RAGE-Aβ Interactions in the Pathophysiology of Alzheimer’s Disease

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Received 28 September 1997; revised 21 November 1997; accepted 21 November 1997

Abstract

RAGE is a cell surface molecule primarily identified for its capacity to bind advanced glycation end-products and amphoterin. Immunocytochemical studies demonstrated that in Alzheimer’s disease (AD) the expression of RAGE is elevated in neurons close to neuritic plaque beta-amyloid (Aβ) deposits and in the cells of Aβ containing vessels. Cross-linking of surface bound Aβ 1-40 to endothelial cells, yielded a band of 50 kDa identified as RAGE. Using the soluble extracellular domain of recombinant human RAGE, we found that Aβ binds to RAGE with a Kd = 57 ± 14 nM, a value close to those found for mouse brain endothelial cells and rat cortical neurons. The interaction of Aβ with RAGE in neuronal, endothelial, and RAGE-transfected COS-1 cells induced oxidative stress, as assessed by the TBARS and MTT assays. ELISA demonstrated a 2.5 times increase of RAGE in AD over control brains. Activated microglia also showed elevated expression of RAGE. In the BV-2 microglial cell line, RAGE bound Aβ in a dose dependent manner with a Kd of 25 ± 9 nM. Soluble Aβ induced the migration of microglia along a concentration gradient, while immobilized Aβ arrested this migration. Aβ-RAGE interaction also activated NF-κB, resulting in neuronal up-regulation of macrophage-colony stimulating factor (M-CSF) which also induced microglial migration. Taken together, our data suggest that RAGE-Aβ interactions play an important role in the pathophysiology of Alzheimer’s disease.

1. Introduction

The neuropathology of Alzheimer’s disease (AD) is characterized by the presence of large quantities of beta-amyloid (Aβ) peptide polymerized into 10-12 nm filaments, which are deposited at the center of the neuritic plaques in the cerebral cortex and in the walls of cerebral and leptomeningeal vessels [1]. A smaller pool of water-soluble amyloid has recently been described in both the normal and AD brain, capable of diffusing along the narrow extracellular space of the neuropil [2]. The 40 to 42 amino acid long Aβ peptide is derived from the proteolytic processing of beta-amyloid precursor protein (βAPP) by the action of the β- and γ-secretases, the latter hydrolyzing the βAPP in the middle of its single transmembrane domain [1]. The Aβ peptide has a hydrophilic N-terminus and a hydrophobic C-terminus folded into a β-sheet structure which is prone to aggregation resulting in insoluble filaments very resistant to proteolytic degradation [3]. Both the insoluble fibrillar amyloid and the water soluble oligomeric Aβ are toxic to neurons and microglia [4-6].

During a survey for potential membrane receptors for Aβ in the AD brain we observed that the receptor for advanced glycation end-products (RAGE) was overexpressed in neurons, microglia and endothelial cells [7]. The 382 amino acid long type I transmembrane protein RAGE is a member of the immunoglobulin superfamily of cell surface molecules primarily identified for its capacity to bind advanced glycation end-products (AGE) and also as a receptor for amphoterin, a
DNA binding protein [8]. The expression of RAGE and amphoterin are increased during the fetal and the early postnatal period in cortical and hippocampal neurons, as these molecules are involved in neuritic outgrowth [9].

In this paper we demonstrate that in AD, RAGE immunoreactivity is increased in neurons and microglia associated with Aβ containing neuritic plaques and in vascular cells exhibiting deposits of amyloid. It is also demonstrated that Aβ binds RAGE in a dose-dependent manner on endothelial cells, rat cortical neurons and rat microglia as well as on RAGE transfected COS-1 cells. The binding of Aβ to RAGE elicits cellular oxidative stress resulting in activation of NF-κB. The Aβ-RAGE interaction also induces microglial activation and migration. The binding of Aβ to neuronal RAGE evokes the secretion of macrophage-colony stimulating factor (M-CSF) which activates and recruits microglia in the vicinity of the AD lesions.

2. Materials and Methods

Mouse endothelial cells [10], rat primary cortical neurons [11], and COS-1 cells transfected with RAGE cDNA [12] were prepared and grown as described. The measurement of TBARS generation [13] and MTT reduction assay [7] were published elsewhere. The Aβ was radiiodinated according to manufacture protocol (Iodobead, Pierce, Rockville, IL) and purified using gel filtration chromatography. Cellular RAGE binding to Aβ studies were described previously [7]. In short, cultures were washed with phosphate-buffer saline and 125I-labelled Aβ (100 nM) was added either alone or in the presence of 100-fold unlabelled Aβ to the cultures at 4°C for 3.5 h for endothelial cells or 3 h for neurons and COS-1 cells. Unbound materials were washed away and elution buffer (0.1 M NaCl with Nonidet P-40, 1 %) was incubated with cultures for 5 min at 37°C prior to scintillation counting. For the recombinant RAGE-Aβ binding assay, purified sRAGE (5 μg/well) was coated on a microtiter plate and 125I-labelled Aβ (100 nM) was added. sRAGE was cloned from a human lung cDNA library and expressed in baculovirus [9].

RT-PCR detection of RAGE mRNA in PC12 and cerebellar granular cells was carried out using primers (forward: 5'-TGACCTGTGCCACCATCTGCC-3' and reverse: 5'-CCACATGATCCATGCTGAGT-3') corresponding to rat RAGE cDNA bases 817-836 and 1383-1402, respectively. Total cellular RNA was isolated using TriReagent (Molecular Research Center, Cincinnati, OH) and PCR carried out with 30 cycles (30 sec. at 94°C, 30 sec. at 60°C, and 30 sec. at 72°C) in a total volume of 50μl. PCR products were analyzed on a 1 % agarose gel stained with ethidium bromide.

Immunohistological studies were performed on paraffin sections of formalin-fixed brain tissues. Rabbit anti-human RAGE IgG (20 μg/ml) [7], rabbit anti-human p50 IgG (5 μg/ml, Santa Cruz, Biotechnology), rabbit anti-human M-CSF IgG (5 μg/ml, Santa Cruz, Biotechnology), rabbit anti-human c-fms IgG (5 μg/ml, Santa Cruz, Biotechnology), mouse anti-human CD68 (Dako) were used as primary antibodies. Sections were visualized using the avidin-biotin alkaline phosphatase method (Sigma). For double stained sections, peroxidase-conjugated IgG was used as the secondary antibody (Sigma). To quantify the concentrations of RAGE in brains, the technique of Yan et al. [7] was used.

All the experiments described in this paper were carried out with Aβ 1-40 which was obtained from California Peptide Research (Napa, CA). Aβ 1-40 was purified by HPLC and its composition was characterized by automatic amino acid analysis and mass spectrometry.

3. Results

3.1. Aβ induces cellular oxidative stress by binding to cell surface receptor

Exposure of primary cultures of mouse endothelial cells and rat cortical neurons to increasing doses of Aβ 1-40 caused cellular oxidative stress, resulting in a dose-dependent generation of thiobarbituric acid-reactive substances (TBARS). Pre-treatment of the cells with the antioxidants probucol or N-acetyl cysteine inhibited the oxidative stress caused by Aβ (Fig. 1A and 1B). Oxidative stress was also arrested when the cells were exposed for 30 min. to trypsin prior to treatment

![Fig. 1. Aβ-induced cellular oxidative stress. Mouse endothelial cells (A) and cortical neurons (B) were incubated with various concentrations of Aβ 1-40 and the cellular oxidative stress was measured by the TBARS assay. Where indicated, cultures were pre-treated with trypsin, N-acetylcysteine (NAC) or probucol (7).](image-url)
with Aβ (Fig. 1). Cultured endothelial and cortical neurons bound radiolabelled Aβ 1-40 in dose-dependent manner. The calculated Kd were 40 ± 10 nM and 55 ± 15 nM, respectively. To investigate the potential receptor for Aβ on the cellular surface, radiolabelled Aβ 1-40 was cross-linked to both endothelial cells and cortical neurons with disuccinimidyl suberate. In both cases this reaction generated a single band on a non-reduced 10 % gel of approximately 50-55 kDa. From previous studies, it was known that a receptor with similar physicochemical characteristics was present in large quantities in the endothelial cells of the lung. Since in the brain the amount of the putative receptor was present in small amounts, we decided to isolate this molecule from lung tissue. Two main Aβ binding bands were observed with MW of 35 and 50 kDa. Thirty cycles of automatic Edman-degradation established the 50 kDa Aβ binding molecule as the previously identified receptor for amyloid B and AGE. Both, the 35 and 50 kDa molecules reacted positively with anti-Aβ IgG, the shorter protein corresponding to the soluble N-terminal domain of RAGE localized to the extracellular space. Purified soluble RAGE when bound to microtiter plates demonstrated a dose-dependent Aβ binding specificity with a Kd of 57 ± 14 nM. This binding can be inhibited by either anti-Aβ IgG or soluble RAGE. Furthermore, AD derived Aβ also suppressed the radiolabelled synthetic Aβ binding to the microtiter plate bound RAGE. In addition, COS-1 cells transfected with RAGE demonstrated specific binding of radiolabelled Aβ with a Kd of 25 ± 6 nM. In an attempt to more specifically establish the Aβ amino acid sequence engaged in the RAGE interaction, we also investigated the binding of Aβ fragments to RAGE. As can be seen in fig. 2, Aβ peptides residues 17-42 and 12-42 demonstrated specific binding equal to that of Aβ 1-42. However, Aβ peptide residues 13-20 showed a partial binding specificity (67 %), suggesting that the amino acid sequence of residues 17-20 may be particularly involved in the RAGE-Aβ interaction.

3.2. Binding of Aβ to RAGE elicits oxidative stress

A number of experiments demonstrated that the Aβ-RAGE interaction results in severe cellular stress. A larger amount of TBARS were generated when Aβ 1-40 was added to RAGE transfected COS-1, a response not observed in mock-transfected cells (Table I). Moreover, Aβ treated endothelial cells also produced TBARS, this reaction was blocked by either anti-Aβ IgG or an excess amount

<table>
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<tr>
<th>Table I</th>
<th>TBARS generated by Aβ in COS-1 RAGE transfected cells.</th>
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<tr>
<td>Aβ (μM)</td>
<td>0.125</td>
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<tr>
<td>Mock-transfected</td>
<td>111.9 ± 7.1</td>
</tr>
<tr>
<td>RAGE-transfected</td>
<td>135.7 ± 11.9</td>
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Fig. 5. Up-regulation of RAGE in neurons near deposits of Aβ in Alzheimer disease brain. Expression of RAGE (red) increased in hippocampal neurons of AD (A); age matched control (B). Neuritic plaque cores are stained in black. *In situ* hybridization demonstrated the up-regulation of RAGE in AD neurons (C) which was not observed in control neurons (D).

Fig. 6. Double staining for RAGE (brown) and Aβ (black) in microglia and blood vessels of Alzheimer disease brain. (A) Microglia specifically stained with CD68 (arrow heads). (B) Consecutive section stained with anti-RAGE IgG demonstrating the presence of RAGE in microglia within the boundaries of the neuritic plaques (see arrow heads). (C) Colocalization of RAGE and Aβ in blood vessel walls.
3.5. Aβ-RAGE induces microglia activation

Activated microglia and microgliosis represent some of the pathological hallmarks of AD. The function of these cells in mediating the sustained inflammatory response and neuronal damage observed in AD is well documented [14]. Since RAGE is also up-regulated in microglia, we further investigated the role of this receptor in relation to Aβ. Binding studies of Aβ to RAGE on the mouse microglial cell line BV-2 indicated a dose dependent response with a Kd of 25 ± 9 nM. This interaction can be prevented by excess soluble RAGE or anti-RAGE IgG.

3.6. Aβ-RAGE elicits neuronal expression of macrophage-colony stimulating factor

Immunocytochemical studies have demonstrated that in the vicinity of the neuritic plaques, neurons contain molecules indicating oxidative stress, among these: heme oxygenase-1, malonaldehyde lysine epitopes and nuclear location of the p50 subunit of NF-xB [15]. Because of the influence of activated microglia in the neuritic plaques we investigated the possibility of NF-xB up-regulating the expression of molecules like macrophage-colony stimulating factor (M-CSF). Indeed, those neurons close to the neuritic plaques which were positive for the NF-xB also express M-CSF. Furthermore, activated microglia identified by the CD68 antibody also were positive for c-fms, the receptor for M-CSF.

Neuroblastoma cells treated with Aβ responded by increasing the expression of M-CSF. This cellular reaction was inhibited by anti-RAGE IgG and by N-acetylcysteine. The activation of M-CSF was specifically mediated by NF-xB as demonstrated by competition experiments [15]. In addition, following treatment with Aβ, a promoter-reporter (CAT) construct carrying the NF-xB binding site from the M-CSF gene when transiently transfected into neuroblastoma cells increased the expression of M-CSF [15]. These experiments suggest that the binding of Aβ to RAGE causes cellular oxidative stress which promotes the NF-xB mediated transcription of M-CSF, ultimately leading to the increased synthesis and secretion of this factor.

3.7. M-CSF provokes microglia migration, expression of macrophage scavenger receptor and is elevated in the cerebrospinal fluid in AD

The M-CSF secreted by neuroblastoma cells treated with Aβ elicits microglial migration. For these experiments the Aβ 1-40 was bound to microbeads, which were removed by centrifugation prior to transferring the conditioned media into the chemotaxis chambers containing BV2 cells. The migration of cells was arrested by the presence of increasing doses of anti M-CSF IgG. The conditioned media from neuroblastoma cells treated with albumin coupled to the microbeads, did not induce microglial migration [15]. Immunocytochemical studies have demonstrated that the macrophage scavenger receptor (MSR) is elevated in the AD brain [15]. The exposure of BV2 cells to M-CSF also increased the ex-
pression of the type-I MSR, as demonstrated by both Northern blot and Western blot analysis [15].

The presence of the increased levels of M-CSF in the cerebrospinal fluid suggest neuronal distress. ELISA Quantitation of M-CSF in the cerebrospinal fluid, obtained post-mortem from 51 individuals with AD, showed an average value of 675 pg/ml. In contrast, the average value from 16 control cases amounted to only 130 pg/ml, five times less than in AD (p < 0.01). However, the M-CSF is not diagnostic of AD since it was also elevated in other neurodegenerative disorders, such as Parkinson’s disease, and amyotrophic lateral sclerosis where it averaged 550 pg/ml (n = 10) and 230 pg/ml (n = 15), respectively. The average values for multiple sclerosis amounted to only 43 pg/ml (n = 15), a surprisingly low value for a disease with an important inflammatory component.

**4. Discussion**

A large body of evidence supports the pathological function of the Aβ peptide in AD. Aβ 1-40 and Aβ 1-42 are either aggregated in the form of insoluble amyloid fibrils concentrated in neuritic plaques and vascular deposits or in dimeric/oligomeric water soluble forms [3]. The biochemical mechanisms involved in Aβ cellular toxicity are multiple. It has been suggested that Aβ, especially in its fibrillar form, can destabilize the plasma membrane by direct contact due to the fusogenic properties of these peptides [16]. Soluble amyloid peptides appear to be capable of forming transmembrane pores resulting in a breakdown of cellular homeostasis [17]. The toxic effects of Aβ could be mediated by the activation of a signal transduction mechanism [18] or by the internalization of Aβ by endocytosis or pinocytosis. It has also been proposed that in addition to the production of Aβ by the endosomal/lysosomal pathway, cytotoxic Aβ can also be generated in the endoplasmic reticulum and early Golgi [19-21]. Recently, it has been reported that Aβ can elicit neurotoxic responses in microglia [5]. Although the exact biochemical reactions involved in neuronal killing by Aβ in AD still remain to be elucidated, alterations resulting in membrane continuity and the generation of free radicals [22-24] have been proposed as two of the major causes for neuronal necrosis and apoptosis.

In AD immunocytochemical studies have revealed that in neurons close to the neuritic plaques there are signs of free radical injury manifested by the expression of heme oxygenase type-I, malondialdehyde lysine epitopes and nuclear presence of the p50 subunit of the NF-κB [15]. Likewise, oxidative stress markers are also localized in cerebral vessels loaded with amyloid fibrils [24]. Cells in those areas of the brain affected by AD pathology appear to express elevated quantities of RAGE which can bind soluble and insoluble Aβ in a dose-dependent manner. This binding can be blocked by the presence of an excess amount of soluble RAGE or anti-RAGE IgG. The interaction of Aβ with RAGE also induces the generation of free radicals, as demonstrated by an increased generation of TBARS and by the decreased capacity to reduce MTT, resulting in the activation of NF-κB which leads to the increased expression and secretion on M-CSF. Binding of this factor to the c-fms receptor on the surface of microglia results in a gradient-dependent chemotaxis and cell proliferation, as well as elevated synthesis of MSR. The recruitment of microglia into the boundaries of the neuritic plaques is a cornerstone in the architecture of these lesions since microglia are in intimate apposition to the centrally located deposits of fibrillar Aβ. Once in contact with the insoluble dense cores of Aβ, microglia could be functioning as an insulating wall, possibly preventing further damage to the neuropil by fibrillar amyloid. The ability of microglia to move against an increasing gradient of M-CSF may be potentially beneficial for the phagocytosis of water soluble oligomeric Aβ. Interestingly, our recent investigations [25] indicate that RAGE has a higher affinity for Aβ 1-42 (Kd = 20 nM) than for the considerably more soluble Aβ 1-40 (Kd = 70 nM), and that RAGE transfected PC12 cells increase the association of Aβ fibrils to the cellular surface [25]. Serendipitously, we observed that addition of soluble RAGE to nascent short Aβ 1-40 fibrils arrested additional fibrillogenesis, a phenomenon not seen in the absence of soluble RAGE [25]. Furthermore, long Aβ 1-40 fibrils apparently dissociate in the presence of soluble RAGE [25], an ability which may or may not be beneficial to the AD brain since dissociated amyloid may be as toxic as the aggregated form. Our preliminary observation suggesting the sequence of Aβ residues 17-20 (Leu-Val-Phe-Phe) as one of the Aβ domains potentially involved in binding to RAGE, along with the ability of soluble RAGE to inhibit Aβ polymerization has a precedent. Tjernberg et al. [26] reported that a pentapeptide comprising the Aβ sequence of residues 16-20 was capable of inhibiting Aβ fibrillogenesis.

From the aforementioned experiments, it is obvious that both membrane bound RAGE and soluble RAGE, when associated to Aβ 1-40 or Aβ 1-42 either soluble or fibrillar, can actively participate in the pathophysiology of AD.

**Acknowledgments**

This work was supported in part by NIH grants AG-11925, AG-00690 and AG-14103.

**Reference**


