF-κB can lead to the transcription of genes expressing TNF-α, IL-1, IL-6, monocyte chemoattractant protein-1 (MCP-1) and nitric oxide (NO) synthase (reviewed in (15)).

To further explore the microglia and astrocyte-mediated inflammatory pathways, in the present study the capability of Aβ to induce NF-κB, TNF-α and superoxide anions in microglia and astrocytes was investigated. In addition the production of these pro-inflammatory molecules was also investigated in cocultures of these cells in order to reveal the intercellular relationship regarding Aβ-induced central nervous system (CNS) inflammation.

Materials and Methods

Cells

Monocytes were derived from peripheral blood mononuclear cells by Ficoll-Paque (Pharmacia Biotech, Sweden) density gradients and purified by centrifugal elutriation as described previously (32). Cells were seeded at a concentration of 2 x 10^6 cells / ml in Teflon Erlenmeyer flasks (Nalgene, USA) and grown as suspension at 37 °C in a humidified atmosphere of 5% CO^2 / 95% air. Culture medium was composed of Iscove's

modified Dulbecco's medium (Gibco Life Technologies, The Netherlands) supplemented with 10% (v/v) heat-inactivated human AB serum, 2 mM L-glutamine, 19 mM sodium bicarbonate, 10 μg/ml gentamicin and 0.5 μg/ml ciprofloxacin. After 7 days, monocyte-derived macrophages (MDMs) were harvested from the flasks, washed with Hanks' balanced salt solution (HBSS) and used for the experiments.

The human astrocytoma cell line U-373 MG was grown in a humidified atmosphere of 5% CO^2 / 95% air in Dulbecco's modified eagle medium / Nutrient mixture Ham F-10 (Gibco Life Technologies), 1:1, supplemented with 10% (v/v) fetal calf serum, 10 IU / ml penicillin, 10 IU / ml streptomycin and 1.2 mM L-glutamine.

Primary human adult astrocytes were kindly provided by Dr. C.J.A. de Groot and were cultured as described earlier (33). In short, cells were seeded into poly-L-lysine (PLL; 15 μg/ml; Sigma Chemical Co., The Netherlands) coated 80 cm^2 flasks at a density of 2 x 10^4 cells/ml in Dulbecco's modified eagle medium / Nutrient mixture Ham F-10, 1:1, supplemented with 10% (v/v) fetal calf serum, 10 IU / ml penicillin, 10 IU / ml streptomycin. For each new passage confluent cultures of astrocytes were harvested using 0.25% trypsin in 0.02% EDTA.

Amyloid-β peptides

The synthetic Aβ peptides Aβ(1-42) and Aβ(1-16) (Bachem AG, Switzerland), prepared as stock solutions in sterile water at a concentration of 500 μM, were stored at –20 ºC. FITC-labeled Aβ(1-42) was prepared by incubating Aβ(1-42) at a concentration of 10 mg/ml with 0.1 mg/ml FITC in PBS (pH = 7.4) for 1 h at 22 ºC. Subsequently, using a 1 kDa Spectra/Pordialyzation membrane, Aβ(1-42)-FITC was dialyzed overnight against PBS to remove unbound FITC, diluted to a concentration of 220 mM and stored at –20 ºC.

Preparation of nuclear extracts

MDMs or astrocytoma cells (1 x 10^6 cells / sample) were incubated with 10 μM Aβ(1-42) for 1 h at room temperature. Hereafter nuclear extracts were prepared as described previously (34). Briefly, cells were pelleted, washed with TRIS-buffered saline and resuspended in an ice-cold hypotonic buffer. Cells were allowed to swell on ice for 15 min, after which a solution of Nonidet NP-40 was added. After centrifugation the pellet was vigorously shacked in an ice-cold hypertonic buffer. Nuclear extracts were centrifuged and the supernatant was aliquoted and stored at –70 °C until use.

P50 and p65 positive controls were obtained as described before (35). In short, COS-1 cells were grown in 10 cm dishes and transfected with 20 μg of expression plasmid by the calcium-phosphate precipitation method. Cells were harvested 48 h post-transfection after which whole cells extracts were made. Extracts were stored at –70 °C until use.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 μg protein per sample) were incubated with a double stranded ^{32}P-labeled probe containing the NF-κB binding motif from the HIV-long terminal repeat

(HIV-LTR, 5'-agcttcagaGGGGACTTTCCgagagg-3'). Incubation was carried out at room temperature for 30 min in 20 µl (total volume) of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5% glycerol, 1 mM DTT, 2 µg poly(dI-dC), 1 µg BSA, 100 mM NaCl and 1 ng probe. Hereafter, samples were loaded on a 5% polyacrylamide gel and run until free probe was at the end of the gel. Films were exposed to vacuum dried gels at –70 °C in cassettes containing intensifying screens.

TNF-α ELISA

Macrophages were grown in teflon flasks for six days after which they were seeded in a 48-well plate (4 x 10^5 / well). For coculture experiments astrocytes were grown in 24-well plates. When confluent an equal amount of macrophages was added. Cells then were incubated with 10 µM Aβ(1-42) for indicated times at 37 °C in a humidified atmosphere of 5% CO^2 / 95% air. TNF-α concentration in the supernatant was quantified with an enzyme-linked immunosorbent assay (ELISA). In short, TNF-α was captured by monoclonal anti-human TNF-α antibody and detected with a biotinylated anti-human TNF-α antibody according to the manufacturer's instructions (Pelikine compact human TNF-a ELISA kit, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands)

Superoxide anion production

Superoxide production was measured as lucigenin-enhanced chemiluminescence (36). Briefly, cells were transferred to polystyrene vials (2 x 10^5 / vial), placed into a luminometer (Packard Instruments, Belgium) and incubated for 30 min at 37 °C in HBSS containing 250 nM bis-N-methylacridinum (Lucigenin; Sigma Chemical Co., The Netherlands) to assess the spontaneous release of superoxide. Subsequently, Aβ peptides were added and chemoluminescence (expressed as millivolts, mV) was monitored for 30 min.

Flow cytometry

For Aβ(1-42)-FITC binding studies, 2 x 10^5 cells were incubated at 37 ºC with HBSS containing different concentrations Aβ(1-42)-FITC. In addition, 2 x 10^5 cells were first preincubated with 50 µM unlabeled Aβ(1-16) at 37 ºC for 30 min after which cells were washed with HBSS and incubated with 10 µM Aβ(1-42)-FITC. After 30 min, cells were washed with HBSS and fixed for 15 min with PBS containing 2% paraformaldehyde. Thereafter, flow cytometry analysis was performed by analyzing 10000 cells per experimental condition on a FACSscan flow cytometer (Becton Dickson and Co., USA) equipped with a computer-assisted data analysis system.

Statistical analysis

The non-parametric Wilcoxon signed-rank test for two related samples was used to compare TNF-α secretion in unstimulated and Aβ(1-42)-stimulated macrophages (Fig 1). The Wilcoxon test was also used to compare TNF-α secretion in Aβ(1-42)-stimulated macrophages and Aβ(1-42)-stimulated macrophages cocultured with astrocytoma cells (Fig 2A) and primary human adult astrocytes (Fig 2B). Reported p-values are two-sided. All statistical analyses were performed using SPSS for Windows (8.0.0).

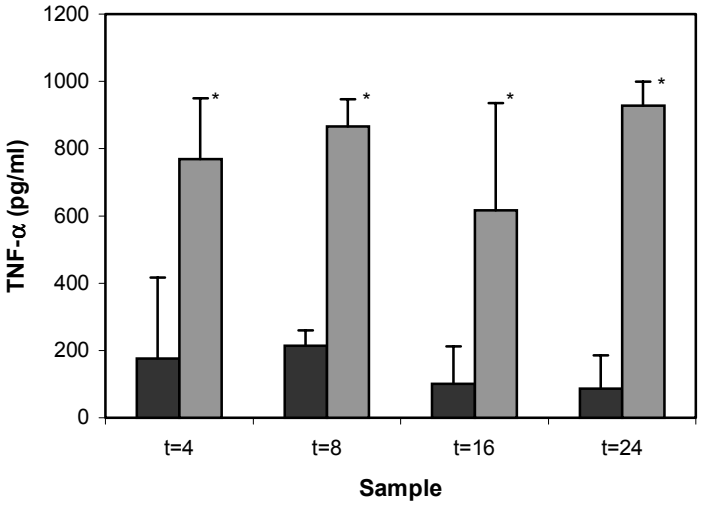

Figure 1.

TNF-α secretion by Aβ(1-42)-stimulated macrophages. Unstimulated and Aβ(1-42)-stimulated macrophages were incubated for 4, 8, 16 and 24 hours after which supernatant samples were taken and TNF-α levels were quantified. Each bar represents mean ± SEM from seven independent experiments each using cells from different donors. Asterisks indicate statistically significant differences in TNF-α secretion from stimulated macrophages compared to unstimulated macrophages.

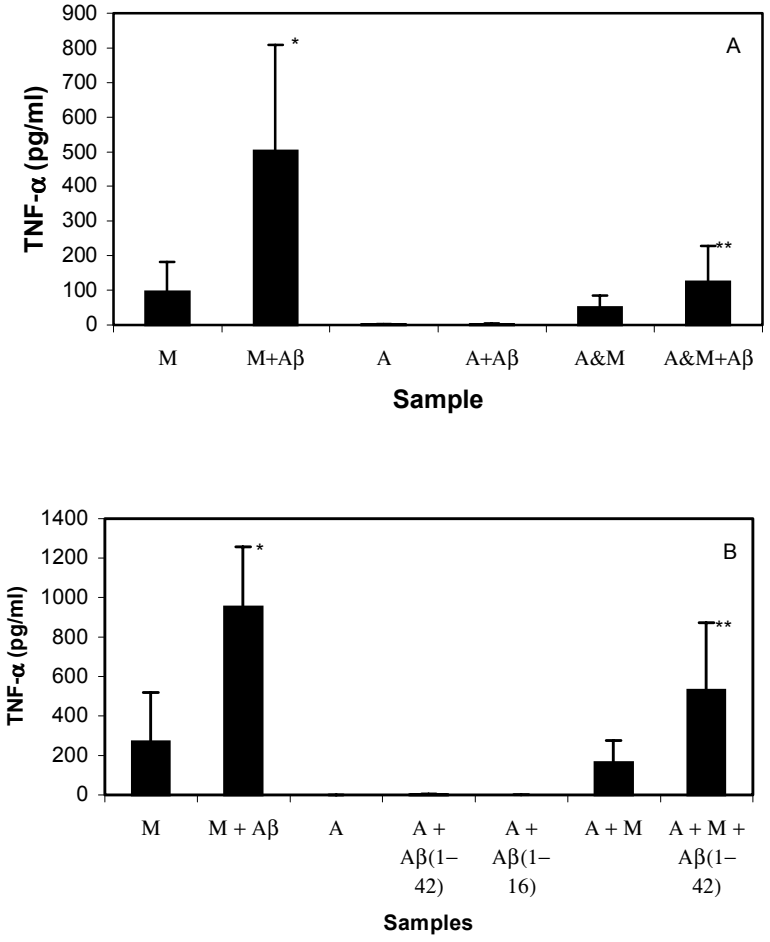

Figure 2.

TNF-α secretion by Aβ(1-42)-stimulated macrophages cocultured with (A) human U-373 astrocytoma cells and (B) primary human adult astrocytes. Unstimulated and 10 μM Aβ(1-42)-stimulated macrophages were cocultured for 6 hours with astrocytes after which supernatant-samples were taken and TNF-α levels were quantified. Each bar represents mean ± SEM from eight independent experiments each using macrophages from different donors. Asterisks indicate statistically significant difference in TNF-α secretion from stimulated macrophages compared to unstimulated macrophages. Double asterisks indicate statistically significant difference in TNF-α secretion from Aβ(1-42)-stimulated macrophages cocultured with (A) human U-373 astrocytoma cells and (B) primary human adult astrocytes compared to Aβ(1-42)-stimulated macrophages.

Results

TNF-α secretion by Aβ(1-42)-stimulated primary human macrophages is attenuated by astrocytes

To investigate whether Aβ(1-42) is capable of inducing primary human macrophages to produce pro-inflammatory cytokines and thus being indirectly neurotoxic MDMs were stimulated with 10 μM Aβ(1-42) for 4, 8, 16 and 24 hours and TNF-α concentrations were measured in the supernatant. As indicated in Figure 1 there is a significant ($p=0.018$) increase in TNF-α secretion at 4, 8, 16 and 24 hours as compared to unstimulated control cells. Maximum TNF-α secretion was measured after 24 hours of incubation. Our results are supported by reports published earlier by Fiala et al. (37) and Klegeris et al. (19) where TNF-α secretion was measured in human monocytes and human monocytic THP-1 cells respectively. Figure 2A shows TNF-α levels of human macrophages and human astrocytoma cells incubated with 10 μM Aβ(1-42) for 6 hours, Figure 2B shows TNF-α levels of human macrophages and primary human adult astrocytes incubated with 10 μM Aβ(1-42) for 6 hours. Whereas macrophages can produce significant levels of TNF-α, astrocytoma cells and primary human adult astrocytes do not when stimulated with Aβ(1-42). When stimulated macrophages are cocultured with either unstimulated astrocytoma cells or unstimulated primary human adult astrocytes there is a significant decrease in TNF-α secretion by these macrophages compared to Aβ(1-42)-stimulated macrophages ($p=0.028$ and $p=0.032$ respectively).

Theoretically, several mechanisms may account for the observed decrease in TNF-α levels in the macrophage-astrocyte cocultures. Among them are the ability of astrocytes to bind Aβ(1-42) and in such a way interfere with Aβ(1-42) activation of macrophages, the ability to bind TNF-α and in such way decrease the levels of TNF-α in the supernatant, the ability to produce macrophage deactivating molecules such as IL-10 and TGF-β or other molecules such as soluble TNF-α receptor. To study the binding of Aβ(1-42) to human astrocytes, astrocytoma cells were incubated with different concentrations of FITC-labeled Aβ(1-42). Figure 3 shows that increasing concentrations of FITC-labeled Aβ(1-42) results in an increase in fluorescence intensity of the cells. Moreover, preincubating astrocytoma cells with 50 μM unlabeled Aβ(1-16) for 30 min before incubation with 10 μM Aβ(1-42)-FITC shows a decrease in fluorescence intensity of the cells (Fig 3E). These data suggest that astrocyte binding of Aβ(1-42) interferes with Aβ(1-42) activation of macrophages.

Superoxide anion production of Aβ(1-42)-stimulated primary human macrophages is attenuated by astrocytes

In order to study the effect of Aβ(1-42) on superoxide anion production by human macrophages, astrocytoma cells, primary human adult astrocytes and combined macrophage-astrocytoma and macrophage-primary human adult astrocyte cultures were placed into a luminometer and stimulated with Aβ(1-42). Figure 4A shows that macrophages do produce superoxide anions, Figure 4B shows that astrocytoma cells do not

when stimulated with 10 μM Aβ(1-42). Figure 4E shows that primary human adult astrocytes also do not produce superoxide anions when stimulated with 10 μM Aβ(1-42). There was however a decrease in the respiratory burst of macrophages when cocultured with astrocytoma cells (Fig 4C) or human adult astrocytes (Fig 4F). To investigate this effect astrocytoma cells and human adult astrocytes were pre-incubated with 50 μM Aβ(1-16) for 15 min, prior to the Aβ(1-42) incubation, thereby blocking the binding of Aβ(1-42) to these astrocytes (38). The results, shown in Figure 4C and 4F, show that the attenuation of the respiratory burst by astrocytoma cells and human adult astrocytes was neutralized concluding that binding of Aβ(1-42) to the astrocytes might interfere with Aβ(1-42) activation of macrophages which in turn might result in a decreased superoxide anion production. Primary human adult astrocytes incubated with 50 μM Aβ(1-16) alone did not show any superoxide anion production (data not shown) or TNF-α production (Fig 2B).

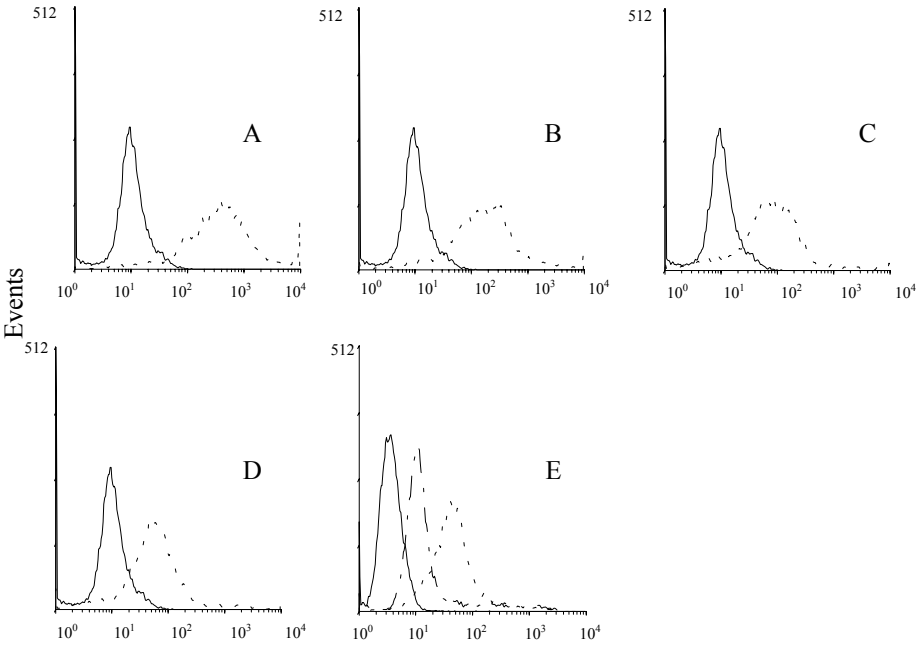

Figure 3.

Binding of FITC-labeled Aβ(1-42) to astrocytoma cells. Fluorescence histogram showing events vs. the log of the fluorescence-intensity of Aβ(1-42)-FITC binding to astrocytoma cells. Superimposed fluorescence histograms of unlabeled (autofluorescence; solid line) and 10 (A), 5 (B), 2.5 (C) and 1.25 (D) μM Aβ(1-42)-FITC-labeled human astrocytoma cells (dotted line). In addition, astrocytoma cells were preincubated with unlabeled Aβ(1-16) before incubation with FITC-labeled Aβ(1-42) (E). Superimposed fluorescence histogram of unlabeled (autofluorescence; solid line), 10 μM Aβ(1-42)-FITC (dotted line) and 10 μM Aβ(1-42)-FITC preincubated with unlabeled 50 μM Aβ(1-16) (broken line). Results are representative of three independent experiments.

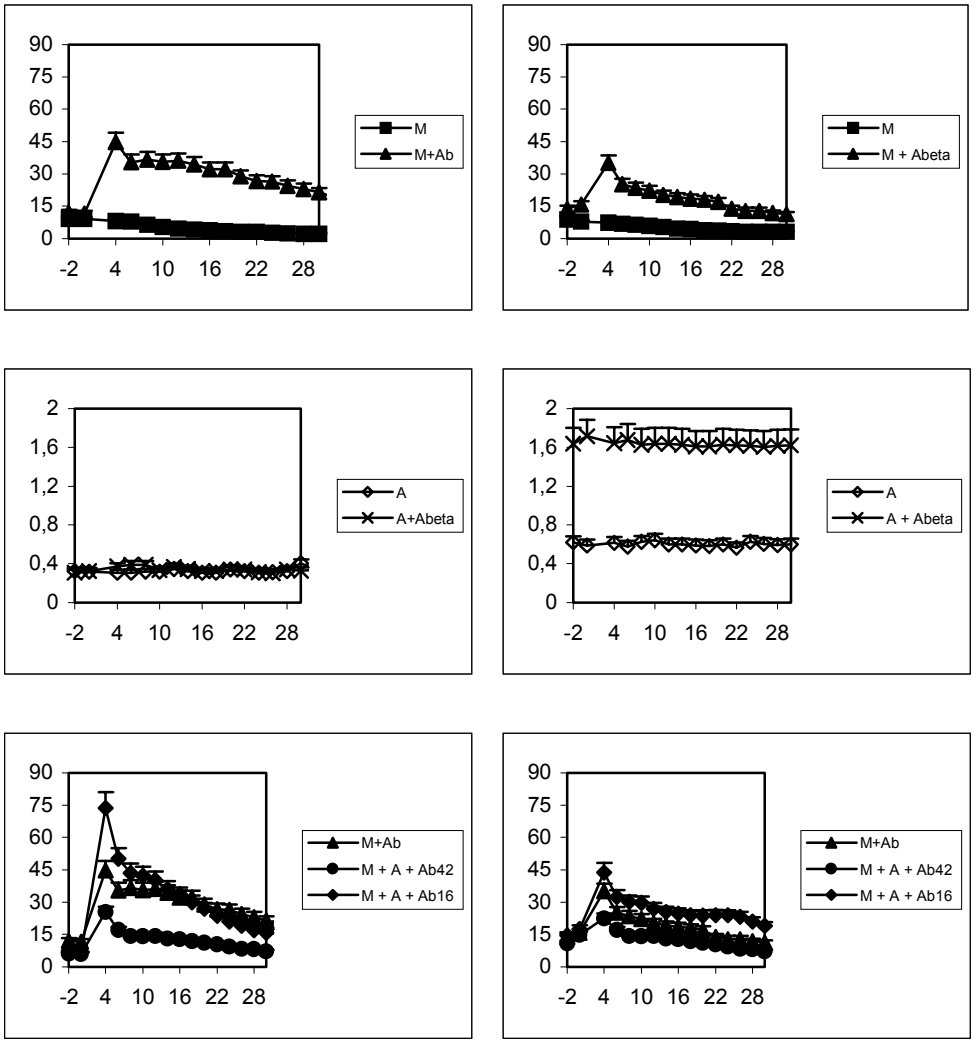

Figure 4.

The effect of coincubation of human astrocytoma cells (A-C) with human macrophages on the Aβ(1-42)-stimulated superoxide anion production and primary human adult astrocytes (D-F) with human macrophages on the Aβ(1-42)-stimulated superoxide anion production. (A) Macrophages are exposed to buffer or 10 μM Aβ(1-42), (B) astrocytoma cells are exposed to buffer or 10 μM Aβ(1-42). (C) To investigate the effect of astrocytes on the respiratory burst of macrophages, astrocytes were preincubated for 15 min with buffer or 50 μM Aβ(1-16) after which they were coincubated with macrophages and exposed to buffer or Aβ(1-42). Also macrophages alone were exposed to 10 μM Aβ(1-42). Experiment was repeated using primary human adult astrocytes. (D) Macrophages are exposed to buffer or 10 μM Aβ(1-42), (E) primary human adult astrocytes are exposed to buffer or 10 μM Aβ(1-42). (F) To investigate the effect of astrocytes on the respiratory burst of macrophages, human astrocytes were preincubated for 15 min with buffer or 50 μM Aβ(1-16) after which they were coincubated with macrophages and exposed Aβ(1-42). Also macrophages alone were exposed to 10 μM Aβ(1-42). Results are representative of five independent experiments each using macrophages from different donors.

Aβ(1-42)-induced NF-κB activity in human astrocytoma cells, human macrophages and cocultures their of

To investigate the role of astrocytes in the inflammatory process occurring around neuritic plaques the activation state of NF-κB in Aβ(1-42)-stimulated human U-373 MG astrocytoma cells was characterized. Therefore cells were stimulated with 10 μM Aβ(1-42) for 3, 6, 12 and 24 hours (3, 12 and 24 hour data not shown). Maximal NF-κB stimulation was seen after 6 hours (Fig 5). Aβ(1-42) stimulation results in the appearance of band A (Fig 5: lane 4) when compared to unstimulated control cells (Fig 5: lane 3). To identify band A we performed a supershift by incubating nuclear extracts and ^{32}P-labeled oligonucleotide probes together with an antibody against either the p50 or the p65 subunits of NF-κB (Fig 5: lanes 5 and 6 respectively). The appearance of bands S1 and S2 indicate that stimulation of astrocytes with Aβ(1-42) results in activation of the NF-κB heterodimer p50/p65.

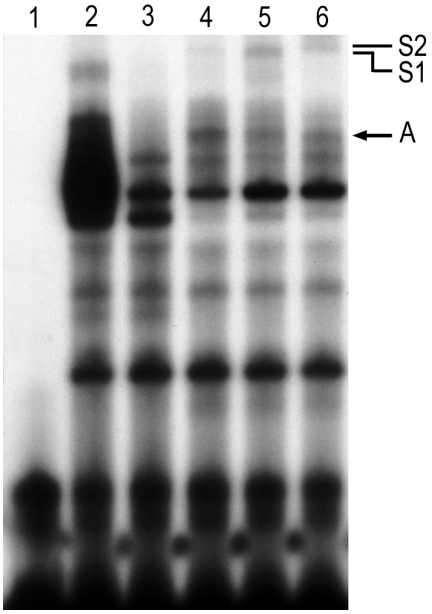

Figure 5.

NF-κB stimulation in Aβ(1-42)-stimulated human U-373 astrocytoma cells. Nuclear extracts from 10 μM Aβ(1-42)-stimulated astrocytes were incubated with ^{32}P-labeled NF-κB oligonucleotide probe for electrophoretic mobility shift assays. Lanes: 1, probe; 2, LPS stimulated astrocytoma cells; 3, unstimulated astrocytoma cells; 4, astrocytoma cells stimulated with 10 μM Aβ(1-42); 5-6, nuclear extracts of Aβ(1-42)-stimulated astrocytoma cells incubated with a monoclonal antibody against NF-κB p50 subunit (5) and p65 subunit (6); NF-κB p50/p65 is indicated by band A, S1 and S2 indicate supershifts of p50 and p65 respectively. Results are representative of three independent experiments.

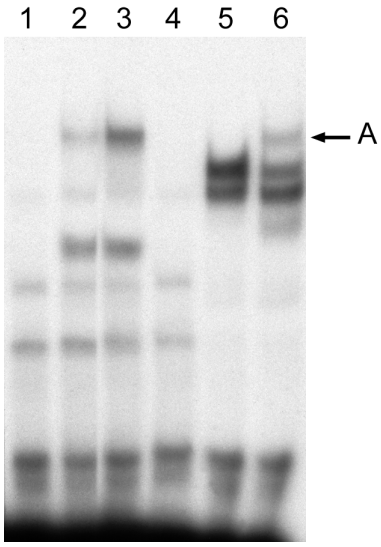

Figure 6.

NF-κB stimulation in Aβ(1-42)-stimulated human U-373 astrocytoma cells. Nuclear extracts from 10 μM Aβ(1-42)-stimulated astrocytes were incubated with ^{32}P-labeled NF-κB oligonucleotide probe for electrophoretic mobility shift assays. Lanes: 1, probe; 2, unstimulated astrocytoma cells; 3, astrocytoma cells stimulated with 10 μM Aβ(1-42); 4, nuclear extracts of Aβ(1-42)-stimulated astrocytoma cells incubated with a 50 fold excess of cold NF-κB probe; 5, p50/p50 positive control; 6, p50/p65 positive control. NF-κB p50/p65 is indicated by band A. Results are representative of two independent experiments.

To confirm these results the same experiment was performed with a new batch of astrocytoma cells. Cells were stimulated with 10 μM Aβ(1-42) for 3, 6, 12 and 24 hours. Maximal NF-κB stimulation was seen after 6 hours (Fig 6). Aβ(1-42) stimulation resulted in the appearance of band A (Fig 6: lane 3) compared to unstimulated astrocytoma cells (Fig 6: lane 2). Nuclear extracts of Aβ(1-42)-stimulated astrocytoma cells incubated with a 50 fold excess of cold NF-κB probe showed a disappearance of band A (Fig 6: lane 4).

As shown in Figure 2B cocultures of primary human adult astrocytes and macrophages produced decreased TNF-α levels as compared to macrophages alone. Although the mechanism behind the decreased levels of TNF-α is related to Aβ(1-42)-astrocyte binding, the astrocytes may also bind at least part of the TNF-α produced by macrophages. To investigate whether TNF-α can bind to astrocytes and stimulate NF-κB activation, human U-373 MG astrocytes were incubated for 6 hours with either 10 μM Aβ(1-42) (Fig 7: lane 2) or two different concentrations of TNF-α or 10 μM Aβ(1-42) together with two different concentrations of TNF-α. Band A in Figure 7 shows that 1000 IU/ml TNF-α (lane 3) as well as 2000 IU/ml TNF-α (lane 4) compared to unstimulated astrocytes (lane 1) clearly stimulate p50/p65 NF-κB heterodimer. Costimulation of Aβ(1-42) together with TNF-α significantly increases NF-κB in the astrocytes (lane 5 and 6).

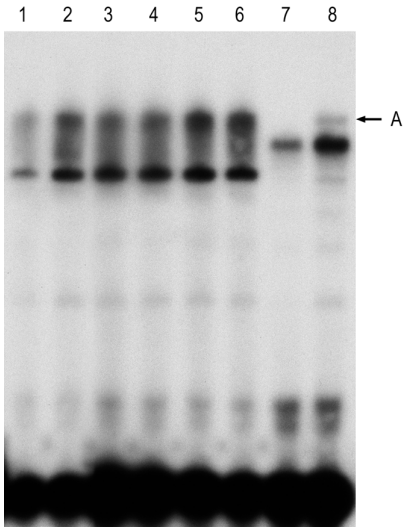

Figure 7.

NF-κB stimulation in U-373 astrocytoma cells costimulated with Aβ(1-42) and TNF-α. EMSA was performed with nuclear extracts of astrocytes stimulated with either 10 μM Aβ(1-42), 1000 / 2000 IU/ml TNF-α or a combination their of. Lanes: 1, unstimulated astrocytes; 2, Aβ(1-42)-stimulated astrocytes; 3-4, astrocytes stimulated with 1000 and 2000 IU/ml TNF-α; 5, astrocytes stimulated with Aβ(1-42) and 1000 IU/ml TNF-α; 6, astrocytes stimulated with Aβ(1-42) and 2000 IU/ml TNF-α; 7, p50/p50 positive control; 8, p50/p65 positive control. Band A represents p50/p65 NF-κB dimer. Results are representative of two independent experiments.

We showed that Aβ(1-42) could induce superoxide anion production as well as TNF-α secretion in human macrophages. To investigate whether NF-κB can be activated by Aβ(1-42) in human macrophages we incubated MDMs with 10 μM Aβ(1-42) for 3,6,12 and 24 hours. Figure 8 shows the 3 hour incubation of macrophages with Aβ(1-42). Aβ(1-42) stimulation (lane 2) does not increase NF-κB activity as compared to unstimulated control cells (lane 1). 6, 12 and 24 hour incubation periods show the same results (data not shown).

As shown in Figure 2B and 4B, cocultures of human astrocytes and Aβ(1-42)-stimulated macrophages produced decreased TNF-α levels as well as superoxide anion levels compared to macrophages alone. Therefore, NF-κB activity in cocultures of Aβ(1-42)-stimulated macrophages and astrocytoma cells was determined (data not shown). Results show no difference of NF-κB activity in cocultures compared to macrophages alone (data not shown).

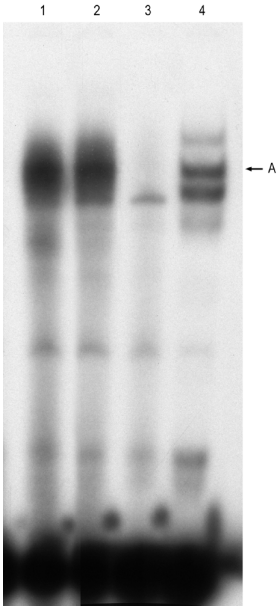

Figure 8.

NF-κB activity in Aβ(1-42)-stimulated macrophages. Lanes: 1, unstimulated macrophages; 2, 10 μM Aβ(1-42)-stimulated macrophages; 3, nuclear extracts of Aβ(1-42)-stimulated macrophages incubated with a 50 fold excess of cold NF-κB probe; 4, p50/p65 positive control. Band A represents NF-κB. Results are representative of three independent experiments.

Discussion

In recent years, it has become increasingly evident that both Aβ and microglial cells play a fundamental role in the pathogenesis of AD. Aβ is not only believed to be direct neurotoxic, by activating microglial cells Aβ might be indirect neurotoxic. Although various receptors are reported to bind Aβ as a ligand (39-41), a specific receptor for Aβ on macrophages has not been found yet. However, it is suggested that the 1-16 domain of the peptide is believed to be responsible for binding to the macrophage, thereby activating numerous intracellular signal transduction pathways leading to transcription and production of pro-inflammatory products (38,42,43). Induction of TNF-α has been shown in THP-1 monocytic cells and in rat and murine microglial cells (19,44-46). The induction of monocytic activation has been achieved by many investigators using micromolar concentration of Aβ (28,37,45,47-50). Indeed, stimulation of THP-1 cells with nanomolar concentration did not result in monocytic activation as measured by the release of pro-inflammatory products (51). This report shows that using primary human monocyte-derived macrophages as a model cell for microglia, activation of these macrophages by Aβ(1-42) not only resulted in the production of superoxide anions but also preceded the release of

TNF-α. Although TNF-α is reported to be neuroprotective under certain conditions (52), the ability of Aβ to induce the release of TNF-α and superoxide anions in macrophages and the capability of TNF-α and reactive oxygen species to amplify the inflammatory reaction by numerous mechanisms in surrounding microglia and astrocytes underscores the importance of understanding the underlying molecular mechanisms in an attempt to slow or prevent injury to the brain.

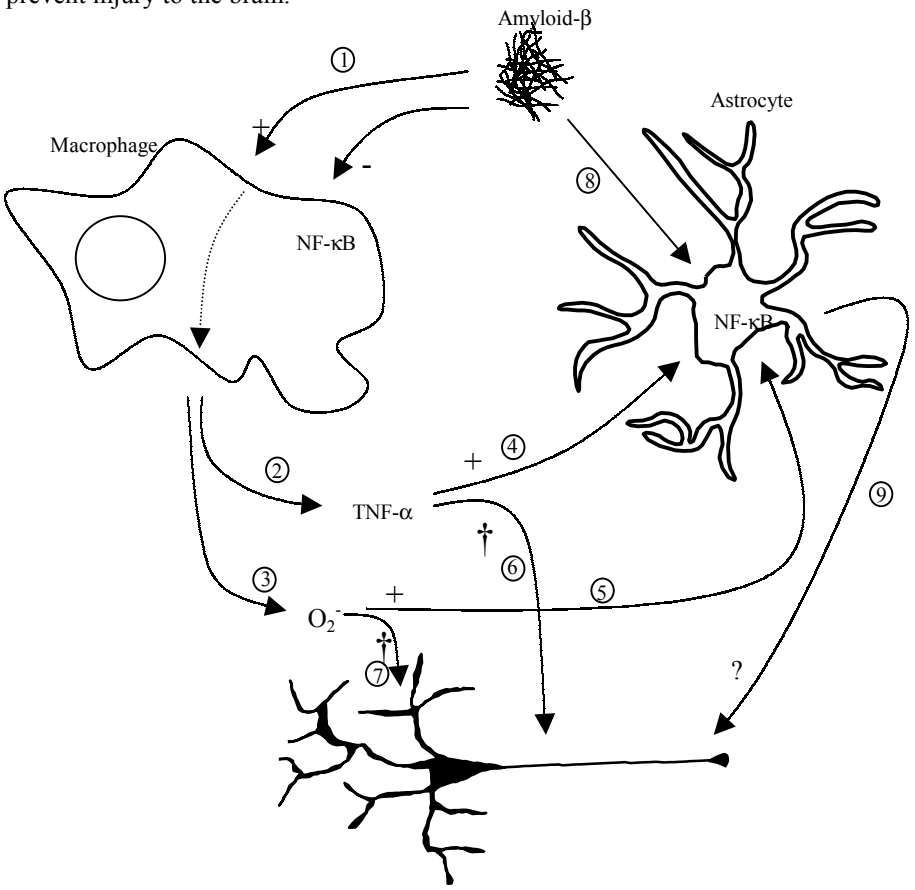

Figure 9.

Proposed mechanism of intercellular pathways eventually leading to neuronal degeneration. Aβ can activate macrophages (1) to produce either TNF-α (2) or superoxide anions (3). TNF-α (4) and superoxide anions (5) can activate astrocytes or can be direct neurotoxic (6 + 7). Aβ may also activate proinflammatory transcription factors (NF-κB) in astrocytes (8). Activation of astrocytes by either TNF-α, superoxide anions or Aβ may lead to the production of proinflammatory neurotoxic products (9). +: able to induce, -: not able to induce, †: neurotoxic.

There have been several studies investigating the intracellular pathways stimulated as a result of Aβ activation of the macrophage. The NF-κB family of proteins is a well-characterized transcription factor, which has gained considerable attention because of its

unique mechanism of activation, its role in cytoplasmic/nuclear signaling and its exquisite responsiveness to pathogenic stimulation of cells. Several studies reported a relationship between Aβ and NF-κB activity in neurons (5,16,29,53) and astrocytes (24). In microglia Bonaiuto et al. showed NF-κB activation upon Aβ-stimulation (29,30). In this study however, a N9 murine microglial cell line was used. This report shows that Aβ was not able to induce NF-κB in primary human macrophages, in contrast to LPS (data not shown). However, incubating human astrocytes with Aβ(1-42) not only shows binding of the peptide to the cells but also shows activation of NF-κB, more specific the heterodimer p50/p65. Because NF-κB is known to be able to stimulate the transcription of TNF-α it can be concluded that because macrophages but not astrocytes showed TNF-α secretion after stimulation with Aβ(1-42) the intracellular pathway following this Aβ(1-42) stimulation in macrophages is different than in astrocytes. Other studies however showed an increased TNF-α secretion upon induction with Aβ (54,55). In these studies rat astrocytes were used, in contrast to the primary human adult astrocytes used in this study. This contradiction indicates that there are species differences regarding Aβ-stimulated TNF-α production. Although the expression of TNF-α is under control of NF-κB additional cellular transcription factors are necessary for the induction of TNF-α expression (56). Indeed, in addition to the results presented in this paper, NF-κB activity is reported to be constitutively present in monocytes (57) and MDMs (58,59). In astrocytes other, inhibitory factors, seem responsible for not producing TNF-α as a consequence of Aβ-stimulated NF-κB activity. Astrocytes do however produce TNF-α when stimulated with LPS and this production is strongly amplified in the presence of Aβ(25-35) while Aβ(25-35) alone does not show this effect (60).

Besides Aβ(1-42)-mediated NF-κB activation, this report also showed that TNF-α could induce NF-κB. Moreover TNF-α in combination with Aβ(1-42) showed an even higher NF-κB activity. Because astrocytes do not produce proinflammatory TNF-α and superoxide anions when stimulated with Aβ, other mechanisms might follow the activation of NF-κB. Akama et al. showed that in astrocytes Aβ stimulation of nitric oxide production occurs through an NF-κB dependent mechanism (24). Another study showed that IL-1β and TNF-α-stimulated alpha 1-antichymotrypsin production in astrocytes occurs via NF-κB (61). Astrocytes are also reported to produce IL-6 and IL-8 after stimulation with Aβ (62). In addition, our results show a neuroprotective role for astrocytes regarding the amount of superoxide anion and TNF-α release by macrophages induced in the presence of Aβ (Fig 9). Taken together, these results not only demonstrate that upon binding and activation of Aβ to astrocytes and macrophages different intracellular mechanisms are responsible for the inflammatory response but also indicates that the interaction between astrocytes and macrophages plays an important role in the growing inflammatory response eventually leading to neurodegeneration.

Acknowledgments

The authors would like to thank Dr. C.J.A. de Groot for supplying the human adult astrocytes and J.W.T. Cohen Stuart for help with statistical analysis. This work was supported by the Netherlands Organization for Scientific Research grant 903-51-141 (to H.S.L.M.N.). H.S.L.M.N. is a Fellow of the Royal Netherlands Academy of Sciences and Art.

References

1. Dickson, D. W. 1997. Neuropathological diagnosis of Alzheimer's disease: a perspective from longitudinal clinicopathological studies. *Neurobiol.Aging 18*:S21.
2. Selkoe, D. J. 1996. Amyloid beta-protein and the genetics of Alzheimer's disease. *J.Biol.Chem. 271*:18295.
3. Haass, C. and D. J. Selkoe. 1993. Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. *Cell 75*:1039.
4. Selkoe, D. J. 1993. Physiological production of the beta-amyloid protein and the mechanism of Alzheimer's disease. *Trends.Neurosci. 16*:403.
5. Behl, C., J. B. Davis , R. Lesley, and D. Schubert. 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell 77*:817.
6. Pike, C. J., N. Ramezan Arab, and C. W. Cotman. 1997. Beta-amyloid neurotoxicity in vitro: evidence of oxidative stress but not protection by antioxidants. *J.Neurochem. 69*:1601.
7. Yankner, B. A., L. K. Duffy, and D. A. Kirschner. 1990. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science 250*:279.
8. Wisniewski, H. M., J. Wegiel, K. C. Wang, M. Kujawa, and B. Lach. 1989. Ultrastructural studies of the cells forming amyloid fibers in classical plaques. *Can.J.Neurol.Sci. 16*:535.
9. McGeer, P. L., S. Itagaki, H. Tago, and E. G. McGeer. 1987. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. *Neurosci.Lett. 79*:195.
10. Rozemuller, J. M., P. Eikelenboom, S. T. Pals, and F. C. Stam. 1989. Microglial cells around amyloid plaques in Alzheimer's disease express leucocyte adhesion molecules of the LFA-1 family. *Neurosci.Lett. 101*:288.
11. Sasaki, A., H. Yamaguchi, A. Ogawa, S. Sugihara, and Y. Nakazato. 1997. Microglial activation in early stages of amyloid beta protein deposition. *Acta Neuropathol.Berl. 94*:316.
12. Meda, L., C. Bonaiuto, P. Baron, L. Otvos, Jr., F. Rossi, and M. A. Cassatella. 1996. Priming of monocyte respiratory burst by beta-amyloid fragment (25-35). *Neurosci.Lett. 219*:91.
13. Klegeris, A. and P. L. McGeer. 1997. beta-amyloid protein enhances macrophage production of oxygen free radicals and glutamate. *J.Neurosci.Res. 49*:229.

14. Tjernberg, L. O., D. J. Callaway, A. Tjernberg, S. Hahne, C. Lilliehook, L. Terenius, J. Thyberg, and C. Nordstedt. 1999. A molecular model of Alzheimer amyloid beta-peptide fibril formation. *J.Biol.Chem. 274*:12619.
15. Baeuerle, P. A. and T. Henkel. 1994. Function and activation of NF-kappa B in the immune system. *Annu.Rev.Immunol. 12*:141.
16. Kaltschmidt, B., M. Uherek, B. Volk, P. A. Baeuerle , and C. Kaltschmidt. 1997. Transcription factor NF-kappaB is activated in primary neurons by amyloid beta peptides and in neurons surrounding early plaques from patients with Alzheimer disease. *Proc.Natl.Acad.Sci.U.S.A. 94*:2642.
17. Giulian, D., L. J. Haverkamp, J. Li, W. L. Karshin , J. Yu, D. Tom, X. Li, and J. B. Kirkpatrick. 1995. Senile plaques stimulate microglia to release a neurotoxin found in Alzheimer brain. *Neurochem.Int. 27*:119.
18. Lorton, D., J. M. Kocsis, L. King, K. Madden, and K. R. Brunden. 1996. beta-Amyloid induces increased release of interleukin-1 beta from lipopolysaccharide-activated human monocytes. *J.Neuroimmunol. 67*:21.
19. Klegeris, A., D. G. Walker, and P. L. McGeer. 1997. Interaction of Alzheimer beta-amyloid peptide with the human monocytic cell line THP-1 results in a protein kinase C-dependent secretion of tumor necrosis factor-alpha. *Brain Res. 747*:114.
20. Mandybur, T. I. and C. C. Chuirazzi. 1990. Astrocytes and the plaques of Alzheimer's disease. *Neurology 40*:635.
21. Pike, C. J., B. J. Cummings, and C. W. Cotman. 1995. Early association of reactive astrocytes with senile plaques in Alzheimer's disease. *Exp.Neurol. 132*:172.
22. Dickson, D. W. 1997. The pathogenesis of senile plaques. *J.Neuropathol.Exp.Neurol. 56*:321.
23. Hu, J., K. T. Akama, G. A. Krafft, B. A. Chromy, and L. J. Van Eldik. 1998. Amyloid-beta peptide activates cultured astrocytes: morphological alterations, cytokine induction and nitric oxide release. *Brain Res. 785*:195.
24. Akama, K. T., C. Albanese, R. G. Pestell, and L. J. Van Eldik. 1998. Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. *Proc.Natl.Acad.Sci.U.S.A. 95*:5795.
25. Vincent, V. A., F. J. Tilders, and A. M. Van Dam. 1997. Inhibition of endotoxin-induced nitric oxide synthase production in microglial cells by the presence of astroglial cells: a role for transforming growth factor beta. *Glia 19*:190.
26. DeWitt, D. A., G. Perry, M. Cohen, C. Doller, and J. Silver. 1998. Astrocytes regulate microglial phagocytosis of senile plaque cores of Alzheimer's disease. *Exp.Neurol. 149*:329.
27. McDonald, D. R., M. E. Bamberger, C. K. Combs, and G. E. Landreth. 1998. beta-Amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. *J.Neurosci. 18*:4451.
28. Combs, C. K., D. E. Johnson, S. B. Cannady, T. M. Lehman, and G. E. Landreth. 1999. Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. *J.Neurosci. 19*:928.
29. Bales, K. R., Y. Du, R. C. Dodel, G. M. Yan, E. Hamilton Byrd, and S. M. Paul. 1998. The NF-kappaB/Rel family of proteins mediates Abeta-induced neurotoxicity and glial activation. *Brain Res.Mol.Brain Res. 57*:63.

30. Bonaiuto, C., P. P. McDonald, F. Rossi, and M. A. Cassatella. 1997. Activation of nuclear factor-kappa B by beta-amyloid peptides and interferon-gamma in murine microglia. *J.Neuroimmunol. 77*:51.
31. Heese, K., C. Hock, and U. Otten. 1998. Inflammatory signals induce neurotrophin expression in human microglial cells. *J.Neurochem. 70*:699.
32. Boven, L. A., L. Gomes, C. Hery, F. Gray, J. Verhoef, P. Portegies, M. Tardieu, and H. S. Nottet. 1999. Increased peroxynitrite activity in AIDS Dementia Complex: implications for neuropathogenesis of HIV-1 infection. *J.Immunol. 162*:4319.
33. De Groot, C. J., C. H. Langeveld, C. A. Jongenelen, L. Montagne, d. van, V, and C. D. Dijkstra. 1997. Establishment of human adult astrocyte cultures derived from postmortem multiple sclerosis and control brain and spinal cord regions: immunophenotypical and functional characterization. *J.Neurosci.Res. 49*:342.
34. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucl.Acids Res. 17*:6419.
35. Wissink, S., S. A. van de, E. Caldenhoven, L. Koenderman, and P. T. van der Saag. 1997. NF-kappa B/Rel family members regulating the ICAM-1 promoter in monocytic THP-1 cells. *Immunobiology 198*:50.
36. Nottet, H. S., L. de Graaf, N. M. de Vos, L. J. Bakker, J. A. van Strijp, M. R. Visser, and J. Verhoef. 1993. Phagocytic function of monocyte-derived macrophages is not affected by human immunodeficiency virus type 1 infection. *J.Infect.Dis. 168*:84.
37. Fiala, M., L. Zhang, X. Gan, B. Sherry, D. Taub, M. C. Graves, S. Hama, D. Way, M. Weinand, M. Witte, D. Lorton, Y. M. Kuo, and A. E. Roher. 1998. Amyloid-beta induces chemokine secretion and monocyte migration across a human blood--brain barrier model. *Mol.Med. 4*:480.
38. Van Muiswinkel, F. L., S. F. Raupp, N. M. de Vos, H. A. Smits, J. Verhoef, P. Eikelenboom, and H. S. Nottet. 1999. The amino-terminus of the amyloid-beta protein is critical for the cellular binding and consequent activation of the respiratory burst of human macrophages. *J.Neuroimmunol. 96*:121.
39. El Khoury, J., S. E. Hickman, C. A. Thomas, L. Cao , S. C. Silverstein, and J. D. Loike. 1996. Scavenger receptor-mediated adhesion of microglia to beta- amyloid fibrils [see comments]. *Nature 382*:716.
40. Yan, S. D., X. Chen, J. Fu, M. Chen, H. Zhu, A. Roher, T. Slattery, L. Zhao, M. Nagashima, J. Morser, A. Migheli, P. Nawroth, D. Stern, and A. M. Schmidt. 1996. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature 382*:685.
41. Lorton, D., J. Schaller, A. Lala, and E. De Nardin. 2000. Chemotactic-like receptors and Abeta peptide induced responses in Alzheimer's Disease. *Neurobiol.Aging 21*:463.
42. Giulian, D., L. J. Haverkamp, J. H. Yu, W. Karshin , D. Tom, J. Li, J. Kirkpatrick, L. M. Kuo, and A. E. Roher. 1996. Specific domains of beta-amyloid from Alzheimer plaque elicit neuron killing in human microglia. *J.Neurosci. 16*:6021.
43. Giulian, D., L. J. Haverkamp, J. Yu, W. Karshin, D. Tom, J. Li, A. Kazanskaia, J. Kirkpatrick, and A. E. Roher. 1998. The HHQK domain of beta-amyloid provides a structural basis for the immunopathology of Alzheimer's disease. *J.Biol.Chem. 273*:29719.
44. Meda, L., M. A. Cassatella, G. I. Szendrei, L. Otvos, Jr., P. Baron, M. Villalba, D. Ferrari, and F. Rossi. 1995. Activation of microglial cells by beta-amyloid protein and interferon- gamma. *Nature 374*:647.
45. Ariga, T. and R. K. Yu. 1999. GM1 inhibits amyloid beta-protein-induced cytokine release. *Neurochem.Res. 24*:219.

46. Galimberti, D., P. Baron, L. Meda, E. Prat, E. Scarpini, R. Delgado, A. Catania, J. M. Lipton, and G. Scarlato. 1999. alpha-MSH peptides inhibit production of nitric oxide and tumor necrosis factor-alpha by microglial cells activated with beta-amyloid and interferon gamma. *Biochem.Biophys.Res.Commun. 263*:251.
47. Bianca, V. D., S. Dusi, E. Bianchini, I. Dal Pra, and F. Rossi. 1999. beta-amyloid activates the O-2 forming NADPH oxidase in microglia, monocytes, and neutrophils. A possible inflammatory mechanism of neuronal damage in alzheimer's disease. *J.Biol.Chem. 274*:15493.
48. Combs, C. K., D. E. Johnson, J. C. Karlo, S. B. Cannady, and G. E. Landreth. 2000. Inflammatory mechanisms in Alzheimer's disease: inhibition of beta- amyloid-stimulated proinflammatory responses and neurotoxicity by PPARgamma agonists. *J.Neurosci. 20*:558.
49. McDonald, D. R., K. R. Brunden, and G. E. Landreth. 1997. Amyloid fibrils activate tyrosine kinase-dependent signaling and superoxide production in microglia. *J.Neurosci. 17*:2284.
50. Yates, S. L., L. H. Burgess, J. Kocsis-Angle, J. M. Antal, M. D. Dority, P. B. Embury, A. M. Piotrkowski, and K. R. Brunden. 2000. Amyloid beta and amylin fibrils induce increases in proinflammatory cytokine and chemokine production by THP-1 cells and murine microglia. *J.Neurochem. 74*:1017.
51. Morelli, L., G. H. Giambartolomei, M. I. Prat, and E. M. Castano. 1999. Internalization and resistance to degradation of Alzheimer's A beta 1-42 at nanomolar concentrations in THP-1 human monocytic cell line. *Neurosci.Lett. 262*:5.
52. Barger, S. W., D. Horster, K. Furukawa, Y. Goodman , J. Krieglstein, and M. P. Mattson. 1995. Tumor necrosis factors alpha and beta protect neurons against amyloid beta-peptide toxicity: evidence for involvement of a kappa B-binding factor and attenuation of peroxide and Ca2+ accumulation. *Proc.Natl.Acad.Sci.U.S.A 92*:9328.
53. Lezoualc'h, F., Y. Sagara, F. Holsboer, and C. Behl. 1998. High constitutive NF-kappaB activity mediates resistance to oxidative stress in neuronal cells. *J.Neurosci. 18*:3224.
54. Akama, K. T. and L. J. Van Eldik. 2000. Beta-amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1beta- and tumor necrosis factor-alpha (TNFalpha)-dependent, and involves a TNFalpha receptor-associated factor- and NFkappaB-inducing kinase-dependent signaling mechanism. *J.Biol.Chem. 275*:7918.
55. Sutton, E. T., T. Thomas, M. W. Bryant, C. S. Landon, C. A. Newton, and J. A. Rhodin. 1999. Amyloid-beta peptide induced inflammatory reaction is mediated by the cytokines tumor necrosis factor and interleukin-1. *J.Submicrosc.Cytol.Pathol. 31*:313.
56. Newell, C. L., A. B. Deisseroth, and G. Lopez-Berestein. 1994. Interaction of nuclear proteins with an AP-1/CRE-like promoter sequence in the human TNF-alpha gene. *J.Leukoc.Biol. 56*:27.
57. Frankenberger, M., A. Pforte, T. Sternsdorf, B. Passlick, P. A. Baeuerle, and H. W. Ziegler-Heitbrock. 1994. Constitutive nuclear NF-kappa B in cells of the monocyte lineage. *Biochem.J. 304 (Pt 1)*:87.
58. Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. Nabel. 1989. Activation of HIV gene expression during monocyte differentation by induction of NF- kappa B. *Nature 339*:70.
59. Conti, L., J. Hiscott, M. Papacchini, A. Roulston, M. A. Wainberg, F. Belardelli, and S. Gessani. 1997. Induction of relA(p65) and I kappa B alpha subunit expression during differentiation of human peripheral blood monocytes to macrophages. *Cell Growth Differ. 8*:435.
60. Forloni, G., F. Mangiarotti, N. Angeretti, E. Lucca, and M. G. De Simoni. 1997. Beta-amyloid fragment potentiates IL-6 and TNF-alpha secretion by LPS in astrocytes but not in microglia. *Cytokine 9*:759.

61. Lieb, K., B. L. Fiebich, H. Schaller, M. Berger, and J. Bauer. 1996. Interleukin-1 beta and tumor necrosis factor-alpha induce expression of alpha 1-antichymotrypsin in human astrocytoma cells by activation of nuclear factor-kappa B. *J.Neurochem. 67*:2039.

62. Gitter, B. D., L. M. Cox, R. E. Rydel, and P. C. May. 1995. Amyloid beta peptide potentiates cytokine secretion by interleukin-1 beta-activated human astrocytoma cells. *Proc.Natl.Acad.Sci.U.S.A. 92*:10738

.

5

Amyloid-β-induced chemokine production in primary human macrophages and astrocytes

Hessel A. Smits
Annemarie Rijsmus
Joyce H. van Loon
Jesse W.Y. Watt
Jan Verhoef
Leonie A. Boven
Hans S.L.M. Nottet

Eijkman-Winkler Institute, section Neuroimmunology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Submitted.

Abstract

In recent years extensive research has revealed an insight into the mechanisms as thought to exist in brains of people suffering from Alzheimer's disease. These mechanisms are eventually responsible for the loss of neurons. One of the pathological hallmarks in those brains is the neuritic plaque. These plaques are surrounded by microglia as well as astrocytes. Amyloid-beta together with the pro-inflammatory products released by these microglia and astrocytes are held responsible for the damage done. There are far more glial cells surrounding the plaque than located elsewhere in the brain. Chemotaxis might be responsible for attracting these cells towards the neuritic plaque. Using primary monocyte-derived macrophages and primary adult astrocytes as a model, the production of the chemoattractant CC-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES in the presence and absence of amyloid-beta 1-42, 1-40 or 42-1 was investigated. Within 6 hours amyloid-beta (1-42) was able to stimulate the production, as measured by RT-PCR, of MIP-1α and MIP-1β mRNA in macrophages and MCP-1 in astrocytes. Cocultures showed in unstimulated as well as in amyloid-beta-stimulated cells an increase in MIP-1α, MIP-1β and MCP-1 mRNA. ELISAs of supernatant samples of macrophages and astrocytes stimulated for 24 hours also showed an increase in MIP-1α and MIP-1β in macrophages and MCP-1 in astrocytes. Cocultures, however, showed an increase in MIP-1α, MIP-1β and MCP-1 when stimulated and no increase when unstimulated. These results show that CC-chemokines might play an important role in attracting glial cells to the neuritic plaque and therefore in the continuation of the inflammatory response.

Introduction

Alzheimer's disease (AD) is the most abundant form of dementia. Brains of people suffering from AD are characterized by depositions composed of aggregated amyloid-beta (Aβ) proteins and several other molecules (reviewed in (1,2)). These depositions are reported to be enclosed by activated microglia (3) and astrocytes (4,5). The depositions and the glial cells in their turn are surrounded by degenerating neurons. In total this is called the neuritic plaque. In contrast to the diffuse plaque which is found both in normal and AD brains. There is less Aβ present in these diffuse plaques and Aβ is not as aggregated as in neuritic plaques. Moreover, these diffuse plaques are reported not to be surrounded by microglia and astrocytes and neurons surrounding these diffuse plaques are not degenerating.

The cause of neuronal loss has not been fully determined yet. There are, however, strong suggestions that the Aβ protein can be direct neurotoxic and that surrounding microglia and astrocytes may play an important role in the inflammatory response. Studies have shown that Aβ stimulation of THP-1 monocytes, rat microglia and primary human macrophages results in the production of an array of pro-inflammatory molecules (6-16). There are also several studies reporting the ability of Aβ-stimulated monocytes and

macrophages to produce anti-inflammatory cytokines (17). Taken together, all these studies have shown the complexity of the situation as it exists in the neuritic plaque. There are numerous glial cells present around each plaque, each residing in its own micro-environment and each capable of producing pro- and/or anti-inflammatory products. It is the balance of pro-inflammatory products and anti-inflammatory products that may be essential in the degenerative process. In AD it is eventually leading to the degeneration of the neurons surrounding the plaque.

Influencing this balance may help in slowing the disease. Therefore a lot of effort has been put in understanding the intracellular pathways leading to the release of these pro-inflammatory products. The group of Landreth has done extensive research on the early events after Aβ stimulation of the microglia (18-21). They showed the involvement of the tyrosine kinases Lyn and Syk and more downstream the activation of the MAPK family members ERK1 and ERK2 in the Aβ stimulation of monocytes and microglia. Akama et al. showed NF-κB to be important in the Aβ-stimulated NO release in astrocytes (22,23). Klegeris et al. showed protein kinase C (PKC) to be involved in the Aβ-stimulated tumor necrosis factor-alpha (TNF-α) release in THP-1 monocytes (8,24). These reports also show that using specific enzyme inhibitors these pathways can be blocked, resulting in a decreased production of the pro-inflammatory products. This in turn may lead to a decreased neurodegeneration.

Diffuse plaques are not surrounded by microglia and astrocytes. This in contrast to neuritic plaques. The extensive research on the production of pro- and anti-inflammatory products concentrates on the latter. Another possibility of changing the balance between pro- and anti-inflammatory products is questioning the recruitment of the microglia and astrocytes towards the plaque. There are far more microglia and astrocytes concentrated around these plaques than located elsewhere in the brain. Little is known about the mechanism responsible for attracting so much glial cells. The research presented in this paper focuses on the involvement of proteins with chemotactic capabilities in recruiting these cells to the plaque. One group of chemotactic proteins are the chemokines. Chemokines constitute a superfamily of chemotactic cytokines and are produced by a wide variety of cells, including T-cells, monocytes, endothelial cells, microglia and astrocytes (25,26). In addition to their chemotactic effects in the immune system, chemokines modulate a number of biological responses, including enzyme secretion, cellular adhesion, cytotoxicity, tumor cell growth, degranulation and T-cell activation (27). This superfamily of about 40 members is divided into two major subfamilies on the basis of the structural conservation of two N-terminal cysteine residues. This division depends on the presence (CXC) or absence (CC) of an additional amino acid between the first two residues. Two other classes of chemokines have been described: lymphotactin (C) and fractalkine (CX_3C). The former one lacks cysteine residues one and three of the typical chemokines structure (28) and the latter one exhibits three amino acids between the first two cysteines and is also the only membrane-bound chemokine through a mucin-like stalk (29). The chemokines mediate their effects via G protein-coupled receptors of the seven transmembrane domain rhodopsin-type superfamily (30,31). These receptors are named CXCR, CCR, CR and CX_3CR respectively.

In many central nervous system (CNS) diseases such as multiple sclerosis (MS), brain trauma, infections, or focal ischemia, the blood-brain barrier is breached, and leukocyte infiltration is found at the lesion sites (32). In AD however, the blood-brain barrier is intact and no infiltration of inflammatory cells is present. Because of the continual inflammation in the AD brain, resident CNS cells have to be involved, and indeed many CNS cells are reported to be able to produce chemokines and express chemokine receptors (33,34). Regarding Aβ-induced chemokine production however, only a small number of papers have been published. The CC-chemokines MIP-1α and MCP-1 have been found to be produced by Aβ-stimulated monocytes after 48 h of cultivation (35). Also Aβ(25-35)-stimulated U373 astrocytoma cells have been reported to have an increased MCP-1 production and rat astrocytes stimulated with 50 μM Aβ(1-42) for 6 h have been found to produce MCP-1 and RANTES mRNA (36,37). In rat astrocytes, the CC-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES mRNA expression can also be induced by either IFN-γ or TNF-α.

This paper shows the results of the mRNA production of the CC-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES by primary human monocyte-derived macrophages as a model for microglia cells, by primary adult astrocytes and by cocultured MDMs and astrocytes when stimulated by Aβ(1-42), Aβ(1-40) for 2, 4, 6 and 8 hours. Moreover, chemokine supernatant levels in cultured MDMs, astrocytes and cocultures thereof are determined.

Materials and Methods

Cells

Monocytes were derived from peripheral blood mononuclear cells by Ficoll-Paque (Pharmacia Biotech, Sweden) density gradients and purified by centrifugal elutriation as described previously (38). Cells were seeded at a concentration of 2 x 10^6 cells / ml in Teflon Erlenmeyer flasks (Nalgene, USA) and grown as suspension at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Culture medium was composed of Iscove's modified Dulbecco's medium (Gibco Life Technologies, The Netherlands) supplemented with 10% (v/v) heat-inactivated human AB serum, 2 mM L-glutamine, 19 mM sodium bicarbonate, 10 μg/ml gentamicin and 0.5 μg/ml ciprofloxacin. After 7 days, monocyte-derived macrophages (MDMs) were harvested from the flasks, washed with Hanks' balanced salt solution (HBSS) and used for the experiments.

Primary human adult astrocytes were kindly provided by Dr. C.J.A. de Groot and were cultured as described earlier (39). In short, cells were seeded into poly-L-lysine (PLL; 15 μg/ml; Sigma Chemical Co., The Netherlands) coated 80 cm^2 flasks at a density of 2 x 10^4 cells/ml in Dulbecco's modified eagle medium / Nutrient mixture Ham F-10, 1:1, supplemented with 10% (v/v) fetal calf serum, 10 IU / ml penicillin, 10 IU / ml streptomycin. For each new passage confluent cultures of astrocytes were harvested using 0.25% trypsin in 0.02% EDTA.

Amyloid-β peptides

Lyophilized synthetic Aβ(1-42) peptide was obtained from Genosphere Biotechnologies (Paris, France). Lyophilized synthetic Aβ(1-40) and Aβ(42-1) peptides were purchased from Bachem AG (Bubendorf, Switzerland). Peptides were initially dissolved at a concentration of 1 mM in sterile double-distilled pyrogen-free water, after which they were aliquoted and stored at –20 °C.

RT-PCR detection of chemokines

Macrophages and astrocytes and cocultures hereof were homogenized and lysed in 1 ml TRIzol (Gibco Life Technologies, Gaithersburg, MD) according to the manufacturer's guidelines. Total RNA was isolated and dissolved in diethylpyrocarbonate (DEPC)-treated water and 1 μg of RNA was used for the synthesis of complementary DNA. Subsequent PCR reactions were performed as described previously (38). For semi-quantification every primer pair was tested at different cycle numbers to determine the linear range. MDM GAPDH mRNA levels were measured at 23 cycles, whereas cDNA had to be subjected to 25 cycles to be in the linear range to detect MIP-1α, MIP-1β, and MCP-1 and 35 cycles for RANTES. Astrocyte GAPDH mRNA levels were measured at 23 cycles, whereas MCP-1 mRNA levels were quantified at 25 cycles. MIP-1α, MIP-1β and RANTES mRNA levels were not detectable in astrocytes. In cocultures of MDMs and astrocytes GAPDH mRNA levels were measured at 23 cycles. MIP-1α, MIP-1β and MCP-1 cDNA were subjected to 25 cycles. RANTES mRNA could not be detected in cocultures.

Aliquots of 5 μl of the biotinylated PCR product were semi-quantitatively analyzed using a fluorescent digoxigenin detection ELISA kit (Roche) according to manufacturer's protocol as described previously (38). All data were normalized against GAPDH mRNA levels. The sequences of the primers and probes are depicted in Table I.

Chemokine ELISA

Macrophages were grown in teflon flasks for six days after which they were seeded in a 48-well plate. Astrocytes were grown in a poly-L-lysine coated 48-well plate. Cocultures of macrophages and astrocytes were grown in a poly-L-lysine coated 48-well plate. Next day, cells were stimulated with 10 μM Aβ-peptides for 6, 12, 24, 36 and 48 hours after which supernatant samples were taken. MIP-1α, MIP-1β, MCP-1 and RANTES concentrations were quantified with a sandwich enzyme-linked immunosorbent assay (ELISA). In short, chemokines were captured by a specific monoclonal antibody according to the manufacturer's instructions (R&D Systems Europe, Abingdon, United Kingdom).

Statistical analysis

In all experiments Student-t test was used to compare unstimulated or Aβ(42-1)-stimulated macrophages, astrocytes or cocultures thereof and Aβ(1-42)-stimulated macrophages, astrocytes or cocultures thereof. Reported p-values are two-sided.

Results

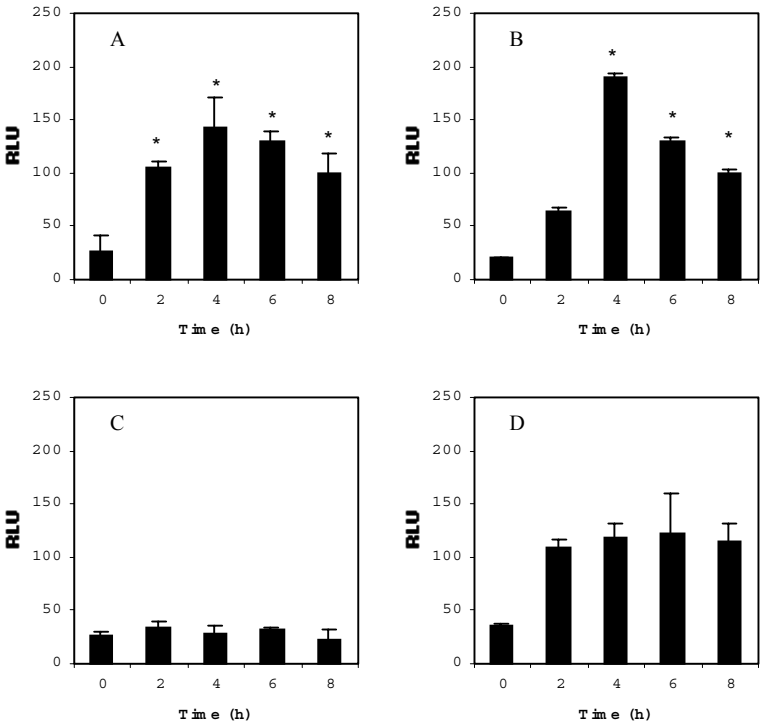

Figure 1

MIP-1α (A), MIP-1β (B), MCP-1 (C) and RANTES (D) production of Aβ(1-42)-stimulated MDMs. Unstimulated and Aβ(1-42)-stimulated MDMs were incubated for 2, 4, 6 and 8 hours after which total RNA was isolated and PCRs were performed using specific biotinylated primers against mentioned chemokines and GAPDH. Biotinylated PCR products were then semi-quantitatively analyzed using a fluorescent digoxigenin detection ELISA kit. All data were normalized against GAPDH mRNA levels. The sequences of the primers and probes are depicted in Table I. PCRs and ELISAs were performed at least three times for each RNA isolation performed. Total RNA was isolated three times using MDMs of different donors each time. Asterisks indicate statistically significant differences in MIP-1α and MIP-1β mRNA production compared to unstimulated macrophages.

CC-chemokine production of Aβ(1-42) and Aβ(1-40)-stimulated macrophages

In order to study the effect of Aβ on the CC-chemokine production of primary human macrophages, MDMs were first stimulated with 10 μM Aβ(1-42) or 10 μM Aβ(1-40) for 0, 2, 4, 6 and 8 hours where after mRNA production was determined. Figure 1 shows the effect of Aβ(1-42) on the MIP-1α (A), MIP-1β (B), MCP-1 (C) and RANTES (D) mRNA production. There is a clear induction of MIP-1α ($p=0.020$) and MIP-

1β (p=0.025), both with a maximum at 4 hours. There is however no obvious induction of MCP-1 and there seems to be a trend in the induction of RANTES mRNA (p=0.110). Aβ(1-40)-stimulated MDMs showed, in contrast to the effect of Aβ(1-42), no induction of either MIP-1α, nor MIP-1β, MCP-1 or RANTES (data not shown).

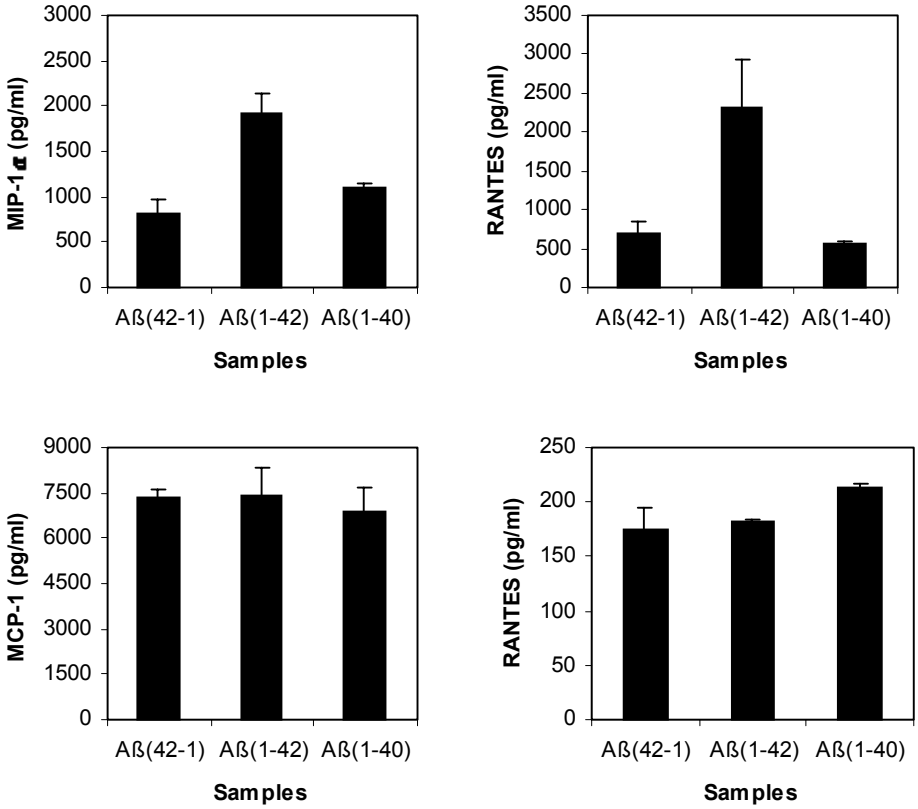

Figure 2

MIP-1α (A), MIP-1β (B), MCP-1 (C) and RANTES (D) secretion by Aβ(42-1)-stimulated, Aβ(1-42)-stimulated and Aβ(1-40)-stimulated MDMs. Cells were incubated for 6, 12, 24, 36 and 48 hours after which supernatant samples were taken and chemokine levels were quantified. 24 h time point is shown. Experiment was done twice. Asterisks indicate statistically significant differences in MIP-1α and MIP-1β supernatant levels compared to Aβ(42-1)-stimulated macrophages.

To look whether the induction of mRNA would lead to the expression of these chemokines, MDMs were stimulated for 6, 12, 24, 36 and 48 hours. Figure 2 shows the MIP-1α (A), MIP-1β (B), MCP-1 (C) and RANTES (D) supernatant levels of 10 μM Aβ(1-42)-stimulated and 10 μM Aβ(1-40)-stimulated MDMs after 24 h of induction. Aβ(42-1)-stimulated macrophages were used as a control. There is a clear expression of MIP-1α (p=0.024) and MIP-1β (p=0.034) of Aβ(1-42)-stimulated macrophages. However, there is

no obvious induction of MCP-1 or RANTES. Both Aβ(1-40) and Aβ(42-1) showed no stimulated expression of the mentioned chemokines.

The data of the RT-PCR and the ELISA show that Aβ(1-42) activates the macrophage, leading to the production and expression of MIP-1α and MIP-1β.

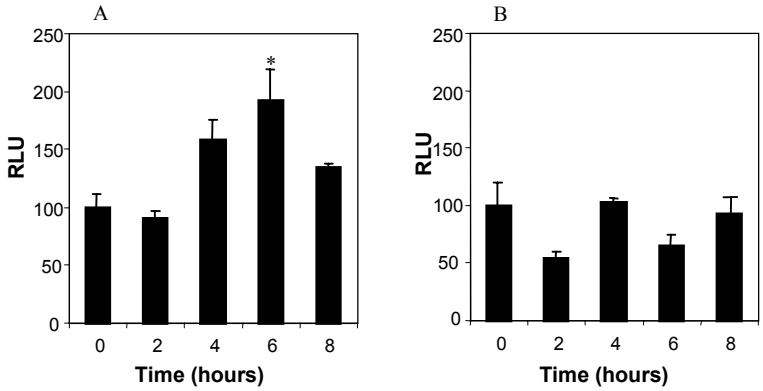

Figure 3

MCP-1 production of Aβ(1-42) and Aβ(1-40)-stimulated (A and B respectively) astrocytes. Unstimulated Aβ(1-42) and Aβ(1-40)-stimulated astrocytes were incubated for 2, 4, 6 and 8 hours after which total RNA was isolated and PCRs were performed using specific biotinylated primers against MCP-1 and GAPDH. Biotinylated PCR products were then semi-quantitatively analyzed using a fluorescent digoxigenin detection ELISA kit. All data were normalized against GAPDH mRNA levels. The sequences of the primers and probes are depicted in Table I. PCRs and ELISAs were performed at least three times for each RNA isolation performed. Total RNA was isolated three times. Asterisk indicate statistically significant differences in MCP-1 mRNA production levels compared to unstimulated astrocytes.

CC-chemokine production of Aβ(1-42) and Aβ(1-40)-stimulated astrocytes

To investigate the CC-chemokine production of Aβ-stimulated astrocytes, primary adult astrocytes were stimulated with either 10 μM Aβ(1-42) or 10 μM Aβ(1-40) for 0, 2, 4, 6 and 8 hours after which mRNA production was determined. Figure 3 shows the effect of Aβ(1-42) (A) and Aβ(1-40) (B) on the MCP-1 chemokine production in astrocytes. There is a clear induction, with a maximum after 6 hours, after stimulation with Aβ(1-42). No upregulation of the MCP-1 mRNA production could be detected after stimulation with Aβ(1-40). MIP-1α, MIP-1β and RANTES were not detectable after stimulation with Aβ(1-42) or Aβ(1-40) (data not shown).

The expression of MCP-1 was determined by stimulating primary adult astrocytes for 6, 12, 24, 36 and 48 hours after which supernatant samples were taken and CC-chemokine levels were determined. Figure 4 shows the MCP-1 supernatant levels of 10 μM Aβ(1-42)-stimulated and 10 μM Aβ(1-40)-stimulated astrocytes after 24 h of induction. Aβ(42-1)-stimulated astrocytes were used as control. There is a clear expression of MCP-1

of Aβ(1-42)-stimulated astrocytes. Both Aβ(1-40) and Aβ(42-1) showed no stimulated expression of MCP-1.

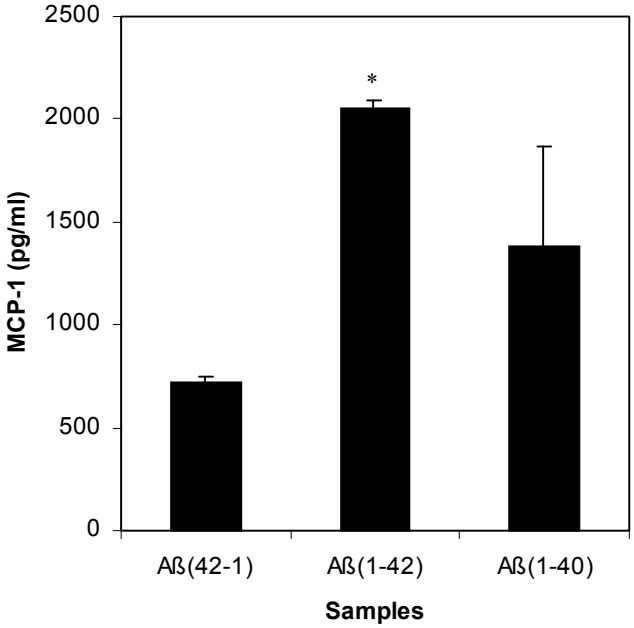

Figure 4

MCP-1 secretion by Aβ(42-1)-stimulated, Aβ(1-42)-stimulated and Aβ(1-40)-stimulated astrocytes. Astrocytes were incubated for 6, 12, 24, 36 and 48 hours after which supernatant samples were taken and chemokine levels were quantified. 24 h time point is shown. Asterisk indicate statistically significant differences in MCP-1 supernatant levels compared to Aβ(42-1)-stimulated astrocytes.

CC-chemokine production in Aβ-stimulated cocultures of macrophages and astrocytes

In order to elucidate the effect of these cells on each other regarding the production of CC-chemokines we cocultured MDMs and astrocytes and determined the production and expression of MIP-1α, MIP-1β, MCP-1 and RANTES in the presence or absence of 10 μM Aβ(1-42). Figure 5 shows the mRNA production after 0, 2, 4, 6 and 8 hours stimulation. In unstimulated as well as in stimulated cocultures there is an increase in the production of MIP-1α (A), MIP-1β (B), and MCP-1 (C). However, maximum mRNA production in Aβ-stimulated cocultures was seen after 2 hours, in contrast to unstimulated cocultures where the maximum mRNA production was seen after 4 hours. We could not detect an increase in RANTES mRNA production (data not shown).

To link the expression and production of MIP-1α, MIP-1β and MCP-1, MDMs and astrocytes were cocultured for 6, 12, 24, 36 and 48 hours in the presence or absence of 10 μM Aβ(1-42), 10 μM Aβ(1-40) or 10 μM Aβ(42-1). Hereafter supernatant samples were

taken and CC-chemokine levels were determined 24 h after stimulation. Figure 6 shows the MIP-1α (A), MIP-1β (B) and MCP-1 (C) supernatant levels.

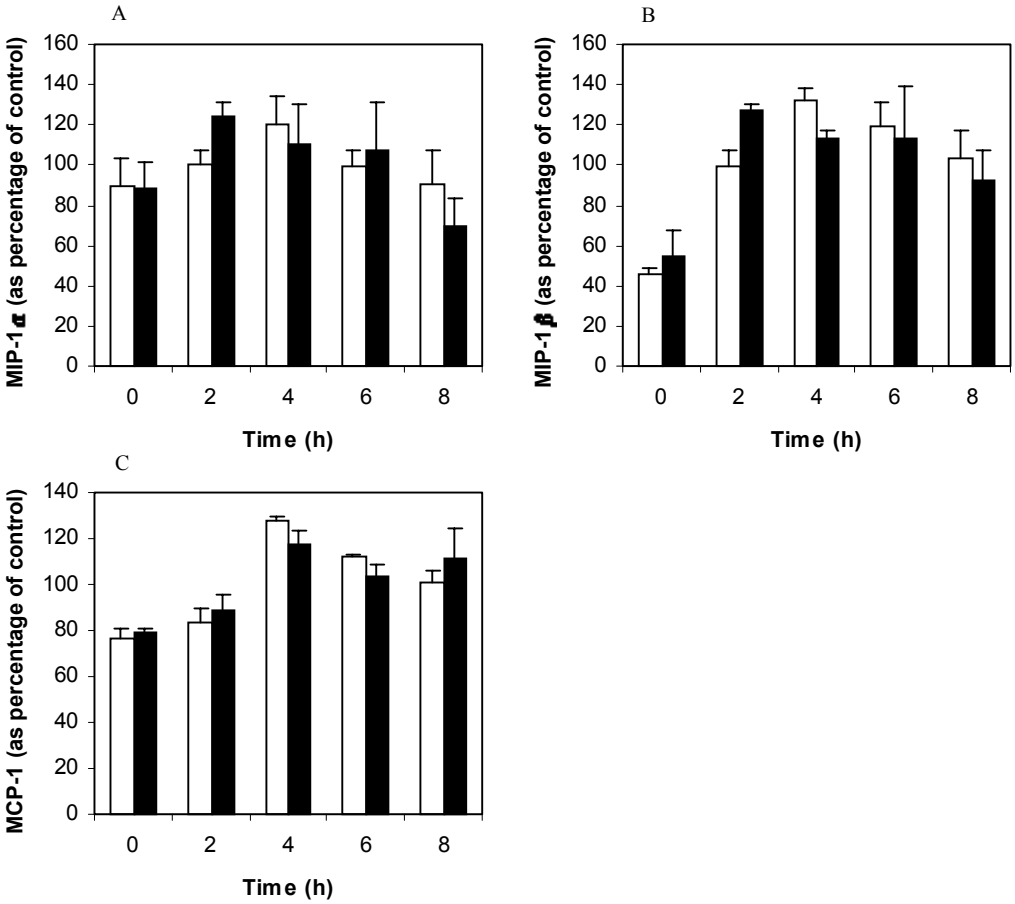

Figure 5

MIP-1α (A), MIP-1β (B), and MCP-1 (C) production of Aβ(1-42)-stimulated cocultures of MDMs and astrocytes. Unstimulated and Aβ(1-42)-stimulated cocultures were incubated for 2, 4, 6 and 8 hours after which total RNA was isolated and PCRs were performed using specific biotinylated primers against mentioned chemokines and GAPDH. Biotinylated PCR products were then semi-quantitatively analyzed using a fluorescent digoxigenin detection ELISA kit. All data were normalized against GAPDH mRNA levels. The sequences of the primers and probes are depicted in Table I. PCRs and ELISAs were performed at least three times for each RNA isolation performed. Total RNA was isolated three times using MDMs of different donors each time.

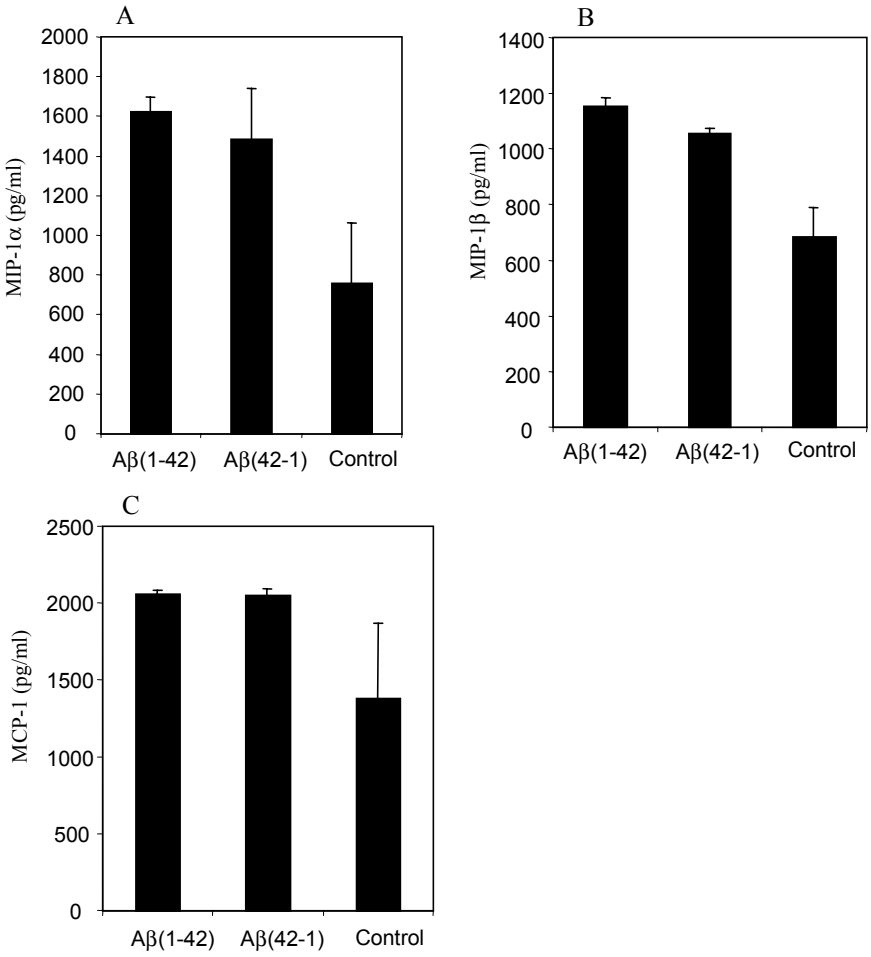

Figure 6

MIP-1α (A), MIP-1β (B) and MCP-1 (C) secretion by Aβ(42-1)-stimulated, Aβ(1-42)-stimulated and Aβ(1-40)-stimulated cocultures of MDMs and astrocytes. Cocultures were incubated for 6, 12, 24, 36 and 48 hours after which supernatant samples were taken and chemokine levels were quantified. 24 h time point is shown.

Discussion

Trying to dissolve possible sources of inflammation in AD, one can only take into account CNS resident cells. Neuritic plaques in AD are surrounded by activated microglia and astrocytes and a large amount of the literature on AD has shown the possibility of these glial cells to produce inflammatory products when stimulated with Aβ (For review see (40)). It seems to be very likely that these cells are involved in the continuation of the inflammatory response, eventually leading to neuronal damage.

Because of the amounts of microglia and astrocytes surrounding neuritic plaques, active chemotaxis might be responsible for attracting those cells. Microglia as well as astrocytes have been shown to have chemotactic capabilities to several factors (41-43). Microglia has been shown to be chemotactic towards Aβ(25-35) and MIP-1α, MIP-1β and MCP-1 (41,42) and astrocytes have been shown to migrate towards MIP-1α (43). Here we show that primary human monocytes-derived macrophages used as a model for microglia are able to produce and express CC-chemokines MIP-1α and MIP-1β. In addition, primary human adult astrocytes are capable of producing and expressing MCP-1. Coculturing MDMs and astrocytes revealed an increased mRNA production of MIP-1α, MIP-1β and MCP-1 in unstimulated as well as Aβ(1-42)-stimulated cells. ELISA's showed increased supernatant levels of all three in Aβ-stimulated cocultures.

Due to the limited availability of microglia studying the interactions of Aβ and microglia has proven to be a difficult task. Human adult microglia would be the ideal cell type to use as an *in-vitro* model in studying microglia-mediated inflammation. Because of the limited availability of these cells and the difficulty of maintaining these cells in culture, different cell types have been used instead. The human monocytic cell line THP-1 has often been used as a model for microglia. A recent paper of Szczepanik and colleagues showed THP-1 cells to produce IL-1α, IL-1β, IL-6, TNF-α and MCP-1 when stimulated with Aβ (17). Using THP-1 cells Yates et al. showed an increase in MIP-1α and MIP-1β when stimulated with Aβ(1-40) and LPS (44). More close to microglia are human blood monocytes. These cells are able to produce MIP-1α, MIP-1β and MCP-1 when stimulated with 50 μg/ml Aβ(1-42) for 48 h (35). In our opinion primary human monocytes-derived macrophages are cells most closely resembling microglia. We found MIP-1α and MIP-1β to be produced and due to the high production of MCP-1 in unstimulated cells no induction of MCP-1 by Aβ(1–42) could be found.

As a model for astrocytes several cell types have been used. Primary rat astrocytes have been shown to produce MCP-1 and RANTES upon stimulation with 50 μM Aβ(1-42) or 50 μM Aβ(1-40) (37). The human astrocytoma cell line U373MG also produces MCP-1 when stimulated with 30 μM Aβ(1-42) (36). Oh et al. showed U373MG also to be able to produce MCP-1 and RANTES (45) when stimulated with TNF-α. Using 10 μM Aβ(1–42) we show that primary human adult astrocytes are only capable of producing MCP-1. High concentration of Aβ used by others and the use of different cells might account for the difference in detecting RANTES.

The overall balance of pro- and anti-inflammatory products in brains of people suffering from Alzheimer's disease might play an important role in the degenerative process. Glial cells and more important microglia are thought to play a major role in the production of pro-inflammatory products. Because microglia are not present around diffuse plaques, chemo-attractive products might be responsible of attracting these cells towards the neuritic plaque. Using primary human cells we show here that CC-chemokines MIP-1α, MIP-1β and MCP-1 could be very good candidates accomplishing this task.

Target (Product size)		Sequence (5'-3')
GAPDH	Sense	CCATGGAGAAGGCTGGGG
(195 bp)	Antisense	CAAAGTTGTCATGGATGACC
	Probe	CTGCACCACCAACTGCTTAGC
MIP-1α	Sense	TGCATCACTTGCTGCTGACACG
(333 bp)	Antisense	CAACCAGTCCATAGAAGAGG
	Probe	CTGACTACTTTGAGACGAGC
MIP-1β	Sense	CCAAACCAAAAGAAGCAAGC
(310 bp)	Antisense	AGAAACAGTGACAGTGGACC
	Probe	ACATCTCCTCCATACTCAGG
MCP-1	Sense	GCGAGCTATAGAAGAATCACC
(230 bp)	Antisense	ATAAAACAGGGTGTCTGGGG
	Probe	GACAAGCAAACCCAAACTCC
RANTES	Sense	CTTTGTCACCCGAAAGAACC
(352 bp)	Antisense	GTTTCATCATGTTGGCCAGG
	Probe	TTGCTCTTGTCCTAGCTTGG

Table I. Sequences of the oligonucleotide primers and probes in reverse transcriptase polymerase chain reaction.

Acknowledgements

This work was supported by Grant No. 903-51-104 from the Dutch Organisation for Scientific Research (NWO) to H.S.L.M.N.

References

1. Dickson, D. W. 1997. The pathogenesis of senile plaques. J.Neuropathol.Exp.Neurol. 56:321.
2. Cummings, J. L., H. V. Vinters, G. M. Cole, and Z. S. Khachaturian. 1998. Alzheimer's disease: etiologies, pathophysiology, cognitive reserve, and treatment opportunities. Neurology 51:S2.
3. McGeer, P. L., S. Itagaki, H. Tago, and E. G. McGeer. 1987. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci.Lett. 79:195.
4. Mandybur, T. I. and C. C. Chuirazzi. 1990. Astrocytes and the plaques of Alzheimer's disease. Neurology 40:635.
5. Pike, C. J., B. J. Cummings, and C. W. Cotman. 1995. Early association of reactive astrocytes with senile plaques in Alzheimer's disease. Exp.Neurol. 132:172.
6. Giulian, D., L. J. Haverkamp, J. Li, W. L. Karshin, J. Yu, D. Tom, X. Li, and J. B. Kirkpatrick. 1995. Senile plaques stimulate microglia to release a neurotoxin found in Alzheimer brain. Neurochem.Int. 27:119.
7. Lorton, D., J. M. Kocsis, L. King, K. Madden, and K. R. Brunden. 1996. beta-Amyloid induces increased release of interleukin-1 beta from lipopolysaccharide-activated human monocytes. J.Neuroimmunol. 67:21.
8. Klegeris, A., D. G. Walker, and P. L. McGeer. 1997. Interaction of Alzheimer beta-amyloid peptide with the human monocytic cell line THP-1 results in a protein kinase C-dependent secretion of tumor necrosis factor-alpha. Brain Res. 747:114.
9. Heese, K., C. Hock, and U. Otten. 1998. Inflammatory signals induce neurotrophin expression in human microglial cells. J.Neurochem. 70:699.
10. Ariga, T. and R. K. Yu. 1999. GM1 inhibits amyloid beta-protein-induced cytokine release. Neurochem.Res. 24:219.
11. Klegeris, A., D. G. Walker, and P. L. McGeer. 1994. Activation of macrophages by Alzheimer beta amyloid peptide. Biochem.Biophys.Res.Commun. 199:984.
12. Meda, L., C. Bonaiuto, P. Baron, L. Otvos, Jr., F. Rossi, and M. A. Cassatella. 1996. Priming of monocyte respiratory burst by beta-amyloid fragment (25-35). Neurosci.Lett. 219:91.
13. Klegeris, A. and P. L. McGeer. 1997. beta-amyloid protein enhances macrophage production of oxygen free radicals and glutamate. J.Neurosci.Res. 49:229.
14. Tjernberg, L. O., D. J. Callaway, A. Tjernberg, S. Hahne, C. Lilliehook, L. Terenius, J. Thyberg, and C. Nordstedt. 1999. A molecular model of Alzheimer amyloid beta-peptide fibril formation. J.Biol.Chem. 274:12619.
15. Smits, H. A., F. L. Van Muiswinkel, N. M. de Vos, J. Verhoef, and H. S. Nottet. 1999. The amyloid-beta (1-42)-induced respiratory burst of primary human macrophages is enhanced in the presence of amyloid-beta (25- 35). In Alzheimer's Disease and Related Disorders. K.Iqbal, D.F.Swaab, B.Winblad and H.M.Wisniewski, eds. John Wiley & Sons Ltd, Sussex, p. 457.
16. Baeuerle, P. A. and T. Henkel. 1994. Function and activation of NF-kappa B in the immune system. Annu.Rev.Immunol. 12:141.

17. Szczepanik, A. M., S. Funes, W. Petko, and G. E. Ringheim. 2001. IL-4, IL-10 and IL-13 modulate Abeta(1-42)-induced cytokine and chemokine production in primary murine microglia and a human monocyte cell line. J.Neuroimmunol. 113:49.
18. Combs, C. K., D. E. Johnson, S. B. Cannady, T. M. Lehman, and G. E. Landreth. 1999. Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. J.Neurosci. 19:928.
19. Combs, C. K., D. E. Johnson, J. C. Karlo, S. B. Cannady, and G. E. Landreth. 2000. Inflammatory mechanisms in Alzheimer's disease: inhibition of beta- amyloid-stimulated proinflammatory responses and neurotoxicity by PPARgamma agonists. J.Neurosci. 20:558.
20. McDonald, D. R., K. R. Brunden, and G. E. Landreth. 1997. Amyloid fibrils activate tyrosine kinase-dependent signaling and superoxide production in microglia. J.Neurosci. 17:2284.
21. McDonald, D. R., M. E. Bamberger, C. K. Combs, and G. E. Landreth. 1998. beta-Amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. J.Neurosci. 18:4451.
22. Akama, K. T., C. Albanese, R. G. Pestell, and L. J. Van Eldik. 1998. Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. Proc.Natl.Acad.Sci.U.S.A. 95:5795.
23. Akama, K. T. and L. J. Van Eldik. 2000. Beta-amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1beta- and tumor necrosis factor-alpha (TNFalpha)-dependent, and involves a TNFalpha receptor-associated factor- and NFkappaB-inducing kinase-dependent signaling mechanism. J.Biol.Chem. 275:7918.
24. Klegeris, A. and P. L. McGeer. 2000. Interaction of various intracellular signaling mechanisms involved in mononuclear phagocyte toxicity toward neuronal cells. J.Leukoc.Biol. 67:127.
25. Schall, T. J. and K. B. Bacon. 1994. Chemokines, leukocyte trafficking, and inflammation. Curr.Opin.Immunol. 6:865.
26. Schluger, N. W. and W. N. Rom. 1997. Early responses to infection: chemokines as mediators of inflammation. Curr.Opin.Immunol. 9:504.
27. Luster, A. D. 1998. Chemokines--chemotactic cytokines that mediate inflammation. N.Engl.J.Med. 338:436.
28. Kelner, G. S., J. Kennedy, K. B. Bacon, S. Kleyensteuber, D. A. Largaespada, N. A. Jenkins, N. G. Copeland, J. F. Bazan, K. W. Moore, and T. J. Schall. 1994. Lymphotactin: a cytokine that represents a new class of chemokine. Science 266:1395.
29. Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, A. Zlotnik, and T. J. Schall. 1997. A new class of membrane-bound chemokine with a CX3C motif. Nature 385:640.
30. Wells, T. N. C., A. E. I. Proudfoot, C. A. Power, M. Lusti-Narasimhan, S. Alouani, A. J. Hoogewerf, and M. C. Peitsch. 1996. The Molecular Basis of the Chemokine/Chemokine Receptor Interaction-Scope for Design of Chemokine Antagonists. Methods 10:126.
31. Murphy, P. M. 1996. Chemokine receptors: structure, function and role in microbial pathogenesis. Cytokine Growth Factor Rev. 7:47.
32. Ghirnikar, R. S., Y. L. Lee, and L. F. Eng. 1998. Inflammation in traumatic brain injury: role of cytokines and chemokines. Neurochem.Res. 23:329.
33. Xia, M. Q. and B. T. Hyman. 1999. Chemokines/chemokine receptors in the central nervous system and Alzheimer's disease. J.Neurovirol. 5:32.

34. Dorf, M. E., M. A. Berman, S. Tanabe, M. Heesen, and Y. Luo. 2000. Astrocytes express functional chemokine receptors. J.Neuroimmunol. 111:109.
35. Fiala, M., L. Zhang, X. Gan, B. Sherry, D. Taub, M. C. Graves, S. Hama, D. Way, M. Weinand, M. Witte, D. Lorton, Y. M. Kuo, and A. E. Roher. 1998. Amyloid-beta induces chemokine secretion and monocyte migration across a human blood--brain barrier model. Mol.Med. 4:480.
36. Prat, E., P. Baron, L. Meda, E. Scarpini, D. Galimberti, G. Ardolino, A. Catania, and G. Scarlato. 2000. The human astrocytoma cell line U373MG produces monocyte chemotactic protein (MCP)-1 upon stimulation with beta-amyloid protein. Neurosci.Lett. 283:177.
37. Johnstone, M., A. J. Gearing, and K. M. Miller. 1999. A central role for astrocytes in the inflammatory response to beta-amyloid; chemokines, cytokines and reactive oxygen species are produced. J.Neuroimmunol. 93:182.
38. Boven, L. A., L. Gomes, C. Hery, F. Gray, J. Verhoef, P. Portegies, M. Tardieu, and H. S. Nottet. 1999. Increased peroxynitrite activity in AIDS Dementia Complex: implications for neuropathogenesis of HIV-1 infection. J.Immunol. 162:4319.
39. De Groot, C. J., C. H. Langeveld, C. A. Jongenelen, L. Montagne, d. van, V, and C. D. Dijkstra. 1997. Establishment of human adult astrocyte cultures derived from postmortem multiple sclerosis and control brain and spinal cord regions: immunophenotypical and functional characterization. J.Neurosci.Res. 49:342.
40. Akiyama, H., S. Barger, S. Barnum, B. Bradt, J. Bauer, G. M. Cole, N. R. Cooper, P. Eikelenboom, M. Emmerling, B. L. Fiebich, C. E. Finch, S. Frautschy, W. S. Griffin, H. Hampel, M. Hull, G. Landreth, L. Lue, R. Mrak, I. R. Mackenzie, P. L. McGeer, M. K. O'Banion, J. Pachter, G. Pasinetti, C. Plata-Salaman, J. Rogers, R. Rydel, Y. Shen, W. Streit, R. Strohmeyer, I. Tooyoma, F. L. Van Muiswinkel, R. Veerhuis, D. Walker, S. Webster, B. Wegrzyniak, G. Wenk, and T. Wyss-Coray. 2000. Inflammation and Alzheimer's disease. Neurobiol.Aging 21:383.
41. Meda, L., C. Bonaiuto, G. I. Szendrei, M. Ceska, F. Rossi, and M. A. Cassatella. 1995. beta-Amyloid(25-35) induces the production of interleukin-8 from human monocytes. J.Neuroimmunol. 59:29.
42. Peterson, P. K., S. Hu, J. Salak-Johnson, T. W. Molitor, and C. C. Chao. 1997. Differential production of and migratory response to beta chemokines by human microglia and astrocytes. J.Infect.Dis. 175:478.
43. Tanabe, S., M. Heesen, M. A. Berman, M. B. Fischer, I. Yoshizawa, Y. Luo, and M. E. Dorf. 1997. Murine astrocytes express a functional chemokine receptor. J.Neurosci. 17:6522.
44. Yates, S. L., L. H. Burgess, J. Kocsis-Angle, J. M. Antal, M. D. Dority, P. B. Embury, A. M. Piotrkowski, and K. R. Brunden. 2000. Amyloid beta and amylin fibrils induce increases in proinflammatory cytokine and chemokine production by THP-1 cells and murine microglia. J.Neurochem. 74:1017.
45. Oh, J. W., L. Schwiebert, and E. N. Benveniste. 1999. Cytokine regulation of cc and cxc chemokine expression by human astrocytes. J.Neurovirol. 5:82.

6

Migration of monocytes in response to CC-chemokine production of primary human macrophages and astrocytes

Hessel A. Smits
Karla Rutten
Jan Verhoef
Hans S.L.M. Nottet

Eijkman-Winkler Institute, section Neuroimmunology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Submitted.

Abstract

In this study the capability of amyloid-β(1–42) to induce chemotaxis of monocytes towards macrophages, astrocytes, neurons and cocultures their of was investigated. Moreover, the involvement of CC-chemokines was studied. Results show a concentration-dependant increase in chemotaxis of monocytes towards amyloid-β-stimulated macrophages. Neutralizing antibodies showed that MIP-1α, MIP-1β and MCP-1 are involved in this migration of monocytes. We found however no increased chemotaxis of monocytes towards amyloid-β-stimulated astrocytes. Cocultures showed an increased chemotaxis of monocytes when compared to cells alone. Amyloid-β-stimulated cocultures showed no increased chemotaxis when compared to unstimulated cells. Amyloid-β-stimulated cocultures of neurons and astrocytes even showed a decreased chemotaxis.

Introduction

In Alzheimer's disease (AD) degradation of neurons is eventually responsible for the clinical aspects. This degradation of neurons is mostly concentrated around neuritic plaques. These plaques are one of the hallmarks of AD and mainly consist of the aggregated protein amyloid-β (Aβ). *In-vitro* studies have shown that Aβ can be directly neurotoxic and thus is it therefore suggested to be partially responsible for the neurodegeneration (1-4). However, neuritic plaques are also reported to be surrounded by microglia, or so called brain macrophages, and astrocytes and these cells are known for their ability to produce multiple inflammatory products. Several groups have shown that Aβ is able to stimulate these cells to produce various pro- and anti-inflammatory products (reviewed in (5)). The released inflammatory molecules can not only be damaging for the neurons but can also stimulate nearby microglia and astrocytes and in this manner continue the inflammatory response. Each cell is in its own environment a target for Aβ and numerous other signals, eventually resulting in an unequal balance between pro-inflammatory and anti-inflammatory responses.

A lot of effort has been put in resolving the intracellular pathways in microglia and astrocytes leading to the production of these pro-inflammatory products (6-10). These reports also show that using specific enzyme inhibitors these pathways can be blocked, resulting in a decreased production of the pro-inflammatory products. This in turn may lead to a decreased neurodegeneration. However, little is known about the recruitment of microglia and astrocytes towards the plaque. Diffuse plaques are in contrast to neuritic plaques only surrounded by a few astrocytes and not by microglia (11). The concentration of glial cells around a neuritic plaques is found to be higher than elsewhere in the brain (12-14). Understanding this mechanism might help in shifting the balance between pro- and anti-inflammatory molecules to the latter. Using primary human monocyte-derived macrophages as a model for microglia we have shown that upon Aβ stimulation these cells produce and express MIP-1α and MIP-1β (Smits et al., submitted). Aβ-stimulated primary

human astrocytes showed an increased MCP-1 production and expression. MIP-1α, MIP-1β and MCP-1 are chemoattractant proteins belonging to the family of CC-chemokines. The chemokine system in humans is a superfamily of approximately 50 ligands and 20 G protein-coupled receptors. These ligands are divided into four families on the basis of the pattern of conserved cysteine residues in the aminoterminus of the proteins. Glial cells present around the neuritic plaque might attract other glial cells by producing the mentioned CC-chemokines. In this way they might be responsible for the continuation of the inflammatory process. The neurons however are the target cells. Their role in the inflammatory process is not very clear. Neurons are capable of producing chemokines and expressing chemokine receptors (reviewed in (15) and (16)).

In order to study the role of neurons in the chemotaxis of glial cells and to study whether CC-chemokines produced by glial cells are indeed chemoattractive, this study reports the capability of Aβ (1-42)-induced macrophages, astrocytes to attract monocytes. Also cocultures of Aβ (1-42)-induced macrophages and astrocytes, astrocytes and neurons and macrophages and neurons were studied in their ability to attract monocytes. Moreover the CC-chemokines involved were identified using neutralizing antibodies.

Materials and methods

Materials

The synthetic Aβ peptide Aβ (1-42) was obtained from Genosphere Biotechnologies (Paris, France). Gentamicin sulfate was purchased from Sigma (Zwijndrecht, The Netherlands). Iscove's modified Dulbecco's medium was obtained from Gibco Life Technologies (Breda, the Netherlands). Ciprofloxacin was bought from Bayer Pharmaceuticals (Mijdrecht, The Netherlands) and Ficoll-Paque from Pharmacia Biotech (Uppsala, Sweden). MIP-1α, MIP-1β and MCP-1 neutralizing antibodies were obtained from PeproTech (London, UK).

Amyloid-β

Lyophilized synthetic Aβ(1-42) peptide, initially dissolved at a concentration of 1 mM in sterile double-distilled pyrogen-free water, was aliquoted and stored at –20 °C.

Cells

Monocytes were derived from peripheral blood mononuclear cells by Ficoll-Paque density gradients and purified by centrifugal elutriation as described previously (17). Cells were seeded at a concentration of 2 x 10^6 cells / ml in Teflon Erlenmeyer flasks (Nalgene, USA) and grown as suspension at 37 °C in a humidified atmosphere of 5% CO^2 / 95% air. Culture medium was composed of Iscove's modified Dulbecco's medium supplemented with 10% (v/v) heat-inactivated human AB serum, 2 mM L-glutamine, 19 mM sodium bicarbonate, 10 μg/ml gentamicin and 0.5 μg/ml ciprofloxacin. After 7 days, monocyte-

derived macrophages (MDMs) were harvested from the flasks, washed with Hanks' balanced salt solution (HBSS) and used for experiments.

Monocytes used for transwell experiments were isolated as described and labeled for 30 min with 10 µM BCECF AM (2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethylester, Molecular Probes, USA). After washing $1x10^6$ monocytes were put in the upper compartment of a Costar Transwell cell culture chamber.

The human astrocytoma cell line U373 MG was obtained from ATCC and was grown in a humidified atmosphere of 5% CO^2 / 95% air in Dulbecco's modified eagle medium / Nutrient mixture Ham F-10 (Gibco Life Technologies), 1:1, supplemented with 10% (v/v) fetal calf serum, 10 IU / ml penicillin, 10 IU / ml streptomycin and 1.2 mM L-glutamine.

The human neuronal cell line SK-N-MC was obtained from ATCC and was grown in a humidified atmosphere of 5% CO^2 / 95% air in Eagle's minimal essential medium, supplemented with 10% (v/v) fetal calf serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 19 mM sodium bicarbonate, 5 µg/ml ciprofloxacin and 10 µg/ml gentamicin.

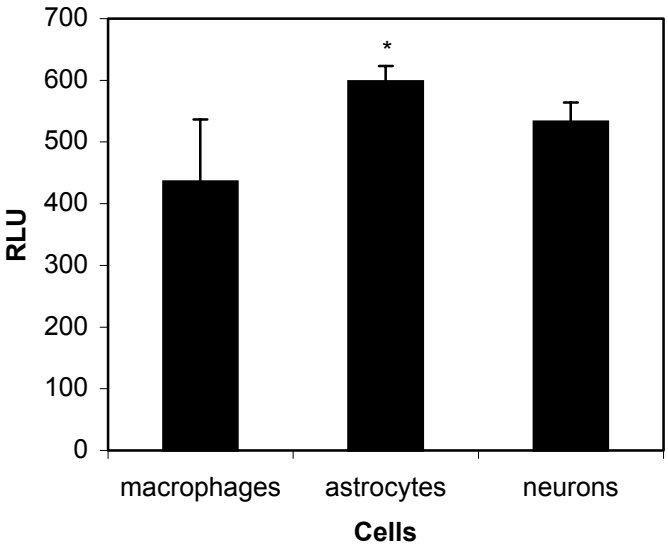

Figure 1

Chemotaxis of monocytes towards unstimulated macrophages, astrocytes and neurons. Cells were incubated for 24 h in the lower compartment of a Costar Transwell cell culture chamber after which labeled monocytes in the upper compartment separated by a microporous membrane with a pore size of 5 µM were allowed to migrate. After 1 h the fluorescence was measured in the lower compartment using an excitation of 485 nm and an emission of 530 nm. *, statistically significant difference in chemotaxis of monocytes towards unstimulated astrocytes from unstimulated macrophages.

Chemotaxis

Costar Transwell cell culture chambers (Costar, The Netherlands) were used for measuring the chemotaxis of labeled monocytes in the upper compartment towards non- and Aβ-stimulated macrophages, astrocytes or neurons or cocultures their of in the lower compartment. 1 x 10^6 cells were stimulated, as shown in Figures 1 to 4, for 24 h in the lower compartment using culture medium as described above, in a humidified atmosphere of 5% CO^2 / 95% air. Next, unstimulated or stimulated cells in the lower compartment were cultured with 1 x 10^6 labeled monocytes in the upper compartment separated by a microporous membrane with a pore size of 5 μM. After 1 h the fluorescence was measured in the lower compartment using an excitation of 485 nm and an emission of 530 nm.

Statistical Analysis

In all experiments Student-t test was used to compare unstimulated or Aβ(42-1)-stimulated macrophages, astrocytes or cocultures thereof and Aβ(1-42)-stimulated macrophages, astrocytes or cocultures thereof. Reported p-values are two-sided.

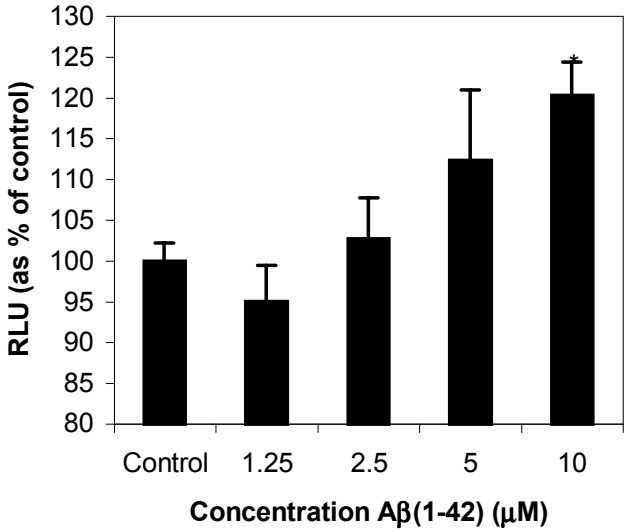

Figure 2A

Chemotaxis of monocytes towards Aβ(1-42)-stimulated macrophages. Cells were stimulated with indicated concentrations Aβ for 24 h in the lower compartment of a Costar Transwell cell culture chamber after which labeled monocytes in the upper compartment separated by a microporous membrane with a pore size of 5 μM were allowed to migrate. After 1 h the fluorescence was measured in the lower compartment using an excitation of 485 nm and an emission of 530 nm. *, statistically significant difference in chemotaxis of monocytes towards 10 μM Aβ(1-42)-stimulated macrophages compared to unstimulated macrophages.

Results

When stimulated with 10 μM Aβ(1-42), macrophages are capable of producing MIP-1α, MIP-1β and MCP-1 (Smits et al., submitted). To study whether or not these CC-chemokines are involved in the chemoattractance of other glial cells towards the neuritic plaque, first the capability of unstimulated macrophages, astrocytes and neurons to attract monocytes was tested. Astrocytes showed a significant ($p<0.01$) increased capability of attracting monocytes compared to macrophages and neurons (Fig. 1).

Next macrophages were stimulated with 1.25, 2.5, 5 and 10 μM Aβ(1-42) for 24 h after which the capability of attracting monocytes was tested. With an increasing concentration of Aβ, an increasing chemotaxis of monocytes was measured (Fig. 2A). Macrophages stimulated with 10 μM Aβ(1-42) showed a significant ($p<0.02$) increased capability of attracting monocytes when compared to unstimulated macrophages.

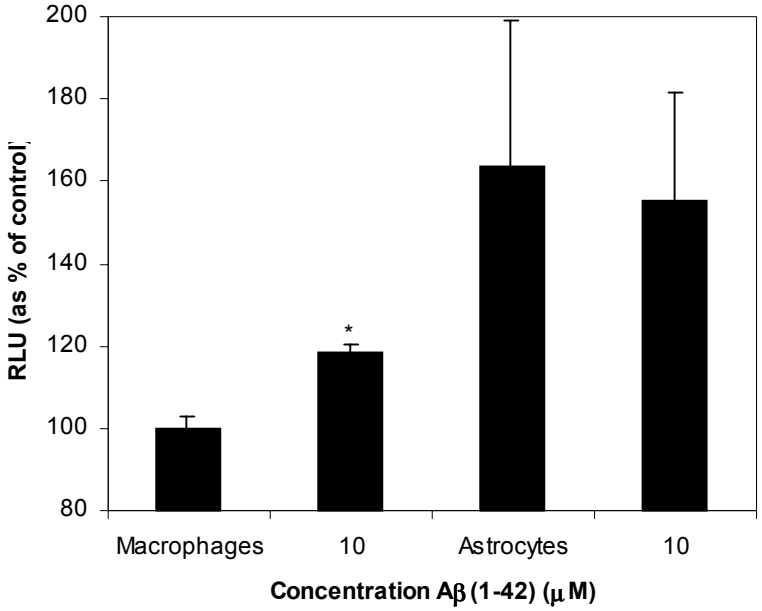

Figure 2B

Comparison of the chemotaxis of monocytes towards unstimulated and Aβ(1-42)-stimulated macrophages and astrocytes. Unstimulated and 10 μM Aβ(1-42) stimulated cells were incubated for 24 h in the lower compartment of a Costar Transwell cell culture chamber after which labeled monocytes in the upper compartment separated by a microporous membrane with a pore size of 5 μM were allowed to migrate. After 1 h the fluorescence was measured in the lower compartment using an excitation of 485 nm and an emission of 530 nm. *, statistically significant difference in chemotaxis of monocytes towards Aβ(1-42)-stimulated macrophages compared to unstimulated macrophages.

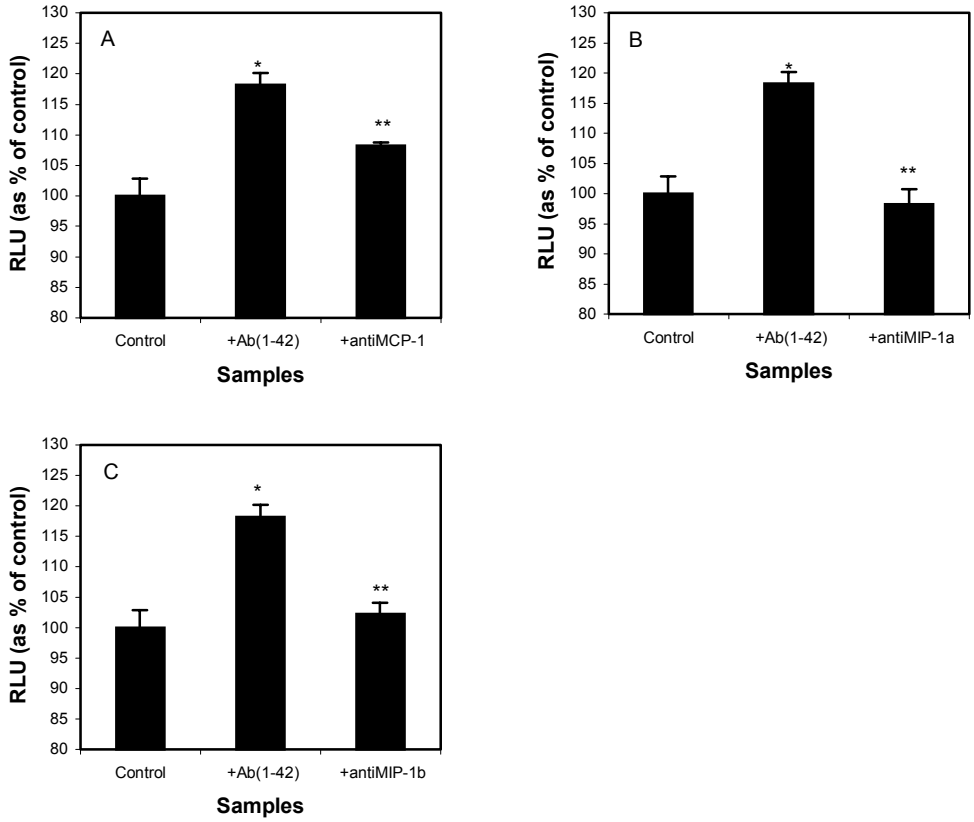

Figure 3

Chemotaxis of monocytes towards unstimulated macrophages, 10 μM Aβ(1-42)-stimulated macrophages and 10 μM Aβ(1-42)-stimulated macrophages incubated with anti-MCP-1 (A), anti-MIP-1α (B) or anti-MIP-1β (C) antibody. Cells were incubated for 24 h in the lower compartment of a Costar Transwell cell culture chamber after which labeled monocytes in the upper compartment separated by a microporous membrane with a pore size of 5 μM were allowed to migrate. After 1 h the fluorescence was measured in the lower compartment using an excitation of 485 nm and an emission of 530 nm. *, statistically significant difference in chemotaxis of monocytes towards Aβ(1-42)-stimulated macrophages compared to unstimulated macrophages. **, statistically significant difference in chemotaxis of monocytes towards Aβ(1-42)-stimulated macrophages incubated with anti-MCP-1 (A), anti-MIP-1α (B) or anti-MIP-1β (C) antibody compared to Aβ(1-42)-stimulated macrophages.

Astrocytes are, when stimulated with 10 μM Aβ(1-42), capable of producing MCP-1 (Smits et al., submitted). As shown in Fig. 1 unstimulated astrocytes already attract more monocytes than unstimulated macrophages do, so the next question was whether stimulated astrocytes attract more monocytes than unstimulated astrocytes like seen in macrophage cultures. Therefore non- and Aβ(1-42)-stimulated macrophages were compared with non-

and 10 μM Aβ(1-42)-stimulated astrocytes. As shown in Fig. 2B 10 μM Aβ(1-42) is able to activate macrophages is such a way that enhanced chemotaxis of monocytes occurs. Unstimulated astrocytes showed an even higher capability of attracting monocytes. Stimulation of astrocytes with 10 μM Aβ(1-42) did not evoke a chemotactic response.

To identify the CC-chemokines involved in the attractance of monocytes towards macrophages and astrocytes, macrophages and astrocytes were stimulated with Aβ(1-42) and incubated for 24 h together with neutralizing antibodies directed against either MIP-1α, MIP-1β or MCP-1 using concentrations according to the manufacture's instructions. Using anti-MCP-1 antibody, Fig. 3A shows that there is a significant ($p<0.03$) decrease in the chemotaxis of monocytes. Fig. 3B shows that there is an even stronger decrease of the chemotaxis when using anti-MIP-1α antibody ($p<0.01$). The decrease in chemotaxis of monocytes is not that strong ($p<0.02$) when using a neutralizing MIP-1β antibody (Fig. 3C). The effect of Aβ(1-42) is almost completely abolished when compared to the effect of the MIP-1β neutralizing antibody.

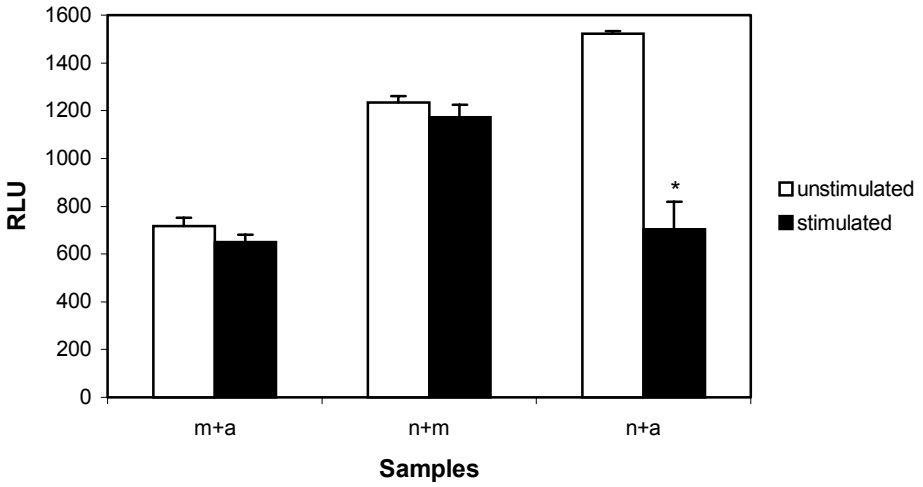

Figure 4

Chemotaxis of monocytes towards unstimulated and stimulated with 10 μM Aβ(1-42) cocultured macrophages and astrocytes (m+a), neurons and macrophages (n+m) and neurons and astrocytes (n+a). Cells were incubated for 24 h in the lower compartment of a Costar Transwell cell culture chamber after which labeled monocytes in the upper compartment separated by a microporous membrane with a pore size of 5 μM were allowed to migrate. After 1 h the fluorescence was measured in the lower compartment using an excitation of 485 nm and an emission of 530 nm. *, statistically significant difference in chemotaxis of monocytes towards Aβ(1-42)-stimulated cocultures of neurons and astrocytes compared to unstimulated cocultures of neurons and astrocytes.

Neuritic plaques are not just surrounded by only microglia and degenerating neurons or only astrocytes and degenerating neurons, the three cells are present at the same time. Therefore macrophages and astrocytes, macrophages and neurons and astrocytes and neurons were cocultured and exposed to 10 μM Aβ(1-42) (Fig. 4). When coculturing macrophages and astrocytes, more monocytes are attracted to the lower compartment of the Transwell then when these cells are cultured apart (Fig. 1). Aβ(1-42) doesn't influence the chemotaxis when compared to unstimulated cocultures. Coculturing neurons and macrophages increases the chemotaxis of monocytes substantial (Fig. 4). Here Aβ(1-42) doesn't influence the chemotaxis either. We saw an even higher chemotaxis of monocytes when coculturing neurons and astrocytes (Fig. 4). When incubated with Aβ(1-42) the chemotaxis decreased dramatically when compared to unstimulated cocultures ($p<0.01$).

Discussion

In AD neuronal injury is partially caused by neuritic plaques. Unraveling the cascade of events leading to the degeneration of neurons is however very complicated. Next to Aβ being neurotoxic itself, it has been shown by different groups that macrophages and astrocytes are able to produce pro- and anti-inflammatory molecules upon stimulation with Aβ (18-23). The presence of these cells makes the situation much more difficult. Macrophages are obviously not able to clear the plaque. This might be due to the early presence of astrocytes around plaques. Astrocytes are already present near diffuse plaques and because of this early presence they probably protect the aggregated Aβ protein against macrophages at a later stage. Once present macrophages turn out to enhance neurotoxicity.

The results presented here show that macrophages are able to attract monocytes and that Aβ-stimulated macrophages show a significant increased ability to attract monocytes. The use of neutralizing antibodies showed particularly MIP-1α and MIP-1β being responsible. MCP-1 antibodies revealed a modest but significant decrease in the chemotaxis of monocytes. Upon Aβ stimulation macrophages are not only able to produce and express MIP-1α and MIP-1β but are, together with MCP-1, produced in such amounts that monocytes are attracted. Interestingly astrocytes are capable of attracting an even higher amount of monocytes. Here, Aβ had no influence. Unstimulated neurons also showed the capability of attracting monocytes.

Coculturing macrophages and astrocytes revealed a slightly higher chemotaxis of monocytes compared to astrocytes alone. It is however not as high as the result from macrophages and astrocytes alone counted together. The presence of chemokine receptors on the surface of astrocytes and macrophages could be an explanation. By binding chemokines they might effectively neutralize these chemokines, in other words disable them from being chemotactic. Aβ is not able to induce an even higher production of chemotactic molecules. There is no significant difference between unstimulated and Aβ-stimulated cocultures.

The highest amount of monocytes were attracted by cocultures of unstimulated astrocytes and neurons. Remarkably the chemotaxis dropped sharply when these cells were

incubated with Aβ(1-42). In the unstimulated situation astrocytes and neurons apparently stimulate each other in the production of chemoattractive molecules, which in turn are able to attract monocytes. The addition of Aβ doesn't change the response of astrocytes, as seen in Fig. 2B. So the result might be due to the effect of Aβ on the neuron. Neurons appear to change directly upon binding of Aβ since only 24 h after addition of Aβ chemotaxis was measured. It is also possible that upon Aβ stimulation the astrocyte is stimulated to produce a factor that inhibits the production of chemokines by the neurons. In both ways the chemokine involved has to bind to the receptors expressed on the monocyte. Monocytes are reported to express the CC-chemokine receptors CCR1, CCR2 and CCR5, the CX_3C receptor and the CXCR4 receptor.

Coculturing unstimulated macrophages and neurons revealed a remarkable increase in the chemotaxis of monocytes when compared to induction of chemotaxis by one of these cells alone. In contrast to cocultured astrocytes and neurons, incubating these cocultures with Aβ brought no significant difference in the chemotaxis. So the effect of Aβ on the chemotaxis of monocytes in cocultured neurons and astrocytes might not be due to the effect of Aβ on the neuron itself. It seems more likely that a factor produced by the astrocytes is responsible. To resolve the question whether the decrease in chemotaxis in cocultured astrocytes and neurons is due to the effect of Aβ on the neuron or due to a inhibiting factor produced by astrocytes remains to be investigated.

When comparing the results of the cocultures with the *in-vivo* situation one should have in mind that in the brain the formation of neuritic plaques is a process of many years and that during this period the involved cells interact with Aβ and each other. Obviously it is very difficult to mimic this situation in a relevant human *in-vitro* model.

Acknowledgements

This work was supported by the Dutch Organization for Scientific Research (NWO) grant 903-51-141 to H.S.L.M. Nottet.

References

1. Behl, C., J. B. Davis, R. Lesley, and D. Schubert. 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77:817.
2. Pike, C. J., A. J. Walencewicz, C. G. Glabe, and C. W. Cotman. 1991. Aggregation-related toxicity of synthetic beta-amyloid protein in hippocampal cultures. Eur.J.Pharmacol. 207:367.
3. Yaar, M., S. Zhai, P. F. Pilch, S. M. Doyle, P. B. Eisenhauer, R. E. Fine, and B. A. Gilchrest. 1997. Binding of beta-amyloid to the p75 neurotrophin receptor induces apoptosis. A possible mechanism for Alzheimer's disease. J.Clin.Invest. 100:2333.
4. Yankner, B. A., L. K. Duffy, and D. A. Kirschner. 1990. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. Science 250:279.

5. Akiyama, H., S. Barger, S. Barnum, B. Bradt, J. Bauer, G. M. Cole, N. R. Cooper, P. Eikelenboom, M. Emmerling, B. L. Fiebich, C. E. Finch, S. Frautschy, W. S. Griffin, H. Hampel, M. Hull, G. Landreth, L. Lue, R. Mrak, I. R. Mackenzie, P. L. McGeer, M. K. O'Banion, J. Pachter, G. Pasinetti, C. Plata-Salaman, J. Rogers, R. Rydel, Y. Shen, W. Streit, R. Strohmeyer, I. Tooyoma, F. L. Van Muiswinkel, R. Veerhuis, D. Walker, S. Webster, B. Wegrzyniak, G. Wenk, and T. Wyss-Coray. 2000. Inflammation and Alzheimer's disease. Neurobiol.Aging 21:383.
6. Akama, K. T. and L. J. Van Eldik. 2000. Beta-amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1beta- and tumor necrosis factor-alpha (TNFalpha)-dependent, and involves a TNFalpha receptor-associated factor- and NFkappaB-inducing kinase-dependent signaling mechanism. J.Biol.Chem. 275:7918.
7. Combs, C. K., D. E. Johnson, J. C. Karlo, S. B. Cannady, and G. E. Landreth. 2000. Inflammatory mechanisms in Alzheimer's disease: inhibition of beta- amyloid-stimulated proinflammatory responses and neurotoxicity by PPARgamma agonists. J.Neurosci. 20:558.
8. McDonald, D. R., K. R. Brunden, and G. E. Landreth. 1997. Amyloid fibrils activate tyrosine kinase-dependent signaling and superoxide production in microglia. J.Neurosci. 17:2284.
9. McDonald, D. R., M. E. Bamberger, C. K. Combs, and G. E. Landreth. 1998. beta-Amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. J.Neurosci. 18:4451.
10. Klegeris, A. and P. L. McGeer. 2000. Interaction of various intracellular signaling mechanisms involved in mononuclear phagocyte toxicity toward neuronal cells. J.Leukoc.Biol. 67:127.
11. Mrak, R. E., J. G. Sheng, and W. S. Griffin. 1996. Correlation of astrocytic S100 beta expression with dystrophic neurites in amyloid plaques of Alzheimer's disease. J.Neuropathol.Exp.Neurol. 55:273.
12. Rozemuller, J. M., P. Eikelenboom, S. T. Pals, and F. C. Stam. 1989. Microglial cells around amyloid plaques in Alzheimer's disease express leucocyte adhesion molecules of the LFA-1 family. Neurosci.Lett. 101:288.
13. Rozemuller, J. M., P. Eikelenboom, and F. C. Stam. 1986. Role of microglia in plaque formation in senile dementia of the Alzheimer type. An immunohistochemical study. Virchows Arch.B Cell Pathol.Incl.Mol.Pathol. 51:247.
14. McGeer, P. L., S. Itagaki, H. Tago, and E. G. McGeer. 1987. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci.Lett. 79:195.
15. Xia, M. Q. and B. T. Hyman. 1999. Chemokines/chemokine receptors in the central nervous system and Alzheimer's disease. J.Neurovirol. 5:32.
16. Bacon, K. B. and J. K. Harrison. 2000. Chemokines and their receptors in neurobiology: perspectives in physiology and homeostasis. J.Neuroimmunol. 104:92.
17. Boven, L. A., L. Gomes, C. Hery, F. Gray, J. Verhoef, P. Portegies, M. Tardieu, and H. S. Nottet. 1999. Increased peroxynitrite activity in AIDS Dementia Complex: implications for neuropathogenesis of HIV-1 infection. J.Immunol. 162:4319.
18. Ariga, T. and R. K. Yu. 1999. GM1 inhibits amyloid beta-protein-induced cytokine release. Neurochem.Res. 24:219.
19. Klegeris, A. and P. L. McGeer. 1997. beta-amyloid protein enhances macrophage production of oxygen free radicals and glutamate. J.Neurosci.Res. 49:229.

20. Klegeris, A., D. G. Walker, and P. L. McGeer. 1994. Activation of macrophages by Alzheimer beta amyloid peptide. Biochem.Biophys.Res.Commun. 199:984.
21. Meda, L., C. Bonaiuto, P. Baron, L. Otvos, Jr., F. Rossi, and M. A. Cassatella. 1996. Priming of monocyte respiratory burst by beta-amyloid fragment (25-35). Neurosci.Lett. 219:91.
22. Meda, L., S. Bernasconi, C. Bonaiuto, S. Sozzani, D. Zhou, L. Otvos, Jr., A. Mantovani, F. Rossi, and M. A. Cassatella. 1996. Beta-amyloid (25-35) peptide and IFN-gamma synergistically induce the production of the chemotactic cytokine MCP-1/JE in monocytes and microglial cells. J.Immunol. 157:1213.
23. Smits, H. A., A. J. van Beelen, N. M. de Vos, A. Rijsmus, B. T. van der, J. Verhoef, F. L. Van Muiswinkel, and H. S. Nottet. 2001. Activation of human macrophages by amyloid-beta is attenuated by astrocytes. J.Immunol. 166:6869.

7

Discussion

Ever since Glenner and Wong (1) found Aβ to be the major component of neuritic plaques and McGeer et al. found activated microglia to be concentrated around these plaques (2) one has questioned the cascade of events leading to neuronal death. The research presented in this thesis addresses several issues of this cascade of events. Using primary human monocyte-derived macrophages as model cells for microglia and primary human adult astrocytes as well as U373 astrocytoma's as model cells for astrocytes the role of these cells in the inflammatory process and the continuation of this process was investigated. In summary the results presented in chapter 2 show the effect of Aβ(25-35) on the binding of Aβ(1-42) to macrophages and the consequent superoxide anion production. Aβ(25-35) markedly enhanced the effect of Aβ(1-42). Chapter 3 shows that both ERK 1/2 and p38 MAPK signal transduction pathways as well as PKC are involved in the amyloid-β-stimulated superoxide anion production in macrophages. In contrast, only ERK 1/2 MAPK seems to be involved in TNF-α production, questioning the connection between PKC and ERK 1/2 activation. Chapter 4 shows that Aβ is able to induce TNF-α and superoxide anion production in macrophages and that astrocytes do not produce these inflammatory mediators. In cocultures however a decreased production of these putative neurotoxins was seen, probably due to the binding of Aβ to the astrocytes. Aβ is able to induce NF-κB activation in astrocytes. However, due to the already high NF-κB activity in macrophages, an additional effect of Aβ could not be detected. Chapter 5 shows the ability of Aβ(1-42) to stimulate the production, and expression of MIP-1α and MIP-1β in macrophages and MCP-1 in astrocytes and in cocultures their of. In chapter 6 the effectiveness of these CC-chemokines to attract monocytes was investigated. Using neutralizing antibodies it was shown that Aβ-stimulated macrophages produce MIP-1α, MIP-1β and MCP-1 in such amounts that they attract monocytes.

Overall the results show that there are many elements that may be involved in the course of inflammation. One way or another, the cascade of events will lead to a local inflammatory reaction and although inflammatory reactions are almost always a secondary reaction, it is as important as the mechanism that gave rise to it. The inflammatory elements must be produced by local cells because in Alzheimer's disease the blood-brain barrier is intact. In case of the brain, these are the neurons, astrocytes, microglia and oligodendrocytes. Of these four cell types the latter is not involved in the pathology of the neuritic plaque in Alzheimer's disease. A first question then might be why are microglia and astrocytes located around these plaques? As simple as the question is, it is very hard to answer. A more important question might be how do these cells get there? Answering this question could resolve a lot of problems. Chapter 5 and 6 show that CC-chemokines very likely play an important role. As important as these chemokines might be, produced by glial cells and attracting other glial cells, it just shows that glial cells are likely to be responsible for the continuation of the inflammatory process. Trying to intervene in the chemotaxis of glial cells towards the plaque might indeed slow the inflammatory response and therefore the neuronal loss. Problem is that chemokines and other proteins responsible for this chemotaxis are not unique in that they are only functional around the plaque. Therefore, preventing or slowing chemotaxis of glial cells through this mechanism is at this moment

not an option. Identifying a protein unique in attracting glial cells towards the plaque might bring a solution.

CC-chemokines might play an important role in attracting more glial cells towards the neuritic plaque, it still doesn't answer the question why glial cells are located around plaques in the first place, does it? Yet, diffuse plaques are reported to be surrounded by a few reactive astrocytes (3) and not by microglia (4). Furthermore, astrocytes are stated to express CC-chemokine receptors CCR1, CCR2 and CCR5, making them able to bind MCP-1, MIP-1α and MIP-1β (5,6). Chapter 5 shows that astrocytes are able to produce MCP-1 upon incubation with Aβ. Thus astrocytes present around a diffuse plaque are able to attract other astrocytes and in this way may be responsible for the large amount of reactive astrocytes that are eventually present around the neuritic plaque. If the astrocytes around the diffuse plaques produce MCP-1 it apparently does not attract microglia. This might be due to the fact that macrophages/microglia produce high amounts of MCP-1 themselves and therefore are not as reactive towards MCP-1 as astrocytes do. It is not known how the few astrocytes around the diffuse plaque are attracted to this plaque. One possible answer might be that Aβ seems to have chemotactic properties itself (7).

Studying inflammation in Alzheimer's disease is made difficult by the obvious fact that human brains are not as accessible as peripheral organs are. There are two ways to overcome this problem. One way is the use of animal models, the other way is the use of *in-vitro* systems. The use of both methods may be required in understanding the role of the astrocyte in the neuritic plaque. This role seems contradictory. On one hand they appear to prevent macrophages/microglia from clearing the Aβ deposit and, as shown in chapter 4, attenuate the Aβ-induced inflammatory response of these macrophages/microglia. On the other hand they are, *in-vitro* and in-situ, capable of producing numerous inflammatory components themselves (for a list see (8)). In the use of animal models the overexpression of cytokines in transgenic mice using astrocyte-specific promoters revealed profound biological influences of these molecules in the CNS. Using the GFAP promoter it has been shown that for example the overexpression of TNF-α leads to chronic and severe inflammation in the CNS (9,10). It may be apparent that this use of astrocyte-specific promoters doesn't mean that cytokine mechanisms aren't that important in microglia. Microglia clearly express cytokines and chemokines, but are so closely related to peripheral macrophages that it is difficult to imagine a microglia-specific promoter that would not lead to expression of a cytokine or chemokine different than macrophages would do throughout the body. Other animal models are based on human FAD APP and/or FAD PS-1 mutations. Brains of mice carrying both mutant genes develop AD-like deposits composed of Aβ which at an later age are surrounded by activated microglia and astrocytes (11-13). Problems with these animal models are the lack of neurofibrillary tangles and, more important, little or no neuronal loss. However, the development of these models is very important. More complex models may in the future be essential in the testing of therapeutic agents. Developing therapeutic agents on basis of the inflammatory process in AD, thereby

using complex animal models, requires every part of the process to be comprehendible. *In-vitro* systems are ideal in that simplifying complex models is rather easy. The use of *in-vitro* studies in this thesis partially clarified the role of the macrophage/microglia in the production of some pro-inflammatory molecules. Shifting the balance between pro- and anti-inflammatory molecules to the latter might be a good alternative in slowing Alzheimer's disease.

Another alternative and may be the only alternative in stopping or preventing the disease is interfering at the very beginning. β-Secretase and γ-secretase are essential in cleaving APP, thereby giving rise to Aβ. By neutralizing these secretases very early in the disease, Aβ might be prevented from coming into existence. There are however some problems that need to be addressed. First the identification of the secretases, only recently five different groups using three different approaches independently found BACE (also known as Asp2 or Memapsin2) to be β-secretase. In addition, it has been shown that BACE is a membrane-bound aspartyl protease that fulfils all possible criteria for β-secretase (14-18). The identification of γ-secretase may be even more important, since this enzyme is responsible for the Aβ releasing cut. Although there is strong evidence for presenilin-1 being γ-secretase (19-22), the working mechanism is still not clear. There are some reports suggesting that γ-secretase most likely is a complex of proteins of which presenilin-1 is a part of (23-25). Another important issue is whether these secretases have other functions next to processing APP. Presenilins are known to be important in processing Notch for example (26-28). Whether or not these secretases are the ideal targets for drug development, in the development of any drug two more problems need to be solved. First in AD the blood-brain barrier is intact. Drugs being developed must be able to pass this barrier. The second problem is age of onset. For the neuritic plaque to develop and to be neurotoxic takes several years to decades. Therefore people who will get AD when at an older age already need this drug at an early age. In this way the plaque is being prevented from developing. To administer a drug to a person who will get AD at a later age requires the ability to diagnose AD at an early age. And at the moment this is not possible. Therefore more preclinical and clinical research is warranted towards drug development for AD.

References

1. Glenner, G. G. and C. W. Wong. 1984. Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. Biochem.Biophys.Res.Commun. 122:1131.
2. McGeer, P. L., S. Itagaki, H. Tago, and E. G. McGeer. 1987. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci.Lett. 79:195.
3. Mrak, R. E., J. G. Sheng, and W. S. Griffin. 1996. Correlation of astrocytic S100 beta expression with dystrophic neurites in amyloid plaques of Alzheimer's disease. J.Neuropathol.Exp.Neurol. 55:273.

4. Sasaki, A., H. Yamaguchi, A. Ogawa, S. Sugihara, and Y. Nakazato. 1997. Microglial activation in early stages of amyloid beta protein deposition. Acta Neuropathol.(Berl) 94:316.
5. Andjelkovic, A. V., D. Kerkovich, J. Shanley, L. Pulliam, and J. S. Pachter. 1999. Expression of binding sites for beta chemokines on human astrocytes. Glia 28:225.
6. Bacon, K. B. and J. K. Harrison. 2000. Chemokines and their receptors in neurobiology: perspectives in physiology and homeostasis. J.Neuroimmunol. 104:92.
7. Davis, J. B., H. F. McMurray, and D. Schubert. 1992. The amyloid beta-protein of Alzheimer's disease is chemotactic for mononuclear phagocytes. Biochem.Biophys.Res.Commun. 189:1096.
8. Akiyama, H., S. Barger, S. Barnum, B. Bradt, J. Bauer, G. M. Cole, N. R. Cooper, P. Eikelenboom, M. Emmerling, B. L. Fiebich, C. E. Finch, S. Frautschy, W. S. Griffin, H. Hampel, M. Hull, G. Landreth, L. Lue, R. Mrak, I. R. Mackenzie, P. L. McGeer, M. K. O'Banion, J. Pachter, G. Pasinetti, C. Plata-Salaman, J. Rogers, R. Rydel, Y. Shen, W. Streit, R. Strohmeyer, I. Tooyoma, F. L. Van Muiswinkel, R. Veerhuis, D. Walker, S. Webster, B. Wegrzyniak, G. Wenk, and T. Wyss-Coray. 2000. Inflammation and Alzheimer's disease. Neurobiol.Aging 21:383.
9. Akassoglou, K., L. Probert, G. Kontogeorgos, and G. Kollias. 1997. Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. J.Immunol. 158:438.
10. Stalder, A. K., M. J. Carson, A. Pagenstecher, V. C. Asensio, C. Kincaid, M. Benedict, H. C. Powell, E. Masliah, and I. L. Campbell. 1998. Late-onset chronic inflammatory encephalopathy in immune-competent and severe combined immune-deficient (SCID) mice with astrocyte-targeted expression of tumor necrosis factor. Am.J.Pathol. 153:767.
11. Matsuoka, Y., M. Picciano, B. Malester, J. LaFrancois, C. Zehr, J. M. Daeschner, J. A. Olschowka, M. I. Fonseca, M. K. O'Banion, A. J. Tenner, C. A. Lemere, and K. Duff. 2001. Inflammatory responses to amyloidosis in a transgenic mouse model of Alzheimer's disease. Am.J.Pathol. 158:1345.
12. Bornemann, K. D., K. H. Wiederhold, C. Pauli, F. Ermini, M. Stalder, L. Schnell, B. Sommer, M. Jucker, and M. Staufenbiel. 2001. Abeta-induced inflammatory processes in microglia cells of APP23 transgenic mice. Am.J.Pathol. 158:63.
13. Murphy, G. M., Jr., F. Zhao, L. Yang, and B. Cordell. 2000. Expression of macrophage colony-stimulating factor receptor is increased in the AbetaPP(V717F) transgenic mouse model of Alzheimer's disease. Am.J.Pathol. 157:895.
14. Vassar, R., B. D. Bennett, S. Babu-Khan, S. Kahn, E. A. Mendiaz, P. Denis, D. B. Teplow, S. Ross, P. Amarante, R. Loeloff, Y. Luo, S. Fisher, J. Fuller , S. Edenson, J. Lile, M. A. Jarosinski, A. L. Biere, E. Curran, T. Burgess, J. C. Louis, F. Collins, J. Treanor, G. Rogers, and M. Citron. 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286:735.
15. Yan, R., M. J. Bienkowski, M. E. Shuck, H. Miao, M. C. Tory, A. M. Pauley, J. R. Brashier, N. C. Stratman, W. R. Mathews, A. E. Buhl, D. B. Carter, A. G. Tomasselli, L. A. Parodi, R. L. Heinrikson, and M. E. Gurney. 1999. Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature 402:533.
16. Sinha, S., J. P. Anderson, R. Barbour, G. S. Basi, R. Caccavello, D. Davis, M. Doan, H. F. Dovey, N. Frigon, J. Hong, K. Jacobson-Croak, N. Jewett, P. Keim, J. Knops, I. Lieberburg, M. Power, H. Tan, G. Tatsuno, J. Tung, D. Schenk, P. Seubert, S. M. Suomensaari, S. Wang, D. Walker, and V. John. 1999. Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature 402:537.

17. Hussain, I., D. Powell, D. R. Howlett, D. G. Tew, T. D. Meek, C. Chapman, I. S. Gloger, K. E. Murphy, C. D. Southan, D. M. Ryan, T. S. Smith, D. L. Simmons, F. S. Walsh, C. Dingwall, and G. Christie. 1999. Identification of a novel aspartic protease (Asp 2) as beta-secretase. Mol.Cell Neurosci. 14:419.
18. Lin, X., G. Koelsch, S. Wu, D. Downs, A. Dashti, and J. Tang. 2000. Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-amyloid precursor protein. Proc.Natl.Acad.Sci.U.S.A 97:1456.
19. Kimberly, W. T., W. Xia, T. Rahmati, M. S. Wolfe, and D. J. Selkoe. 2000. The transmembrane aspartates in presenilin 1 and 2 are obligatory for gamma-secretase activity and amyloid beta-protein generation. J.Biol.Chem. 275:3173.
20. Xia, W., W. J. Ray, B. L. Ostaszewski, T. Rahmati, W. T. Kimberly, M. S. Wolfe, J. Zhang, A. M. Goate, and D. J. Selkoe. 2000. Presenilin complexes with the C-terminal fragments of amyloid precursor protein at the sites of amyloid beta-protein generation. Proc.Natl.Acad.Sci.U.S.A 97:9299.
21. Yu, G., F. Chen, M. Nishimura, H. Steiner, A. Tandon, T. Kawarai, S. Arawaka, A. Supala, Y. Q. Song, E. Rogaeva, E. Holmes, D. M. Zhang, P. Milman, P. Fraser, C. Haass, and P. George-Hyslop. 2000. Mutation of conserved aspartates affect maturation of presenilin 1 and presenilin 2 complexes. Acta Neurol.Scand.Suppl 176:6-11.:6.
22. Li, Y. M., M. Xu, M. T. Lai, Q. Huang, J. L. Castro, J. DiMuzio-Mower, T. Harrison, C. Lellis, A. Nadin, J. G. Neduvelil, R. B. Register, M. K. Sardana, M. S. Shearman, A. L. Smith, X. P. Shi, K. C. Yin, J. A. Shafer, and S. J. Gardell. 2000. Photoactivated gamma-secretase inhibitors directed to the active site covalently label presenilin 1. Nature 405:689.
23. Yu, G., M. Nishimura , S. Arawaka, D. Levitan, L. Zhang, A. Tandon, Y. Q. Song, E. Rogaeva, F. Chen, T. Kawarai, A. Supala, L. Levesque, H. Yu, D. S. Yang, E. Holmes, P. Milman, Y. Liang, D. M. Zhang, D. H. Xu, C. Sato, E. Rogaev, M. Smith, C. Janus, Y. Zhang, R. Aebersold, L. S. Farrer, S. Sorbi, A. Bruni, P. Fraser, and P. George-Hyslop. 2000. Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. Nature 407:48.
24. Vassar, R. and M. Citron. 2000. Abeta-generating enzymes: recent advances in beta- and gamma-secretase research. Neuron 27:419.
25. Schenk, D. 2000. Alzheimer's disease. A partner for presenilin. Nature 407:34.
26. Zhang, D. M., D. Levitan, G. Yu, M. Nishimura, F. Chen, A. Tandon, T. Kawarai, S. Arawaka, A. Supala, Y. Q. Song, E. Rogaeva, Y. Liang, E. Holmes, P. Milman, C. Sato, L. Zhang, and P. George-Hyslop. 2000. Mutation of the conserved N-terminal cysteine (Cys92) of human presenilin 1 causes increased A beta42 secretion in mammalian cells but impaired Notch/lin-12 signalling in C. elegans. Neuroreport 11:3227.
27. Berezovska, O., C. Jack, P. McLean, J. C. Aster, C. Hicks, W. Xia, M. S. Wolfe, W. T. Kimberly, G. Weinmaster, D. J. Selkoe, and B. T. Hyman. 2000. Aspartate mutations in presenilin and gamma-secretase inhibitors both impair notch1 proteolysis and nuclear translocation with relative preservation of notch1 signaling. J.Neurochem. 75:583.
28. Selkoe, D. J. and M. S. Wolfe. 2000. In search of gamma-secretase: presenilin at the cutting edge. Proc.Natl.Acad.Sci.U.S.A 97:5690.

Nederlandse Samenvatting

De meest voorkomende vorm van dementie is de ziekte van Alzheimer. Kenmerkend is de steeds erger wordende vergeetachtigheid. Deze vergeetachtigheid wordt veroorzaakt door het afsterven van hersencellen. In tegenstelling tot veel andere cellen in het lichaam worden deze hersencellen, met de naam neuronen, niet vervangen na het afsterven. Blijkbaar zijn de neuronen die afsterven betrokken bij het geheugen en treedt na het afsterven van veel neuronen geheugenverlies op.

Een heel specifiek kenmerk in de hersenen van patiënten die lijden aan de ziekte van Alzheimer is de plaque. Deze plaque is een opeenhoping van een eiwit met de naam amyloid-beta (Aβ). Rondom deze plaque vindt het afsterven van neuronen plaats. Er is echter nog een belangrijke component. Het is gebleken dat deze plaques omringd worden door niet-neuronale hersencellen. Deze gliale cellen, genaamd astrocyt en microglia, liggen met velen rondom de plaque. De centrale vraag in dit proefschrift is: wat is de rol van de microglia en de astrocyt in het afsterven van neuronen?

De microglia, ook wel hersenmacrofaag genoemd, is in staat veel verschillende toxische stoffen te maken. Een van die stoffen is het zuurstofradicaal. Hoofdstuk 2 laat zien dat door binding van het eiwit Aβ aan de macrofaag, deze macrofaag gestimuleerd wordt in de productie van deze zuurstofradicalen. Eerder onderzoek heeft al uitgewezen dat verschillende onderdelen van het eiwit Aβ verantwoordelijk zijn voor verschillende functies van het eiwit. In hoofdstuk 2 is de macrofaag ook nog gestimuleerd met een mengsel van Aβ zelf en een van die onderdelen. Dit onderdeel zelf kan niet aan de macrofaag binden, echter door het mengsel van Aβ en dit onderdeel maakt de macrofaag veel meer van het toxische zuurstofradicaal. De experimenten laten zien dat dit komt doordat in het mengsel meer van het Aβ eiwit bindt aan de macrofaag dan wanneer het Aβ alleen aan de macrofaag wordt toegevoegd.

Er worden door macrofagen niet alleen zuurstofradicalen gemaakt wanneer deze worden gestimuleerd met Aβ maar ook andere producten. Hoofdstuk 3 laat zien dat macrofaag stimulatie met Aβ ook resulteert in de productie van TNF-α. Om beter te kunnen begrijpen hoe het komt dat de macrofaag na toevoeging van Aβ TNF-α en superoxide radicalen gaan maken zijn er een aantal experimenten uitgevoerd waar is gekeken naar de signaalwegen in de macrofaag, die bij dit proces betrokken zouden kunnen zijn. Wanneer dit specifieke signaalwegen zijn dan zouden deze eventueel geremd kunnen worden. Dit zou een mogelijkheid kunnen zijn om de macrofaag minder toxische stoffen te laten maken wat weer tot gevolg zou kunnen hebben dat minder neuronen om de plaque afsterven. De resultaten in hoofdstuk 3 laten inderdaad zien dat bepaalde intracellulaire signaalwegen betrokken zijn bij de produktie van TNF-α en superoxide radicalen. In het laboratorium kunnen deze signaalwegen specifiek geblokeerd worden waarbij er een verminderde produktie van TNF-α en superoxide radicalen gemeten werd.

Naast microglia bevinden zich rondom de plaque ook astrocyten. De rol van deze astrocyten is echter nog niet duidelijk. Hoofstuk 4 laat zien dat ze na stimulatie met Aβ in tegenstelling tot macrofagen geen TNF-α en superoxide radicalen maken. Omdat de microglia en astrocyt rondom de plaque naast elkaar aanwezig zijn is dit in het laboratorium ook gedaan. Het samen kweken van macrofagen en astrocyten laat zien dat wanneer gestimuleerd wordt met Aβ er een duidelijk verminderde productie van TNF-α en

superoxide radicalen plaats vindt in vergelijking met macrofagen alleen. Het lijkt er dus op dat de astrocyt de macrofaag remt in de productie van deze toxische stoffen. Verdere resultaten in hoofstuk 4 laten zien dat dit waarschijnlijk komt door binding van Aβ aan de astrocyt in plaats van aan de macrofaag.

Een belangrijke vraag in het ziekteproces is hoe komen zoveel gliale cellen rondom die plaque te liggen. De dichtheid aan gliale cellen rondom de plaque is veel groter dan ergens anders in de hersenen. Nu is van sommige eiwitten bekend dat ze cellen kunnen aantrekken. Enkele van deze eiwitten worden gemaakt door gliale cellen en zijn in staat ander gliale cellen aan te trekken. Deze specifieke groep van attractieve eiwitten worden CC-chemokines genoemd. In hoofdstuk 5 is gekeken naar de mogelijkheid van macrofagen en astrocyten om na activatie met het eiwit Aβ deze CC-chemokines te maken. Zowel op RNA niveau in de cel alsmede op eiwit niveau in het kweekmedium van de macrofagen is aangetoond dat er een verhoogde productie is van twee van deze CC-chemokines, genaamd MIP-1α en MIP-1β. De astrocyt laat een verhoogde productie van een ander CC-chemokine zien, het MCP-1. Omdat deze gliale cellen in patiënten met de ziekte van Alzheimer naast elkaar aanwezig zijn, hebben we dit in het laboratorium nagebootst. Doel hiervan was om te kijken of de astrocyt invloed heeft op de productie van CC-chemokines door de macrofaag en andersom. Dit bleek niet het geval. In kweken van macrofagen en astrocyten samen is in het kweekmedium zowel MIP-1α, MIP-1β alsmede MCP-1 gevonden. Deze experimenten hebben aangetoond dat gliale cellen in staat zijn om CC-chemokines te maken. Deze attractieve eiwitten zouden een belangrijke rol kunnen spelen in het aantrekken van andere gliale cellen naar de plaque.

In hoofdstuk 6 is dit onderzoek voortgezet door te kijken of deze door de macrofaag en astrocyt gemaakte chemokines in zulke hoeveelheden worden gemaakt dat deze in staat zijn om andere cellen daadwerkelijk aan te trekken. In het laboratorium werden monocyten, de voorlopercellen van de macrofaag, gebruikt voor dit doeleinde. De resultaten laten zien dat als macrofagen worden gestimuleerd met een steeds hoger wordende concentratie Aβ er ook steeds meer migratie plaats vindt van monocyten naar de macrofaag. Dat betekent dat macrofagen niet alleen chemokines of andere attractieve eiwitten maken maar dat deze in zulke hoeveelheden gemaakt worden dat er daadwerkelijk cellen in staat zijn naar deze macrofaag toe te migreren. In een zelfde situatie hebben we gekeken naar de astrocyt. Deze maakt onder invloed van Aβ immers MCP-1. Het blijkt dat ook de astrocyt in staat is monocyten aan te trekken. Dit gebeurt echter ook al wanneer de astrocyt niet wordt gestimuleerd door Aβ. Om de factoren die belangrijk zijn bij de migratie van deze monocyten naar de macrofaag te identificeren hebben we gebruik gemaakt van antilichamen. Specifieke antilichamen voor MIP-1α, MIP-1β en MCP-1 kunnen de werking van deze blokkeren. De resultaten laten zien dat er inderdaad een verminderde tot sterk verminderde migratie plaats vindt van monocyten naar de macrofaag wanneer deze antilichamen worden gebruikt. Dat betekent dat MIP-1α, MIP-1β en MCP-1 betrokken zouden kunnen zijn bij de migratie van gliale cellen naar de plaque.

Dankwoord

Het is af. Wat een zeldzaam gevoel geeft het dit proefschrift af te sluiten. Ik had vier jaar geleden niet kunnen bedenken dat het allemaal zo voorspoedig zou verlopen. Dit heb ik aan veel mensen te danken. Deze plaats is bedoeld om al deze mensen een naam te geven.

Als eerste wil ik Prof. Verhoef bedanken. U heeft mij de mogelijkheid gegeven om op het *Eijkman-Winkler* instituut onderzoek te doen. Hans, ik ben je zeer erkentelijk voor de bijdrage die je hebt geleverd aan mijn wetenschappelijke vorming. Van de wat strakkere hand in het begin tot de vrijheid van de afgelopen anderhalf jaar heb je mij altijd goed aangevoeld. Niet in de laatste plaats door mij dan voort te duwen wanneer iets af moest of iets weg moest. Machiel, gedurende de eerste twee jaar heb jij heel wat werk voor mij verzet. Daar ben ik je zeer erkentelijk voor. We hebben heel wat discussies gehad en ik heb het altijd gewaardeerd dat je m'n directheid als positief hebt ervaren. Freek, ondanks dat we elkaar niet zoveel hebben gezien wil ik je bedanken voor de discussies die we samen hebben gehad. Alhoewel je in het begin veel meer aan het woord was dan ik.

Zonder de geweldige hulp van mijn studenten Astrid, Annemarie, Joyce, Jesse en Karla was dit boekje zeer zeker niet op tijd afgekomen. Heel erg Bedankt!

Onze eigen Neuroimmunologiegroep. Leonie, altijd anderhalf jaar verder en altijd leek het jou even gemakkelijk af te gaan. Ik heb veel van je geleerd. Cândida, je Nederlands is gevorderd! Jeena, zeer bedreven in de isolatie van rattencortexen. Tjomme, samen aan de EMSA en altijd zeer kritisch kijkende naar m'n intracellulaire experimenten. Maroeska, al die NF-κB constructen. Helaas geen publicatie opgeleverd. Niki, op dezelfde dag begonnen. Nu even iets belangrijkers. Succes met de laatste loodjes.

Al mijn kamergenoten en ex-kamergenoten: Annemarie B, Annemarie W, Ben, Camiel, Desiree, Gunnar, James, Leonie, Leontine, Mascha, Mireille, Monique, Ruzena, Steven, Wilco en Wouter. We hebben samen heel veel meegemaakt. En ook met z'n elven op één kamer gezeten. Ik heb een onvergetelijk tijd gehad. We hebben veel lol gehad maar we konden ook vertrouwelijke dingen met elkaar delen. Ik zal jullie missen.

Door al dat werk van mijn studenten heb ik zelf niet echt veel op het lab gedaan. Doordat dit met pieken en dalen ging was enige bijsturing en wat hulp als ik weer iets niet kon vinden af en toe wel nodig. Veel collega's hebben mij geholpen. Maar met name Piet is hier verantwoordelijk voor geweest. Op 406 heb ik veel geëlutrieerd. Ook dit deed ik niet altijd even regelmatig. Wanneer het weer een tijd geleden was kon ik echter altijd rekenen op Jelle.

Alle andere nog niet bij naam genoemde collega's van het EWI. De koepels, de borrels, de buitendagen, het is altijd erg gezellig geweest.

Petra en Wim en de andere mensen van de AVD. Petra, zonder jou waren de kaft van dit boekje en de EMSAs die erin staan nooit zo goed geworden. Bedankt.

Mijn paranimfen, Annemarie en Bas. In een stress periode de onmisbare hulpen geweest. Bedankt!

M'n familie, Pap, Mam, Judith, Robert, jullie zijn de basis van mijn bestaan. Zuid-Limburg wordt dichterbij.

En natuurlijk Nathalie. Mijn liefste, mijn evenwicht. Woorden zijn niet genoeg. Volgend jaar wordt bijzonder!!

Curriculum Vitae

De auteur van dit proefschrift werd op 23 augustus 1971 geboren. Na het behalen van het HAVO diploma in 1988 op de S.G. Groenewald te Stein werd vervolgens het VWO diploma 2 jaar later op het S.G. Romboutscollege te Brunssum gehaald. In september van dat zelfde jaar is de auteur begonnen met de studie Biologie aan de Katholieke Universiteit Nijmegen. Tijdens deze studie liep hij een hoofdvakstage bij de afdeling Biochemie onder leiding van Dr. T.H. van Kuppevelt en Prof. Dr. J.H. Veerkamp en een hoofdvakstage bij de afdeling Celbiologie onder leiding van Dr. H. Kraft en Prof. Dr. E.J.J. van Zoelen. In maart 1996 behaalde hij het doctoraal examen Biologie. In oktober 1997 begon hij als onderzoeker in opleiding onder begeleiding van Dr. H.S.L.M. Nottet en Prof. Dr. J. Verhoef aan het onderzoek dat heeft geleid tot dit proefschrift.

List of publications

1) Harry J. Kraft, Sietse Mosselman, **Hessel A. Smits**, Peter Hohenstein, Ester Piek, Qi Chen, Karen Artzt and Everardus J. J. van Zoelen. Oct-4 regulates alternative platelet-derived growth factor-α receptor gene promoter in human embryonal carcinoma cells (1996), *JBC*, Vol. 271, p. 12873-12878.

2) Freek L. van Muiswinkel, Sigrid F.A. Raupp, N. Machiel de Vos, **Hessel A. Smits**, Jan Verhoef, Piet Eikelenboom and Hans S.L.M. Nottet. The amino-terminus of the amyloid-β protein is critical for the cellular binding and consequent activation of the respiratory burst of human macrophages (1999), *J. Neuroimmunol*., Vol. 96, p. 121-130.

3) **Hessel A. Smits**, Leonie A. Boven, Cândida da Fonseca Pereira, Jan Verhoef and Hans S.L.M. Nottet. Role of macrophage activation in the pathogenesis of Alzheimer's disease and AIDS dementia complex (2000), *Eur. J. Clin. Invest.*, Vol. 30, p. 526-535.

4) **Hessel A. Smits**, N. Machiel de Vos, Jesse W.Y. Wat, Tjomme van der Bruggen, Jan Verhoef and Hans S.L.M. Nottet. Intracellular pathways involved in TNF-α and superoxide anion release by Aβ(1-42)-stimulated primary human macrophages (2001), *J. Neuroimmunol.*, Vol. 115, p. 144-151.

5) **Hessel A. Smits**, Astrid J. van Beelen, N. Machiel de Vos, Tjomme van der Bruggen, Annemarie Rijsmus, Jan Verhoef, Freek L. van Muiswinkel and Hans S.L.M. Nottet. Activation of human macrophages is attenuated by astrocytes (2001), *J. Immunol.*, Vol. 166, p. 6869-6876.

6) **Hessel A. Smits**, Annemarie Rijsmus, Joyce H. van Loon, Jan Verhoef, Leonie A. Boven and Hans S.L.M. Nottet. Amyloid-β-induced chemokine production in primary human macrophages and astrocytes (2001), submitted.

7) Jolita V.R.B. Hendriksen, Hans S.L.M. Nottet and **Hessel A. Smits**. Secretases as targets for drug design in Alzheimer's disease (2001), *Eur. J. Clin. Invest.*, in press.

8) **Hessel A. Smits**, Karla Rutten, Jan Verhoef and Hans S.L.M. Nottet. Migration of monocytes in response to CC-chemokine production of primary human macrophages and astrocytes (2001), submitted.

9) **Hessel A. Smits**, Freek L. van Muiswinkel, N. Machiel de Vos, Hans S.L.M. Nottet. Amyloid-β (1-42)-induced respiratory burst of primary human macrophages is enhanced in the presence of amyloid-β (25-35). In: Alzheimer's Disease and Related Disorders: Etiology, Pathogenesis and Therapeutics, pp. 457-461, 1999. K. Iqbal, et al, editors. John Wiley & Sons, England.