Immunohistochemical analysis of human brain suggests pathological synergism of Alzheimer's disease and diabetes mellitus

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A B S T R A C T
It has been extensively reported that diabetes mellitus (DM) patients have a higher risk of developing Alzheimer's disease (AD), but a mechanistic connection between both pathologies has not been provided so far. Carbohydrate-derived advanced glycation end products (AGEs) have been implicated in the chronic complications of DM and have been reported to play an important role in the pathogenesis of AD. The earliest histopathological manifestation of AD is the apparition of extracellular aggregates of the amyloid β peptide (Aβ). To investigate possible correlations between AGEs and Aβ aggregates with both pathologies, we have performed an immunohistochemical study in human post-mortem samples of AD, AD with diabetes (ADD), diabetic and nondemented controls. ADD brains showed increased number of Aβ dense plaques and receptor for AGEs (RAGE)-positive and Tau-positive cells, higher AGEs levels and major microglial activation, compared to AD brain. Our results indicate that ADD patients present a significant increase of cell damage through a RAGE-dependent mechanism, suggesting that AGEs may promote the generation of an oxidative stress vicious cycle, which can explain the severe progression of patients with both pathologies.

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Introduction

Alzheimer's disease (AD) is a progressive dementia affecting a large proportion of the aging population, that leads to accelerated neuronal death and ultimately to irreversible loss of cerebral functions. The principal histological and cytological characteristics in AD neuropathology are the presence of extracellular senile plaques, containing fibrillar aggregates formed by the amyloid β peptide (Aβ), and of intracellular neurofibrillary tangles (NFT), composed of hyperphosphorylated Tau protein (Durany et al., 1999; Heese and Akatsu, 2006; Kar et al., 2004; LaFerla and Oddo, 2005; Mott and Hulette, 2005). Molecular studies show that the presence of Aβ aggregates, particularly in the form of oligomeric assembly intermediates, are highly toxic to the cell (Catalano et al., 2006; Meredith, 2005; Walsh and Selkoe, 2004). Experimental data indicate that insolubility of amyloid plaques is caused by extensive covalent protein cross-linking and that the growth of Aβ aggregates is accelerated by the formation of carbohydrate-derived highly reactive chemical groups, such as advanced glycation end products (AGES) (Loske et al., 2000; Münch et al., 1997a, b, 2003; Prabhakaram and Ortwerth, 1994; Smith et al., 1996; Thome et al., 1996)). AGEs are generated by a non-enzymatic reaction of glucose and other carbohydrates with stable protein deposits (Wells-Knecht et al., 1995), and are potentially toxic for the cell via a variety of mechanisms (Ahmed, 2005; Dukic-Stefanovic et al., 2001; Gasic-Milenkovic et al., 2003; Gella and Durany, 2009; Li et al., 1994; Picklo et al., 2002). AGEs form in various tissues in a time- and concentration-dependent process, and this accumulation is especially prominent in patients with diabetes mellitus (DM) (Baynes, 1991; Giriones et al., 2004). AGEs can enter the cells, where they are metabolised, through the activation of the receptor for AGEs (RAGE) (Sasaki et al., 2001). These receptors are highly expressed in microglial and neuronal cells, and play a key role in brain oxidative stress processes and their pathological consequences (Lue et al., 2001). It has been demonstrated that RAGE are over-expressed in AD and that Aβ is a RAGE ligand (Chen et al., 2007; Origlia et al., 2008). There is accumulated evidence that AGEs are present in senile plaques and could be involved in their development in the brain of AD patients (Kimura et al., 1995; Kuhl et al., 2004; Münch et al., 1997b, 1998, 2002; Richter et al., 2005).

Several studies have shown that many physiological alterations are common to DM and AD, including aging-related processes, high cholesterol levels, metabolic disorders, Aβ aggregation, glycogen synthase kinase 3 overactivity, and deregulated protein phosphorylation, association with cardiovascular disease, increased oxidative stress and inflammation response, correlation with the apolipoprotein
E4 allele, perturbed function of insulin and insulin-like growth factor signalling and AGEs generation (Brands et al., 2004; Carro and Torres-Aleman, 2004; Doble and Woodgett, 2003; Haan, 2006; Hoyer, 2004; Li et al., 2007; de la Monte and Wands, 2006; Qiu and Folstein, 2005; Ristow, 2004).

In order to investigate the role of AGEs in the physiopathology of both diseases and to explore possible new therapeutic strategies, we have performed an immunohistochemical study in post-mortem samples from patients suffering from DM, AD or both disorders, to determine the presence of AGEs, Aβ aggregates, Tau and RAGE. We have focused our study on the hippocampus because it is a brain area extensively related with memory processes and highly affected in AD. The data are discussed within the frame of a possible functional link between DM and AD.

Materials and methods

Human brain samples

The brain samples used in this study were supplied by the human neurological tissue bank at the Hospital Clinic (Barcelona, Spain). The whole procedure was performed in accordance with the Helsinki Declaration in its latest version and with the Convention of the Council of Europe on Human Rights and Biomedicine, and was approved by the Ethical Committee of Barcelona University. The clinical diagnosis of AD was based on NINCDS-ADRDA criteria and confirmed by neuropathological findings (Braak and Braak, 1991). The histological samples were obtained from the cerebellum, hippocampus and cerebral cortex (temporal, frontal and parietal lobes) of post-mortem brain of four groups: nondemented controls (ND; n = 9), diabetic patients (DM; n = 9), Alzheimer's disease patients, (AD; n = 10) and Alzheimer's disease with diabetes patients (ADD; n = 9). The age, gender, post-mortem interval and Braak stage of the subjects are described in Table 1.

Table 1

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ND, nondemented; DM, diabetes mellitus; AD, Alzheimer’s disease; ADD, Alzheimer’s disease and diabetes mellitus.

All samples were fixed in 4% paraformaldehyde solution, embedded in paraffin following standard protocols and finally sectioned at 5 μm.

Immunohistofluorescence technique

Immunohistofluorescence was performed on paraffin-embedded human brain samples. Briefly, the sections were deparaffinised, hydrated and finally washed in phosphate buffer saline (PBS; pH 7.4) containing 0.1% Tween-20 (PBS-TW). Sections were then treated with 55% formic acid, blocked with 10% of foetal bovine serum (FBS) and incubated overnight at 4 °C with monoclonal anti-Aβ1-40 (1:150, Sigma), and polyclonal anti-AGEs (1:1000, gift by Dr. R. Bucala, Yale University). After several washes in PBS-TW, sections were incubated for 1 h at 25 °C with the corresponding secondary antibody: goat anti-mouse Alexa 488 or goat anti-rabbit Alexa 594 (both 1:1000, Invitrogen). Sections were then washed in PBS in dark conditions and processed for thioflavin T (ThT) staining.

Thioflavin T histochemistry technique

The sections were washed in distilled water, stained in Nissl solution and submerged for 3 min in 0.1 M HCl containing 0.5% ThT and washed in distilled water. The stain was developed during 20 min in 1% acetic acid and finally the sections were washed in distilled water and mounted.

Immunohistochemistry technique

Briefly, the sections were deparaffinised, rehydrated and washed in PBS-TW, and then they were treated with 55% formic acid, 2% hydrogen peroxide, blocked with 10% FBS and incubated overnight at 4 °C with monoclonal or polyclonal anti-Aβ1-40 (1:300 and 1:100, respectively, Sigma) or monoclonal anti-Tau (1:500, Millipore) or polyclonal anti-RAGE (1:500, Fitzgerald Industries Intl.). After that, sections were sequentially incubated with biotinylated goat anti-rabbit antibody (1:200, Sigma) and avidin–bixin–alkaline phosphatase or avidin–bixin–HRP complex (1:200, Vector Labs). Alkaline phosphatase (EC 3.1.3.1.) activity was developed with 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitro-blue tetrazolium chloride substrate solution (Roche) until the reaction products were visualized (blue colour). Peroxidase (EC 1.11.1.7) activity was developed with 0.05% 3,3′-diaminobenzidine (Sigma) and 0.01% hydrogen peroxide until the reaction products were visualized (brown colour).

Tomato lectin histochemistry technique

Following the Aβ immunohistochemistry developed with alkaline phosphatase (see Immunohistosterechnique section), sections were washed in PBS-TW and incubated with lectin from Lycopersicum esculentum (6 mg/L, Sigma) during 2 h at 37 °C. After that, sections were washed in PBS-TW, incubated for 1 h at 25 °C with avidin-peroxidase and developed with 0.05% 3,3′-diaminobenzidine and 0.01% hydrogen peroxide until the reaction products were visualized. Finally the sections were mounted with Mowiol medium.

Plaques and cell counting

Samples were analysed in a light microscope Eclipse 901 (Nikon) and images were captured with a digital camera (Nikon). In all samples the plaques positively stained for Aβ were counted in five sequential sections. The mean section area of all samples was calculated (for cortical areas, 3.28±0.52 mm², and for hippocampus, 2.45±0.37 mm²) and the results obtained were presented as number of Aβ1-40 immunopositive plaques per area. In the same manner, the number of positive RAGE and Tau cells was counted in three
Fig. 1. (A) Microphotographs of Aβ immunolabeling in several brain areas from human post-mortem samples of nondemented, ND (a–d), diabetes mellitus, DM (e–h), Alzheimer’s disease, AD (i–l) and AD with diabetes, ADD (m–p) patients. The brain areas analyzed are frontal (a, e, i and m), parietal (b, f, j and n) and temporal (c, g, k and o) cortices (CX) and CA1 hippocampus (d, h, l and p). White arrows in microphotographs show diffuse plaques, black arrows show core plaques and black arrowheads show dense plaques. (B) Quantification of total Aβ+ positive plaques in cortical areas (frontal, parietal and temporal) and hippocampus from ND, DM, AD and ADD patients. Statistical significance: *: p < 0.05.
sequential sections of different hippocampal regions and cortical areas and the results were expressed as number of positive cells per area.

Statistical analysis

The data were expressed as the mean ± SD. Statistical analyses were carried out using the one-way analysis of variance with significance set at \( p < 0.05 \). A Newman–Keuls post-hoc test was performed to determine differences between groups when a significant main effect was found. All statistical analysis was carried out using GraphPad Prism 4.0 (GraphPad Software, Inc.).

Results

**AÎ³ immunostained senile plaques in human brain**

Because different types of senile plaques likely have different pathogenic significance, an accurate description and classification of plaques in AD and ADD patients was performed in the present study. The micrographs selected for the figures in the immunohistochemical analysis are the most representative of all ND, DM, AD and ADD cases studied (Table 1). According to AÎ³ staining, ND and DM patients did not show plaques (Fig. 1A, a–d and e–h, respectively), with the exception of two hippocampus samples from ND1 and ND3 patients (data not shown), in agreement with previous observations reporting that senile plaques may also develop in nondemented elderly humans (Crystal et al., 1988; Dickson et al., 1992; Troncoso et al., 1996). In AD samples, a high density of large amyloid plaques was observed in hippocampus, especially in CA1 and CA3 regions (Fig. 1A, l). A high number of plaques were also found in the upper layers of frontal, parietal and temporal cortices (Fig. 1A, i–k), whereas less plaques were found in deeper layers of these cortical areas. In frontal and parietal cortices the size of the amyloid plaques was smaller than in temporal cortex and hippocampus. ADD samples showed also an elevated number of plaques similar to that observed in AD patients (Fig. 1A, m, o and p). Despite the differences in plaque size observed in AD and ADD samples, the number of amyloid plaques was statistically different \( (p < 0.05) \) only in frontal cortex (Fig. 1B). In ADD parietal cortex amyloid plaques were smaller (Fig. 1A, n) in comparison to AD (Fig. 1A, j), whereas the size of amyloid plaques in temporal cortex and hippocampus was larger in ADD (Fig. 1A, o and p) than in AD patients (Fig. 1A, k and l). Temporal cortex was the region with more AÎ³-positive plaques \( (p < 0.05) \) in both AD and ADD samples (Fig. 1B). No plaques were observed in the cerebellum of all groups studied (data not shown).

**Quantification of AÎ³1–40 plaques in hippocampus**

Because cognitive function is an important parameter in the evaluation and determination of AD progression, the hippocampus is a critical region in AD pathology, and for that reason we focused our AÎ³ plaque quantification study in that brain area. Based on the morphology of AÎ³ plaques we have quantified two major groups: core and dense plaques (Fig. 2A). As no AÎ³ plaques were observed in the hippocampus of DM patients, the data obtained for this group were statistically analysed together with the ND patients (control group). Both AD and ADD samples presented a significant increase in core and dense plaques compared to the control group (Fig. 2B). The number of core plaques was similar in AD and ADD samples, whereas the presence of dense plaques increased significantly in ADD compared to AD \( (p < 0.05) \).

When the data were classified according to gender (Fig. 2C), the number of dense plaques in women was 2-fold higher than in men for AD patients \((1.5 ± 0.3 \text{ and } 0.8 ± 0.2, \text{ respectively;} p < 0.05)\), but no significant gender differences were detected for core plaques. Similarly, in ADD women patients the number of core and dense plaques \((2.5 ± 0.4 \text{ and } 3.0 ± 0.1, \text{ respectively})\) was higher than in men.
(1.8±0.1 and 1.5±0.6, respectively), and it reached statistical significance for dense plaques (p<0.05).

Multiple staining for AGEs, Aβ and thioflavin T

In order to characterise the Aβ fibril content of senile plaques we stained brain sections with the fluorescent dye thioflavin T (ThT), which binds amyloid fibrils (Krebs et al., 2005; LeVine, 1993). In hippocampus and temporal cortex of AD patients, ThT staining was moderate in dense plaques and strong in core plaques (Fig. 3G). ThT positive core plaques co-localized mostly with AGEs and Aβ, whereas dense plaques showed poor co-localization (Figs. 3H–I, respectively). A similar histological pattern was observed in cortical areas (data not shown).

The intensity of ThT staining was similar in ADD and AD samples but ADD plaques were larger (Fig. 3J). Correlation between AGEs, Aβ and ThT was observed, and an increase in the immunostaining of AGEs and Aβ was evident in core and dense plaques of ADD compared to AD patients (Figs. 3K and L). Both dense and core plaques were larger in ADD than in AD, suggesting a mature plaque stage development (Figs. 3J and K). The hippocampus presented larger number of AGE-positive plaques compared to other brain areas (data not shown). In ADD patients we observed that AGE and Aβ immunostaining was present at all stages of plaque development (Figs. 3K and L).

RAGE immunostaining

In the hippocampus of ND and DM, we did not detect RAGE-positive cells (Fig. 4A, a and b, and c and d, respectively), but in AD patients RAGE expression was detected in all hippocampal regions, especially in hilar cells corresponding to dentate gyrus neurons (Fig. 4A, e) and in CA3 pyramidal neurons (Fig. 4A, f). On the other hand, patients which suffered simultaneously Alzheimer’s disease and diabetes exhibited an increased immunostaining for RAGE protein in hippocampal regions (Fig. 4A, g and h).

Quantitative analysis made in dentate gyrus (Fig. 4B) showed that the number of RAGE positive cells in the granular layer was similar in AD (64.8±8.8), DM (61.0±28.0) and ND (45.6±18.2), whereas a significant increase was observed in ADD patients (139.4±32.8). For hilar cells (Fig. 4C), RAGE staining was low in ND (11.0±2.6) and DM (34.0±8.0) whereas the number of RAGE-positive cells increased significantly in AD (176.2±44.0), and especially in ADD (347.4±40.8).

Tau immunostaining

Abundant neurons were positively labeled for Tau aggregates in frontal cortex (Fig. 5A, a and b) and hippocampus of AD and ADD samples (Fig. 5A, c and d), with the larger stained areas present in ADD.
patients. Quantitative analysis (Fig. 5B) demonstrated that the number of Tau positive cells increased significantly in ADD compared to AD (p < 0.001 and p < 0.01 for hippocampus and frontal cortex, respectively), whereas Tau immunostaining was not detected in cerebellum and other brain areas of ND and DM samples (data not shown).

Microglial reactivity

In AD patients, microglial reactivity colocalized with Aβ deposits inside the plaques, independently of the developmental stage of the amyloid plaque (Figs. 6A–C), but with major microglial reactivity found inside Aβ dense plaques, particularly in hippocampus (Fig. 6A). In contrast, in ADD patients, most microglial cells were found inside the diffuse and dense Aβ plaques and a small number of activated microglia were observed outside Aβ dense plaques (Figs. 6D–F).

Discussion

A relationship between diabetes mellitus and Alzheimer’s disease has not been established, but several studies suggest that patients with diabetes mellitus have a major risk of developing Alzheimer’s disease (Arvanitakis et al., 2004; Luchsinger et al., 2004; Takeuchi and Yamagishi, 2008). AGEs and RAGE have been implicated in the chronic complications of DM, and have been reported to play an important role in the pathogenesis of AD (Brownlee et al., 1984; Monnier and Cerami, 1982; Vlassara et al., 1994). The formation and deposition of AGEs in the tissues and vessels occurs mainly in hyperglycemia (present in DM), oxidative stress (AD and DM) and amyloidosis processes (AD). In the present work we observed several common histological features in patients with both diseases.

Over-expression of Aβ and AGE in ThT plaques in ADD patients

Aβ-positive senile plaques are more prevalent in temporal cortex, whereas Tau immunostaining shows a higher expression in hippocampus, suggesting that the number of plaques is not directly related with the cell damage and the neurodegenerative processes associated. Several studies have shown that the toxicity of senile plaques is related with the presence of Aβ fibrillar forms (Serpell, 2000; Walsh et al., 1999). Our results showed high intensity of ThT staining in hippocampus reinforcing the hypothesis that Aβ fibrillar forms are directly related with neuronal damage. In hippocampus of AD and ADD patients, many plaques present Aβ and AGEs immunolabeling and co-localize with ThT staining. AGE labeling is associated with amyloid plaques (Smith et al., 1994); however, its role in the various developmental stages of plaque pathology is still unknown (Smith et al., 1995). It has been proposed that Aβ-mediated AGE generation is a late event in the evolution of AD pathology and that it results from
free radical generation by Aβ itself (Mattson et al., 1995; Guix et al., 2009). ThT and AGE staining are more intense in core plaques of ADD compared to AD patients, suggesting that ADD plaques are in an earlier developmental stage than in AD patients, as previously reported by Sasaki et al. (1998), and could be more reactive or toxic, and consequently may be implicated in neuronal damage. The Aβ fibrillogenetic pathway proceeds through a series of protofibrillar and fibrillar intermediates of different cytotoxicity (Arimon et al., 2005), and it has been proposed that certain loosely structured fibrillar species might act as reservoirs of highly toxic soluble forms (Bravo et al., 2008).

Another important observation is that in ADD patients the number of dense Aβ-positive plaques in hippocampus increased significantly compared to AD, suggesting an overlap of different temporal features of AD pathology in ADD patients. The increased dense plaques could be due to the presence of AGEs, which stabilize Aβ aggregation. Previous studies report high levels of AGEs in both amyloid plaques and NFTs of AD patients (Sasaki et al., 1998; Vitek et al., 1994).

The number of dense plaques in AD and ADD was higher in women patients, in agreement with epidemiological studies reporting a greater incidence of AD pathology in women (Brookmeyer et al., 1998; Overmyer et al., 1999) even after adjustment for age, which is the most important risk factor for AD (Hy and Keller, 2000). Although the precise relationship of AD with gender is unknown, recent studies suggest that both an increase in Aβ production and a decrease in Aβ degradation may contribute to the higher risk of AD in women (Hirata-Fukae et al., 2008).

Enhancement of neuronal cell damage in ADD patients

Other factors in addition to plaque formation are involved in the AD neurodegenerative process, such as Tau protein hyperphosphorylation and the overexpression of cytokines in the extracellular matrix, which contribute to microglial cell activation (Walker et al., 1995). Binding of AGEs or/and Aβ to AGE receptor in neurons has also been suggested to set off a cascade of events that result in oxidative stress and nuclear transcription factor-κB activation (Akama et al., 1998; Bales et al., 1998). This leads to increased production of macrophage–colony stimulating factor, which activates microglia in the vicinity of Aβ plaques, resulting in the elaboration of potentially neurotoxic mediators (Yan et al., 1997).

In our study, we observed a significant increase of both RAGE-positive cells and the size of aggregated Tau-positive cells in hippocampal regions (hilus, pyramidal cells and granular layer) of ADD patients compared to AD. Furthermore, the increase in aggregated Tau and RAGE proteins, which are indicators of cell damage and neuronal cell death, correlates with high numbers of dense Aβ plaques in ADD patients, but not in AD. These immunohistochemical findings

![Figure 5](image_url)
suggest that the increase of RAGE by AGEs or Aβ results in NF-κB activation, thereby triggering a positive feedback loop in which RAGE expression is up-regulated and thus enhances the binding capacity of the ligands (AGE and Aβ) and mediates inflammation caused by glial cells. These events perpetuate another cycle of oxidative stress, Tau protein hyperphosphorylation and neuronal cell death (Maczurek et al., 2008; Schmidt et al., 1999; Stern et al., 2002; Yan et al., 2008). The up-regulation of RAGE and increase in Tau aggregates is more accentuated in hilar and pyramidal cells than in granular cells. In hippocampus of several neurodegenerative models it has been observed that pyramidal and hilar cells are vulnerable to cell damage whereas granular cells are resistant (Becker et al., 1999; Djebarli et al., 2001; Pollard et al., 1994).

In summary, RAGE-mediated oxidative vicious cycles caused by AGEs and Aβ are likely to be important contributors to neurodegeneration in AD and more severe in diabetic patients with AD, even though their mean ages are slightly low. As long as the neuronal damage is reversible, anti-oxidant medication (e.g., medications interfering with the Aβ and AGE–RAGE pathways) may be interesting therapeutics for AD and DM treatment (Yamagishi et al., 2008).

Conclusions

In the present report we conclude that the classic cellular features of Alzheimer’s disease are more pronounced in diabetic patients, who show increased number of amyloid plaques and, in some brain areas, also increased size. At the same time, these patients present an up-regulation of RAGE and Tau protein, which suggests more brain damage in the hippocampus. Therefore, these findings suggest that in diabetic patients, AD pathology is more severe and the progression of the disease is faster. In addition, we report for the first time that female diabetic patients who develop plaques exhibit an AD phenotype more severe than male patients, independently of the patient’s age. These results suggest that blocking the AGE–RAGE system and using different antioxidants could potentially reduce AGE-mediated oxidative stress and Tau protein hyperphosphorylation in neurons, thus protecting and preventing the accumulation of potentially neurotoxic products and attenuating the progression of Alzheimer's disease.

Disclosure statement

There are no actual or potential conflicts of interest. Appropriate ethical approvals were obtained with regards to the procedures used concerning human subjects.

Acknowledgments

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References


Fig. 6. Tomato lectin histochemistry for microglial cells stained in brown color and Aβ plaques stained in blue color, in CA3 hippocampal region of Alzheimer’s disease, AD (A–C) and AD with diabetes, ADD (D–F). Large arrows show internal microglial cell activation within Aβ plaques and thin arrows show microglial cell activation around Aβ plaques.

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