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Neuroinflammation: Implications for the Pathogenesis and Molecular Diagnosis of Alzheimer's Disease

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During the past few years, an increasing set of evidence has supported the major role of deregulation of the interaction patterns between glial cells and neurons in the pathway toward neuronal degeneration. Neurons and glial cells, together with brain vessels, constitute an integrated system for brain function. Inflammation is a process related with the onset of several neurodegenerative disorders, including Alzheimer's disease (AD). Several hypotheses have been postulated to explain the pathogenesis of AD, but none provides insight into the early events that trigger metabolic and cellular alterations in neuronal degeneration. The amyloid hypothesis was sustained on the basis that Aβ-peptide deposition into senile plaques is responsible for neurodegeneration. However, recent findings point to Aβ oligomers as responsible for synaptic impairment in neuronal degeneration. Amyloid is only one among many other major factors affecting the quality of neuronal cells. Another explanation derives from the tau hypothesis, supported by the observations that tau hyperphosphorylations constitute a common feature of most of the altered signaling pathways in degenerating neurons. Altered tau patterns have been detected in the cerebrospinal fluids of AD patients, and a close correlation was observed between the levels of hyperphosphorylated tau isoforms and the degree of cognitive impairment. On the other hand, the anomalous effects of cytokines and trophic factors share in common the activation of tau hyperphosphorylation patterns. In this context, a neuroimmunological approach to AD becomes relevant. When glial cells that normally provide neurotrophic factors essential for neurogenesis are activated by a set of stressing events, they overproduce cytokines and NGF, thus triggering altered signaling patterns in the etiopathogenesis of AD. A solid set of discoveries has strengthened the idea that altered patterns in the glia-neuron interactions constitute early molecular events within the cascade of cellular signals that lead to neurodegeneration in AD. A direct correlation has been established between the Aβ-induced neurodegeneration and cytokine production and its subsequent release. In effect, neuroinflammation is responsible for an abnormal secretion of proinflammatory cytokines that trigger signaling pathways that activate brain tau hyperphosphorylation in residues that are not modified under normal physiological conditions. Other cytokines such as IL-3 and TNF-α seem to display neuroprotective activities. Elucidation of the events that control the transitions from neuroprotection to neurodegeneration should be a critical point toward elucidation of AD pathogenesis.

Introduction

During several decades, most research advances in Alzheimer's disease (AD) were concentrated on the activity of neuronal cells. However, in the past few years an increasing set of evidence has converged on the major role of glial

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cells and alterations in their function, in the pathway toward neuronal degeneration (1,2). Thus, the axis formed by neurons and glial cells appears to constitute a basic unit in brain function, which together with the role of blood vessels conform a set of interacting elements that define the functional capacity of the brain. The relationships between glial and neuronal cells involve proinflammatory cytokines. Inflammation is a process that has been actively related with the onset of several neurodegenerative disorders, including AD. However, the precise implications of the inflammatory response for neurodegeneration have not been elucidated. A current hypothesis considers that an extracellular insult to neurons could trigger the production of inflammatory cytokines by astrocytes and microglia. These cytokines, namely, IL-1β, TNF-α, and IL-6, could affect the normal behavior of neuronal cells (3). Therefore, dysfunction at this core level may lead to abnormalities such as neurofibrillary degeneration in AD (1). The brain has a marked capacity to activate inflammatory reactions in response to an immune challenge in the periphery (4). Various cytokines are implicated, such as interleukin-1 beta (IL-1β) that responds with a widespread pattern of activities within the brain (5), and interleukin-6, whose overproduction has been considered as a step in the pathway to neuronal degeneration (6,7). The well-known tumor necrosis factor (TNF-α) constitutes an important cytokine that shares these patterns of expression (7).

In spite of these studies, the precise role of cytokines in neurodegenerative processes is not fully understood. In this context, it has been described that cytokines secreted by microglial cells, astrocytes and/or neuronal cells may induce synthesis of certain acute-phase proteins including the amyloid precursor protein AβPP (8,9). On the other hand, the amyloid-β peptide (Aβ) itself can induce the expression of IL-1β, TNF-α, and IL-6 in astrocytes and microglial cells in culture (10,11). IL-6 has been found at an early stage of senile plaque formation (12,13). It was also observed that IL-6 secretion by peripheral blood mononuclear cells was increased in patients with AD as opposed to normal subjects or those suffering from other brain disorders such as vascular dementia (14). In addition to these findings, overexpression of IL-6 in the brain of transgenic mice that overproduce this cytokine (15) is associated with a variety of neuropathological findings, including gliosis and disruption of cholinergic neurotransmission in the hippocampus (16). Thus, a direct correlation has been established between the Aβ-induced neurotoxicity in neurodegenerative conditions and cytokine production.

Cognitive Disorders and Alzheimer's Disease: Links between Neuropathology and Inflammation Pathways

AD is characterized histopathologically by the presence of extracellular senile plaques that consist of Aβ peptide in its fibrillary form, and also neurofibrillary tangles (17,18). The Aβ peptide causes hippocampal and cortical neuronal death *in vitro* and *in vivo* (19,20), and it has been suggested that both Aβ (1–40) and Aβ (1–42) downregulate Bcl-2. This effect may lead to increased neuronal degeneration during the age-dependent stress (21). However, these peptides or their aggregated oligomeric forms (22) also trigger alterations in several signalling mechanisms in neurons, including activation of the protein kinase cdk5/p35 system (23,24); RAGE receptors cascade (25); Rho GTPases (26); alterations in NMDA signalling (6) and others. In the meantime, Aβ (1–42) aggregates appear to be involved in triggering reactivity of glial cells with the consequent release of either NO (1); NGF or proNGF and signalling through the p75 cell death receptor (2). Moreover, an abnormal release of interleukins IL-1 and IL-6 (6) affects tau phosphorylation patterns and other intracellular events linked with neuronal degeneration. In the nervous system, and particularly during development, apoptosis appears to be triggered by trophic factor deprivation. Neuronal apoptosis is likely to occur in AD, a widespread neurodegenerative disorder that results in progressive dementia (17).

Cytokines and Their Dual Roles in Neurodegenerative Disorders

Interleukin-1 (IL-1) and Interleukin-6 (IL-6). The available information indicates that the range of effects of cytokines and other brain neuroimmune modulators oscillates between the opposing actions of neuroprotection and neurodegeneration. In this context, we will analyze some of these major cytokines. Thus, a direct role for IL-1β in Alzheimer pathogenesis was proposed upon observations of the immunological parameters of AD patients. The levels of IL-1β in cerebrospinal fluid of AD patients are significantly higher than in patients with vascular dementia or in healthy controls (27,28). A direct role for IL-1β in promoting neuronal degeneration was evidenced in studies carried out in AD brains (29,30).

IL-1β activates MAPK-p38 *in vitro* and is markedly overexpressed in Alzheimer brains (6). One of the main protein kinases involved in tau hyperphosphorylation in neurodegenerative disorders is the cdk5/p35 complex (17,24), which appears to be upregulated by the effects of both IL-1 and IL-6 (6). The cdk5/p35 complex plays a pivotal role in the development of the nervous system, as it has been shown through engineered targeted mutations of cdk5, p35, and p39 genes producing cortical migration defects (31,32). The function of this critical enzyme, a member of the proline-directed protein kinases (PDPKs) family that also includes the glycogen synthase kinase 3β (gsk3β), is regulated by the neurospecific activators p35 and p39 (33). Both kinases have been implicated in the etiopathogenesis of AD (23,24). However, changes on cdk5 regulation by varying the neuronal specific activator p35 and/or its soluble cytosolic fragment p25 have been correlated

with an increase of tau hyperphosphorylated epitopes of Alzheimer's type *in vitro* and *in vivo* (23,34,35). Aβ peptide induces a marked increase of cdk5 activity paralleled by an abnormal tau hyperphosphorylation in hippocampal cells. Moreover, the ERK kinase and p38 phosphorylate tau protein in epitopes Ser^{202} and Thr^{205}, like the cdk5/p35 system. IL-1β expression is also known to induce iNOS in the hippocampal neurons (36), with the concomitant production of NO that has been demonstrated previously to be involved in neuronal death and damage (37,38).

IL-6 is a pleiotropic inflammatory cytokine secreted by leukocytes and other cell types in acute infection (39). In the nervous system, IL-6 production has been shown to mainly occur in activated glia such as astrocytes and microglial cells (40). IL-6 has been involved in the etiopathology of neurodegenerative diseases such as AD with acute or chronic inflammatory components (41,42), Parkinson's disease, systemic lupus erythematosus, multiple sclerosis, and HIV encephalopathy (43). Under physiological conditions, IL-6 and the IL-6 receptor (IL-6R) are expressed in several regions of the brain, including the hippocampus, striatum, hypothalamus, neocortex, and brainstem and its expression is developmentally regulated (44). The role of IL-6 in neuroinflammation and neurodegeneration is also associated with cognitive impairment. Recently, it has been shown that peripheral lipopolysaccharide (LPS) injection in IL-6 knockout (KO) mice is refractory to develop impairment in working memory, thus providing an additional support to the involvement of IL-6 in AD (45). The mechanisms used by IL-6 in impairing cognitive functions may result from its own activity (6) or be related to production of other cytokines because in the LPS-induced neurodegeneration, mRNA levels of IL-1β and TNF-α in hippocampus are significantly higher in IL-$6^{+/+}$ wild-type animals and very low in IL-$6^{-/-}$ KO mice (45).

Although it has been proposed as a key role for inflammation in the etiopathogenesis of AD, there is no clear correlation between the upregulation of proinflammatory cytokines and tau hyperphosphorylation, one of the hallmarks of AD (17). The links between acute inflammation processes by using IL-6 as a stimulatory signal over the cdk5 kinase system, and the abnormal hyperphosphorylation of tau have been investigated. As indicated, an activation of cdk5/p35 system concomitant with tau hyperphosphorylations at Alzheimer's epitopes was observed in hippocampal cells treated with IL-6 (6). The observed increase of Alzheimer's type epitopes on tau is explained by a sustained increase of intraneuronal levels of the protein kinase cdk5 in response to this cytokine. Further confirmation of cdk5 involvement in this process was based on the findings that inhibition of the kinase activity with butyrolactone-I prevents the appearance of tau of Alzheimer's type in IL-6-treated neurons. Additional studies suggest that an increase of cdk5 activity could be mediated by the signaling cascade described for IL-6 function, namely, the MAPK-p38 signaling pathway. Stimulation of the IL-6 pathway appears to increase the tau epitopes of Alzheimer's type, as demonstrated by using specific inhibitors. These results support the findings of a pathological role for IL-6 in the neuroinflammatory response as related with the pathogenesis of neuronal degeneration.

In addition to activation of the cdk5 system, IL-6 can also activate the JAK/STATs and the MAPK-p38 protein kinases; the latter is also involved in hyperphosphorylation of tau (46). In this regard, Aβ-induced hyperphosphorylation of tau was decreased in presence of the specific inhibitor of MEK-1, PD98059, an event also found in neuroblastoma cells exposed to the same inhibitor (47).

Neuroprotective Roles of IL-3. The cytokine, interleukin (IL)-3, is an important regulator that exhibits pleiotropic activities (48). It is expressed in hematopoietic cells as well in several non-hematopoietic cell types (49). Biological activity of IL-3 is mediated through specific cell surface receptors composed of α and β subunits. IL-3 is known to activate at least three signalling pathways: Jak/STAT, Ras/Raf/mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (PKB) pathway. An important PI 3-kinase target is the serine/threonine kinase Akt/PKB involved in cell survival, an interaction mediated by many growth factors (50). A major role for IL-3 in increasing the activity of Bcl-2, thus activating neuroprotection mechanisms and preventing apoptosis, has been evidenced recently (51). The physiological role of IL-3 receptors in neurons is unknown. It has been suggested that IL-3 can activate neuronal survival pathways (51). IL-3 appears to be a potent inhibitor of neuronal death induced by Aβ (1−42) exposure. These findings are complemented by kinase phosphorylation studies, including the use of specific inhibitors that identified which survival pathways are activated by IL-3. The findings indicate that IL-3r activation induces Jak2 activation via tyrosine phosphorylation, and that this initial event is followed by tyrosine phosphorylation of PI 3-kinase and Akt serine phosphorylation as suggested by the inhibitory effect of AG-490 on the phosphorylation of both proteins (Zambrano et al., unpublished results). These results are consistent with those reported for hematopoietic cells, in which the kinase domain of Jak2 inhibits cell death, and treatment with the Jak2 inhibitor AG-490 reduces phosphorylation of PI 3-kinase, resulting in increased caspase-3 activity and Bax in acute myocardial infarction (52). In addition, activation of neuronal erythropoietin receptors prevents apoptosis by triggering cross-talk between the signalling pathways of Jak2 and the nuclear factor-κB (NF-κB) (53,54).

Several studies have demonstrated the presence of IL-3 in the central nervous system (55,56). Although there is evidence indicating that IL-3 is expressed in some neuronal populations (57), its physiological role in these cells is unknown. Some studies (58) demonstrated that IL-3

significantly facilitates sensory neuron survival and stimulates the formation of the neural network *in vitro*, promotes the process extension of cultured cholinergic neurons (59), and that IL-3 exerts a trophic action on hippocampal neurons, rescuing hippocampal CA1 neurons from lethal ischemic damage (60). However, the mechanism by which IL-3 supports neurons has not yet been determined.

Tumor Necrosis Factor (TNF) Family. In the pathogenesis of AD, TNF-α is produced by activated microglia (61,62), mainly in response to Aβ (1−40) and Aβ (1−42) peptides, oxidative stress (63), glutamate (64), and LPS (65). Its neurotoxic effect involves induction of inflammatory tissue damage as well as inducing neuronal death through its receptor TNF-RI (66−68).

Recent studies showed that serum concentrations of TNF-α were significantly higher in AD patients than in mild dementia patients or in healthy controls (69). Furthermore, AD patients exhibit lower levels of serum IGF-1, a neuroprotective factor, negatively correlated with TNF-α levels. Several studies on genetic polymorphisms as related with risk for AD support a role of TNF-α locus in the pathogenesis of AD (Figure 1). The carrier allele for mutation −308A/G in the promoter region of TNF-α gene is associated with higher intrathecal levels of this cytokine and early onset of disease. The age of disease onset is even earlier if this mutation is carried together with APO ϵ4+ allele (genotype −308A TNF-α/APO ϵ4+). In contrast, the allele carrying the mutation at −863 of TNF-α locus conferred reduced risk for developing AD (70). A contribution to solve the controversial role of TNF-α was provided by Orellana and coworkers (7), who demonstrated a neuroprotective role of this cytokine against neurodegeneration by Aβ (1−42) or oxidative stress in hippocampal neurons, as opposed by the IL-6 effects in promoting neurodegeneration (71).

TRAIL (TNF-related apoptosis-inducing ligand) is a type II integral membrane protein that belongs to the TNF superfamily. It is normally expressed in macrophages and can be induced in neurons by Aβ and astrocytes stimulated with cytokines (72). In addition, TRAIL is specifically expressed in the brain of AD patients and completely absent in the brain of non-demented patients. TRAIL-like immunoreactivity was localized in AD-affected regions such as cerebral cortex, in the proximity of amyloid plaques (73). Neutralization of the TRAIL death pathway protects human neuronal cells from β-amyloid toxicity.

TGF-β. TGF-β is a cytokine with inhibitory functions in several immunological processes such as inflammation and cellular immune responses. The role of this cytokine in neurodegenerative disorders remains controversial. In AD, for example, it has been described that TGF-β1 stimulates astrocytes to generate Aβ (1−40) and Aβ (1−42) peptides. TGF-β may stimulate astrocytes to produce APP, release soluble APPβ, and then generate peptide Aβ. In contrast, other studies indicate that TGF-β stimulates activated microglia to remove Aβ protein from brain to blood vessels. In addition, a decreased intracellular TGF-β signalling mediated by TGF-β II receptor promotes age-dependent neurodegeneration, loss of dendrites, and Aβ accumulation in mouse brain (74). In AD patients, significantly increased TGF-β levels have been found in serum as well as in CSF compared to other forms of dementia. At the population genetics level, *TGFB1* −509 polymorphism has been associated with AD in certain populations only; however, this polymorphism −509 conferred a significant risk of AD when carrying allele *APOE4* of APOE receptor (75).

Regulatory Aspects and the Roles of Polymorphism. The main question is: What are the relationships between cytokine modulation and the cognitive decline in humans?, as well as the relationships between the mechanisms of cytokine control and pathogenesis of AD. Some conflicting results have been published. Whereas some authors point to a major role of IL-1 and IL-6 deregulation in triggering modifications in the normal signaling cascades of neurons, others argue for a lesser involvement of these as molecular factors in the pathogenesis of dementia. In epidemiological studies in the elderly population, it was observed that higher peripheral IL-6 levels exhibited higher rates of cognitive decline over a period of 7 years (76). After studies of elderly patients being investigated in dementia centers, proinflammatory cytokines TNF-α and IL-1β were linked to the onset of AD (77,78). In contrast with these evidences, the Longitudinal Aging Study Amsterdam (LASA) did not detect an association between poor memory performance or slow information processing and cognitive impairment, with the serum levels of IL-6 (79). Despite cumulative information on the involvement of these cytokines in cognitive decline, very little information exists on the prevalence of polymorphisms among the population. The relationship between specific IL-6 genotypes and cognitive function was shown in a study on newborns, and the IL-6-572 C allele (CC/GC genotypes) is associated with cognitive impairment linked to embryogenesis and brain development (80). Further evidence was shown in a study on whole animals, after examining cognitive function in KO mice for Il-6. As compared with wild-type mice, IL-6 KO animals showed better performance in various cognitive trials. These studies suggest that IL-6 may play a role in memory processing (81). On the other hand, IL-1a − 889*1 allele has been shown to be associated with cognitive decline in patients with AD (82). In another set of studies, IL-1β-(1418C > T) and TNF-α- (308G > A) gene polymorphism induce protein expression of the corresponding serum cytokines (83,84). This effect appears to modify cognitive performance through several neuronal actions of peripheral cytokines (85,86). Congruent with these studies, evidence has been found that very little amounts of released cytokines

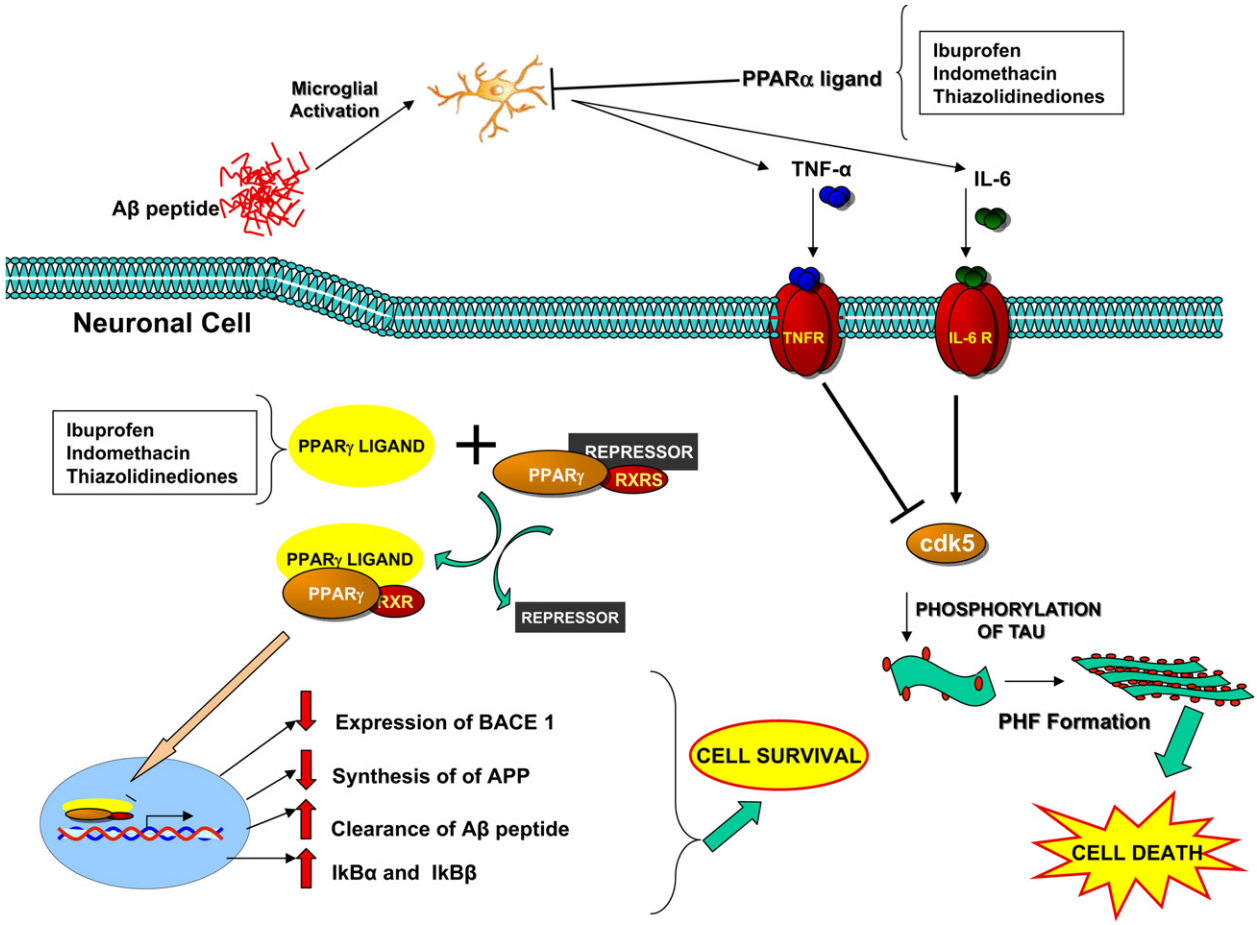

Figure 1. General scheme of an integrated model describing the effects of PPAR ligands on the inflammation pathways and the potential implications for the neurodegenerative process.

have dramatic effects on brain function, especially at the level of memory processing (87).

Chemokines and Chemokine Receptors

Chemokines are a family of low molecular weight proteins produced by a variety of cell types, with the main function of recruiting leukocytes to inflammatory sites. The role of chemokines in neurodegenerative disorders, particularly AD, has become evident in the last few years due to brain postmortem analysis describing specific immunostaining of chemokine receptor CCR1 in AD patients on dystrophic neurites in proximity to senile plaques (88), a pattern not seen in control brains. CCR1 was not detected in glial cells. Also, in AD brains several investigators have observed other chemokine receptors such as CXCR3, CXCR4, CCR3, and CCR5 (89).

In AD patients, CSF leveles of α-chemokine IL-8 and β-chemokine MCP1 are increased in all cases, whereas IP-10 levels are increased in mild but not severe cases (90). Microglial cell cultures obtained from brain of AD patients express significantly higher quantities of IL-1β, IL-8, and MIP3α (CCL20) mRNAs than donors without dementia (91). The notion that chemokine production can be associated with neurotoxic effects is additionally supported by observations in MPK-2 knockout mice (65). MPK2 is a MAPK-activated protein kinase 2, a kinase directly regulated by p38 MAPK. Microglial cells from these KO mice fail to produce proinflammatory cytokines or chemokines such as KC and MIP1-α, in response to LPS and interferon γ. Macrophage and astrocyte cocultures stimulated with Aβ peptide produce MIP1-α, MIP1-β, and MCP1 and neurotoxicity on neuronal cells. Aβ-stimulated microglia from $MK2^{-/-}$ mice do not induce toxicity when cocultured with cortical neurons (65,92).

It is known that chemokine MIP1-α is involved in other neurodegenerative processes in CNS such as Saddhoff disease (93) and that KC chemokine stimulates ERK1/2 and PI-3 kinase signalling pathways and tau hyperphosphorylation (94). In contrast, chemokine production in AD patients may play a neuroprotective role. It has been reported that astrocytes migrate to the sites of Aβ deposition in response to the chemokine MCP-1 and participate in clearance of Aβ deposits. These mechanisms may be critical to understanding chemokine production and astrocyte function in AD pathogenesis (95).

Despite the evidence that chemokines are involved in AD pathogenesis, association between disease susceptibility and polymorphisms of chemokine genes has been unclear. Pola et al. reported that GG polymorphism at position −2518 of MCP1 gene was an independent risk factor for AD in an Italian population (96), whereas two other groups did not find such an association (97,98). Currently, polymorphisms in CCR5 and CXCL1 genes are not associated with AD susceptibility (99,100).

Factor CD40. CD40 belongs to the tumor necrosis factor receptor family and is expressed in antigen-presenting cells. Interaction with its ligand CD40L is critical for optimal T cell-dependent B lymphocyte responses. After engagement with CD40L, CD40 cytoplasmic tail recruits TNFR-associated factor, which activates MAPK, p38MAPK, and NF-κ signaling pathways. The CD40−CD40L has also been implicated in other immune diseases including acute or chronic graft-vs.-host disease and systemic lupus erythematosus. More recently, the CD40−CD40L interaction has been demonstrated to play critical roles in atherosclerosis, where it promotes advanced atherosclerotic lesions and thrombosis, resulting in acute complications (101). These findings highlight that, in addition to its role in refereeing immune cell homeostasis, the CD40−CD40L interaction is an important mediator of chronic inflammation. The role of these receptor-ligand pairs in AD has been recently suggested by the observation that CD40 is constitutively expressed in microglia and astrocytes but its surface expression is upregulated by IFN-γ, TNF-α, and LPS (102). In addition, CD40L is expressed by astrocytes after brain injury. *In vivo* experiments have shown that AD transgenic TgAPP mice crossed with CD40L-deficient mice show reduced or absent phosphor-Tau signal compared to CD40L-expressing control mice (103).

Multiple isoforms of CD40 mRNA, which are generated by alternative splicing, lack the membrane-associated endodomain and constitute the soluble form of CD40 (sCD40) (93). In AD patients, sCD40 are significantly higher than healthy controls; however, further studies are required to establish its role in disease pathogenesis. What has certainly been established so far in various reports is that the CD40−CD40L interaction mediates inflammatory processes of AD pathology and therefore the CD40−CD40L system could be a target for therapeutic intervention and/or molecular diagnosis of AD (104,105).

Neuroinflammation, Nonsteroidal Anti-inflammatory Drugs (NSAIDs), and AD: Do Molecular Findings Provide a Therapeutic Avenue to Stop the Cognitive Decline?

As mentioned above, cellular and molecular studies show that the inflammatory process plays a pivotal role in neuropathological changes occurring at the early stages of AD (106). During the last 10 years, consistent evidence has been published in order to highlight the strong association between AD and abnormal activity of proinflammatory cytokines such as interleukin-1β (IL-1β) (107), IL-6, TGFβ (108), IL-18, and TGFβ1 (109). In addition, *in vitro* experiments have demonstrated that non-selective inhibitors of COX enzyme can preferentially decrease the levels of Aβ 1−42.

The primary action of NSAIDs is inhibition of the COX enzymes, which exist in an inducible form (COX-2) that has been found to be elevated in the AD brain, and a constitutive form (COX-1). Both COX-1 and COX-2 are known to be involved in numerous inflammatory activities, as well as in normal neuronal functions (107). In addition, several retrospective studies have found associations between a reduced risk of AD and the chronic use of NSAIDs (110−113). These studies show statistically significant reductions in the risk of AD after long time use (>2 years) (114−116). In order to validate clinical use of NSAIDs in AD, several randomized clinical trials have been undertaken assessing the efficacy of some NSAIDs in AD treatment (Table 1). The first promising report was published by Rogers and coworkers (117). They reported that oral administration of indomethacin, a nonselective COX inhibitor, for a period of only 6 months appears to protect mild to moderately impaired AD patients from the degree of cognitive decline. This report was followed by a series of reports (118−121) providing evidence that linked the clinical use of NSAIDs and AD. In one of these clinical studies, patients treated with diclofenac and misoprostol did not show significant differences compared to placebo group in the cognitive assessments (119). Naproxen, a nonselective COX inhibitor, was also assessed in a multicentric, randomized, double-blind, placebo-controlled trial without successful results in the treatment or prevention of AD and cognitive decline over a period of 52 weeks (120).

The effect of hydroxychloroquine, a potent inhibitor of the production of IL-1 and IL6, has also been clinically assessed regarding the progression of dementia in early AD; however, this drug did not slow cognitive decline in patients with mild-to-moderate AD (121). Moreover, 5% of the treated patients died as compared to 2% of the placebo group. COX-2, being an inducible enzyme that is increased during inflammatory processes, has been proposed as a potential target for AD treatment. Moreover, some authors have hypothesized that COX-2-selective NSAIDs could be potential therapeutic tools due to the relevant role of chronic inflammation in AD and other dementias (122).

In spite of their safe security profile, COX-2-selective inhibitors such as celecoxib, rofecoxib, and nimesulide have yielded negative results in clinical trials performed so far, showing no statistically significant improvements between patients and placebo controls (120,123−125). It has been demonstrated that the NSAIDs such as ibuprofen, indomethacin, and sulindac sulfide preferentially decrease the

Table 1. Summary of the main clinical trials assessing security profile and efficacy of NSAIDs in Alzheimer's disease

Drug and dosage	Study design	*n* (beginning/ end)	Duration (weeks)	Primary outcome	Secondary outcome	Results and comments	Overall conclusion	Ref.
Indomethacin 100−150 mg/day	Double-blind, placebo-controlled study	28/44	26	Cognitive decline		Improvement in treated-patients1.3% (±1.8%), Placebo-patients declined 8.4% (±2.3%).	Positive	(117)
Diclofenac 50 mg/day (misoprostol)	Randomized, double-blind placebo-controlled trial	27/41	25	ADAS-cog GDS CGIC	MMSE ADAS-oncog ADAS-total IADL, PSMS cGIC	No group differences in any of the outcome measures in an intent-to-treat analysis. Some nonsignificant trends for the placebo group to have deteriorated more than the D/M-treated patients	Negative	(107)
Celecoxib 200 mg BID	Double-blind, randomized, placebo-controlled, comparative study	328/425	52	ADAS-cog CIBIC-Plus	Behave-AD NOSGER MMSE PE QOL MADRS	No significant limited or attenuated symptomatic progression of AD	Negative	(111)
Nimesulide 200 mg/day	Randomized, controlled, parallel-group trial	38/40	12+12	MMSE, ADAS-cog, CDR, ADL, Ham-D, BPRS.		No significant effect on total assessment scores of measures of cognition, clinical status, activities of daily living, affect, and behavior.	Negative	(118)
Rofecoxib 25 mg/day	Multicenter, randomized, double-blind, placebo-controlled trial	177/223	52	ADAS-cog	CDR, CDR-SOB, NPI, QOL-AD	ADAS-cog change was not different in one-year study. Neither of the other measurements was better in the treated group. Side effects such as fatigue, dizziness, and hypertension reported in the active drug	Negative	(120)
Naproxen 440 mg/day	Multicenter, randomized, double-blind, placebo-controlled trial	178/229	52	ADAS-cog	CDR, CDR-SOB, NPI, QOL-AD	ADAS-cog change was not different in one-year study. Neither of other measurements was better in the treated group. Side effects such as fatigue and dizziness were reported in the active drug.	Negative	(120)
Hydroxychloroquine 200 or 400 mg/day	Multicentric double-blind trial	155/168	72	IDDD	ADAS-cog RMBPC	Drug does not slow the rate of decline in minimal or mild AD; 5% of treated patients died compared to 2% of placebo group	Negative	(121)
Rofecoxib 25 mg/day	Randomized, double-blinded, placebo-controlled trial	521/692	52	ADAS-cog CIBIC+	MMSE,14 CDR, 15 ADCS-ADL	No significant differences between treatments were found	Negative	(124)
Rofecoxib 25 mg/day	Randomized, double-blind placebo-controlled trial	801/1457	204	ADAS-cog	Death, institutionalization, CDR, ADCS-ADL, CDR-SOB	No delayed diagnosis of AD in the treated group	Negative	(123)

n, number of patients; D/M, diclofenac/misoprostol.

amyloidogenic Aβ (1–42) peptide produced from a variety of cultured cells (126). Also, only a few non-selective NSAIDs possess Aβ-lowering properties such as ibuprofen and sulindac sulide that reduce Aβ (1–42) secretion, and indomethacin that protects differentiated human neuroblastoma cells from Aβ toxicity (127,128).

In an *in vitro* aggregation study conducted by Hirohata et al. (129), several NSAIDs dose-dependently inhibited formation of fibrillary Aβ (fAβ) from the soluble Aβ (1–40) and Aβ (1–42) and destabilize preformed fAβ, ibuprofen being the most active compound. A comprehensive review of NSAIDs with potential effect on Aβ aggregation has been published by Gasparini et al. (127). Although in the last decade many reports have been published regarding the effect of NSAIDs on the amyloidogenic pathway of APP, the precise mechanism by which NSAIDs decrease Aβ neurotoxicity in human is still unclear. On the other hand, the prescription of this type of drug for prophylactic proposes in AD and other cognitive impairments is even less supported. This potential effect of NSAIDs on AD pathogenesis seems not to be mediated only by inhibition of COX activity (126). NSAIDs can decrease Aβ (1–42) production by direct modulation of γ-secretase activity (130). In fact, it was demonstrated that sulindac sulfide, a COX inhibitor, is a noncompetitive γ-secretase inhibitor that preferentially reduces Aβ (1–42) generation. (131) Moreover, the Aβ (1–42)-lowering activity of NSAIDs is not mediated only by the inhibition of cyclooxygenases and could be dissociated from the anti-inflammatory properties of this class of drugs (132).

It has been suggested that NSAIDs may decrease natural killer lymphocyte activity in vascular dementia. However, this finding would not explain the ability of anti-inflammatory drugs to delay the onset or slow down the progression of AD (133). Another promising hypothesis for the role of NSAIDs in AD is that this drug may interfere with tau hyperphosphorylation and its pathological aggregation. Regarding this issue, studies performed in our laboratory have demonstrated that proinflammatory cytokines modulate the activity of cdk5 (7), a key enzyme in the anomalous phosphorylation of tau. Interestingly, it has recently been demonstrated that acetylsalicylic acid, a non-selective COX inhibitor, decreases tau phosphorylation at serine 422 (134), a posttranscriptional modification relevant to AD pathogenesis.

A subset of NSAIDs has been shown to preferentially reduce the secretion of the highly amyloidogenic, 42-residue amyloid-peptide Aβ (1–42). In this context, we have recently demonstrated that Rho and its effector, Rho-associated kinase, preferentially regulate the amount of Aβ (1–42) produced *in vitro* and that only those NSAIDs effective as Rho inhibitors can lower Aβ (1–42) levels. The administration of Y-27632, a selective Rock inhibitor, preferentially lowered brain levels of Aβ (1–42) in a transgenic mouse model of AD. These findings show that the Rho-Rock pathway can regulate APP processing, and a subset of NSAIDs can reduce Aβ (1–42) by inhibiting Rho activity (135).

In animal models, the most frequently reported finding after administration of NSAIDs is a reduction in the Aβ load in CSF and brain aggregates. Ibuprofen decreases insoluble Aβ deposits and activated microglia. Also, long-term treatment of Tg-2576 transgenic female mice restored the open field behavior (136). In APP/PS-1, a transgenic mouse model of AD that reproduces senile plaques, the COX inhibitor ibuprofen caused a modest reduction of Aβ load (137), while in the similar transgenic mouse model APPV717I, ibuprofen reduced the amount of activated microglia (138). Studies with indomethacin in the transgenic mice model Tg2576 have demonstrated a reduction in hippocampal and cortex Aβ load. (139,140). In the same model, nimesulide, a mixed COX-1/COX-2 inhibitor, did not show any significant effect on the Aβ load. Flurbiprofen, a nonselective COX inhibitor, failed to decrease Aβ load in the APP/PS-1 transgenic mouse model of AD, but the flurbiprofen derivatives NCX-2216 and HCT 1026 caused a dramatic reduction in Aβ and activated microglia in the same transgenic model (141). On the other hand, celecoxib, a potent and specific COX-2 inhibitor, failed to decrease Aβ load in the APP-PS1 transgenic mice (137) and rather increased Aβ in the Tg2576 AD mouse (142).

Overall, so far, clinical studies indicate that NSAIDs cannot clinically improve patients with AD, regardless of the strong evidence of basic pharmacology toward a potential benefit of NSAID in AD pathology. NSAIDs seem to show relative efficacy lowering the risk of AD, but not decreasing the clinical symptoms in mild cognitive impairment or advanced cases of AD. The controversial results yielded from animal or cell culture studies (143) with respect to data from the clinical trials (144) (Table 1) could be explained by several reasons such as (i) the stage of the disease when the therapy with NSAIDs is initiated, (ii) the different types of drugs and dosages used and the levels that the drug reached the CNS (144). It is likely that in patients with advanced stages of AD and high degree of neurodegeneration, the inflammatory process is not pivotal for the clinical symptoms and therefore these patients are refractory to anti-inflammatory drug treatment. These observations demonstrate that NSAIDs could be useful only in early stages of AD. Also the effectiveness of NSAIDs would demand chronic treatment in a long-term period, which increases the possibilities of serious side effects such as hemorrhages, renal impairment, heart-related diseases and death.

Albeit COX-2 induction promotes APP amyloidogenic processing (145) and enhances cognitive deficits in model animals (146), clinical trials with COX-2 inhibitors were all negative for cognitive improvement (Table 1). These findings suggest that COX-2 inhibitors are an inappropriate choice for therapeutic approach toward the treatment of AD

(94) and, apparently, cyclooxygenase-2 would not play a significant role in the pathogenesis of AD, at least in the clinical phase of the disorder (124,147). In addition, this class of drug has side effects, increasing the risk of cardiovascular events (148). Classic NSAIDs show side effects related to gastrointestinal damage when chronically used (149). An interesting alternative seems to be the NO-NSAIDs, which have reduced gastrointestinal toxicity (150) and promising results *in vitro* in mice models, reducing amyloid depositions (151).

Neuroinflammation and Peroxisome Proliferator-activated Receptor Gamma (PPARγ): A New Target for AD Treatment?

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are related to nuclear receptors. This family also includes steroidal transcription factors, non-steroidal receptors and retinoic acid receptors. PPARs have three major isoforms identified to date: α, β/δ, and γ, with splicing variants for all three subtypes (152). The three isotypes of these receptors are encoded by different genes and distributed in various tissues with relative abundance in adipose tissue (153). In general, PPARs are strongly implicated in the control of inflammatory responses and in cholesterol homeostasis (84).

Even though considerable evidence has shown the role for PPARs in adipose differentiation, glucose metabolism and in the regulation of inflammation, their participation in central nervous system inflammation and its potential role as therapeutic targets for the treatment of neurodegenerative syndromes are still partially elucidated. Although the relative abundance of PPAR in brain is not as high as in other tissues (153), there is a consistent amount of evidence demonstrating that PPAR ligands modulate brain inflammatory response in order to decrease the neuronal damage that occurs in neurodegenerative diseases (154).

Levels of expression of PPARγ receptors in postmortem brain sections from AD patients have revealed that PPARγ is expressed in astrocytes and neurons from the frontal cortex. However, in addition to its regular nuclear location, PPARγ has not been localized in senile plaques (157). Specifically, PPARγ has a major role in the neuroinflammation process. Activation of PPARγ by thiazolidinediones induces anti-inflammatory effects at systemic level by inhibiting the induction of proinflammatory cytokines, adhesion molecules, and extracellular matrix proteins or by stimulating the production of anti-inflammatory molecules (155) (Figure 1). On the other side, PPARα agonists inhibit IL-6, TNF-α, and COX-2 expression in cell cultures (156).

Also, PPARγ ligands have been shown to inhibit microglial activation induced by proinflammatory agents such as lipopolysaccharides and Aβ. PPARγ ligands also inhibit expression of iNOS, COX-2, and TNF-α caused by Aβ stimulation in cell cultures (157). Several authors have demonstrated that when neuroblastoma cells are stimulated with proinflammatory cytokines IL-1β, IL-6, interferon-α, and interferon-γ to produce Aβ and APP, pre-incubation with PPARγ ligands such as ibuprofen, indomethacin, or other PPARγ ligands reverses the production of proinflammatory molecules (Figure 1) (158). This effect is partially explained by the downregulation of β-secretase mRNA level expression, since this effect can be inhibited by the addition of PPARγ antagonists (Figure 1). It has also been demonstrated that PPARγ agonists such as thiazolidinediones reverted Aβ-stimulated microglial activation, secretion of proinflammatory products, and microglial-mediated astrocyte proliferation, IL-6 (159). Also, PPARγ ligands inhibit specific gene expression without direct binding to the gene promoter, rather antagonizing the activities of transcription factors STAT1, NF-kB, and AP-1 by trans-repression of several genes, i.e., iNOS and COX-2, which is achieved in part (160).

Interestingly, controversial results appear in the evaluation of the potentially therapeutic role for PPARγ ligands in AD (161). It was shown that the PPARγ ligand 15-deoxy-delta12,14-prostaglandin J2 (15d-PGJ2) induces neurite degeneration and nuclear condensation, consistent with apoptotic cell death in primary cultures of cortical neurons and human neuroblastoma. These effects were reversed by previous incubation with the general caspase inhibitor, Z-VAD. Thus, the activation of PPARγ has a deleterious effect on neurons. In parallel, consistent evidence has been published suggesting that glucose metabolism might be indirectly linked with the pathogenesis of AD (162). A comprehensive review is presented in Craft et al. (163). In regard to glucose metabolism, it is widely known that in elderly subjects, hyperinsulinemia is a risk factor for AD and memory decline. Furthermore, insulin infusion into healthy older subjects increases cerebral spinal fluid levels of Aβ. On the other hand, insulin-dependent glucose transporters have been identified in brain areas controlling memory (163).

In the clinical field, a preliminary study by Watson et al. (164) demonstrated that, relative to the placebo group, subjects receiving rosiglitazone, a PPARγ ligand used for diabetes, exhibited better delayed recall and selective attention (after 6 months). In the same study, plasma Aβ levels were unchanged from baseline for subjects receiving rosiglitazone, but declined for subjects receiving placebo during the past 6 months. These findings are consistent with recent reports that plasma Aβ (1−42) decreases with progression of AD. Even though this evidence is still too weak to recommend rosiglitazone for AD treatment, authors suggested that rosiglitazone may offer a novel strategy for the treatment of cognitive decline associated with AD. The PPARγ Pro12Ala polymorphism, which is associated with an increased risk of type II diabetes (165), has been found to influence plasma 24S-hydroxycholesterol/cholesterol ratios in AD patients. 24S-hydroxycholesterol is the major

product of brain cholesterol metabolism and is released into the blood stream (166).

AD and the Complement Pathway: A ''Pathway'' to Future Pharmacological Treatments and Molecular Diagnosis?

During the last 13 years, strong evidence has been published supporting the fact that the complement system, a major component of inflammatory responses, plays a pivotal role in AD, especially during early subclinical stages of the neurodegenerative process (106,107,113,122). It is known that the membrane attack complex (MAC), formed as a result of the activation of the complement system, is present in dystrophic neurites in AD, which provides a link between β amyloid deposits and neurotoxicity (167,168).

However, there is still controversy because protective roles have been attributed to the complement system in AD by eliminating aggregated and toxic proteins associated with AD. Rogers et al. have recently provided evidence that circulating Aβ (1–42) peptide can be eliminated from the blood stream by complement C3b-dependent adherence to erythrocytes, which is a classical mechanism by which pathogens are recognized as foreign and cleared from the body. Levels of Aβ (1-42) peptide targeted by the complement pathway differ significantly in AD compared to mild cognitive impairment and nondemented elderly controls (169). This fact seems to offer a promising avenue towards a future molecular diagnosis of AD.

It has been demonstrated that tau protein, the main component of NFTs, is a potent antibody-independent activator of the classical complement pathway. Tau is therefore able to increase inflammatory responses and cytokine production. Because Aβ deposits and extracellular NFTs are present during early preclinical until terminal stages of AD, their ability to activate complement provides a mechanism for initiating and sustaining chronic, low-level inflammatory responses that may accumulate over the disease course (170). This supports the idea that the complement system cascade intervention can be a pharmacological approach to treat early stages of AD in the near future.

Perspectives Toward a Potential Molecular Diagnosis of AD

Alzheimer's disease diagnosis is currently carried out through clinical and neuropsychological evaluation, which involves a certain level of uncertainty. Therefore, it is critical to find quantifiable tools to assess an early diagnosis and for early detection of symptoms. During the last several years, a major emphasis has focused on the search for potential and sensitive biomarkers in biological fluids such as plasma, serum or CSF toward a differential diagnosis of AD. CSF reflects the composition of the brain extracellular environment; however, to obtain a CSF sample in a routine fashion is rather invasive (171).

Identification of blood biomarkers is a less invasive procedure and appropriate for monitoring patients during the course of the disease, but no blood tests are available for differential diagnosis of AD that can be used for monitoring the progress of dementia. One aspect that limits the search of reliable blood markers resides in the fact that despite the brain accumulation of cytokines in acute phase, not all proteins pass through the hematoencephalic barrier. Proinflammatory molecules are potential blood markers for AD. One aspect to highlight is that the interpretation of measures of immunological mediators in plasma and serum of AD patients is limited by controversial results. The following are the best-studied immunological factors as potential biomarkers: (i) CD40 soluble (sCD40): a significant increase was found for sCD40 plasma levels in AD patients (172); (ii) TGF-β1: a significant decrease in the TGF-β1 plasma levels was found in AD subjects (172–174). The complex between TGF-β1 and α2-macroglobulin (α2M) was investigated in serum samples, even though this does not seem to be a differential marker for AD because this complex is formed in AD patients as well as in normal controls. Nitric oxide synthase (NOS) was investigated in the leucocytes of AD patients and it was found significantly increased. Thus, the combination of TGF-β1 and NOS measurements in leucocytes can be considered as a potential biomarker for AD (174).

In regard to interleukins, IL-6 plasma levels are significantly increased in MCI and AD as derived from several studies (175–177). However, in a study carried out in 1997, the presence of IL-6, the soluble receptor for IL-6 (sIL-6R) and the soluble form of gp130 protein in the CSF of AD patients, it was shown that none of the parameters analyzed changed with respect to controls (178). In another study, levels of gp130 protein were found significantly reduced in the CSF of AD patients as compared with the normal counterparts (179). A broad study in 2005 indicated that an increment of IL-6 levels in CSF is useful for screening AD and vascular dementia (VD) in certain populations, IL-6 levels are not significantly different between AD and VD, while descending Aβ (1–42) and ascending TNF-α in CSF could be preferable to diagnose AD (180). It was also found that plasma levels of IL-18 as well as TGF-β1 were significantly higher in AD and VD patients as compared with normal counterparts. In patients with VD, the correlation between IL-18 and TGF-β1 reached a borderline positive value. In the non-demented, age-matched subjects, a positive correlation between IL-18 and TGF-β1 levels was observed. The study suggests that plasma levels of both IL-18 and TGF-β1 are potential biomarkers for dementia (181). On the other hand, protein kinases Erk1 and Erk2 level values were inversely correlated with AD, suggesting maximal efficacy for early diagnosis (182).

In another study, interleukin IL-2 did not exhibit significant changes when AD samples were compared with normal controls, even though a higher level of this cytokine

was detected in severe cases of AD (179), suggesting that the increase of IL-2 correlated with advanced stages of the disease. Thus, in a report that investigated cytokine secretion in mononuclear cells, a significant increase in IL-2 and IFN-α in CSF of severe cases of AD was found (183).

The proinflammatory cytokine IL-1 has been reported to be elevated in the CSF, brain tissue and plasma from AD patients (184,185), but another study indicates that serum levels of IL-1α in the early onset of AD subjects are not significantly different than those of healthy elderly controls. Interestingly, the interleukin postulated to have a neuroprotective activity, IL-3, displayed significantly lower levels in blood mononuclear cells of AD patients (177). On the other hand, TNF-α was increased in the CSF fluid of AD patients, but a higher increase was observed in subjects with VD (184). However, in another study, serum levels of these cytokines were decreased in AD patients, a negative correlation was found between serum TNF-α levels and the age of AD patients (67,172).

In conclusion, it is likely that in the near future the early molecular diagnosis of AD will be based on correlation polymorphisms and levels of proinflammatory cytokines such as TNF-α, IL-1β, and sCD40 in CSF and serum.

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