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Advanced glycation end products are mitogenic signals and trigger cell cycle reentry of neurons in Alzheimer's disease brain

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ABSTRACT

Neurons that reenter the cell cycle die rather than divide, a phenomenon that is associated with neurodegeneration in Alzheimer's disease (AD). Reexpression of cell-cycle related genes in differentiated neurons in AD might be rooted in aberrant mitogenic signaling. Because microglia and astroglia proliferate in the vicinity of amyloid plaques, it is likely that plaque components or factors secreted from plaque-activated glia induce neuronal mitogenic signaling. Advanced glycation end products (AGEs), protein-bound oxidation products of sugar, might be one of those mitogenic compounds. Cyclin D1 positive neurons are colocalized with AGEs or directly surrounded by extracellular AGE deposits in AD brain. However, a direct proof of DNA replication in these cells has been missing. Here, we report by using fluorescent in situ hybridization that consistent with the expression of cell cycle proteins, hyperploid neuronal cells are in colocalization with AGE staining in AD brains but not in nondemented controls. To complement human data, we used apolipoprotein E-deficient mice as model of neurodegeneration and showed that increased oxidative stress caused an intensified neuronal deposition of AGEs, being accompanied by an activation of the MAPK cascade via RAGE. This cascade, in turn, induced the expression of cyclin D1 and DNA replication. In addition, reduction of oxidative stress by application of α-lipoic acid decreased AGE accumulations, and this decrease was accompanied by a reduction in cell cycle reentry and a more euploid neuronal genome.

1. Introduction

In Alzheimer's disease (AD) brain and other neurodegenerative disorders, cell cycle events of neurons are closely related to selective cell death in the hippocampus as well as in the frontal, occipital, and temporal cortex (Braak and Braak, 1991). Although terminally differentiated neurons have previously been considered to stay quiescently in the G0 phase, neurons in AD show signs of cell cycle reentry; precisely, they leave the G0 state, pass through G1, and become arrested at the G1/S or the G2/M checkpoint (Arendt, 2000; Kruman, 2004; Nagy, 2000; Yang et al., 2001). For example, cyclin D1, a G1 phase marker, cyclin E, a G1/S phase marker (Hoozemans et al., 2002), and the cdk inhibitor p16, a protein that signals reentry into the cell cycle (McShea et al., 1997), were found to be abnormally expressed in AD neurons. In addition, dysregulation of cell cycle kinases, in particular cdk2 and cdk5, might contribute to the progression of AD (Baumann et al., 1993; Lew and Wang, 1995; Vincent et al., 1997). Whether the detection of cell cycle enzymes is indicative of a true cell cycle or only the dysregulation of enzyme synthesis (Yang et al., 2001) remains open as the direct proof of DNA replication has been lacking. Nevertheless, several groups found direct evidence for attempted cell cycling in AD neurons and proposed that the ultimate death of nerve cells can be attributed to the chromosomal imbalance caused by the polyploidy status of their genome, that is, 3n-4n genome (Iourov et al., 2009; Mosch et al., 2007; Yang et al., 2006). In this cell cycle-like process, neurons survive for many months until they get a second still unknown "hit" (Yang and Herrup, 2005), probably resulting in apoptotic cell death.

Mitogenic signals may force neurons to leave the G0 phase by overstimulation of the p42/44 MAPK pathway resulting in activation of nuclear factor-κB (NF-κB) and in upregulation of NF-κB-dependent cell cycle proteins (Arendt et al., 2000, 1995; Gärtner et al., 1999). A potential source of mitogenic signals is advanced glycation end products (AGEs), which are able to elicit cell cycle reentry of neuronal cells via RAGE dependent MAPK pathway activation, in an immortalized cell line kept in serum deprived medium (Schmidt et al., 2007). Specifically, 2 transfected mouse Neuro2a cell lines (Sajithlal et al.,

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2002), either expressing human full-length RAGE or carrying a mutation that leads to the cytosolic deletion of RAGE were used to explore AGE-dependent signal transduction (Schmidt et al., 2007). However, an in vivo proof of the mitogenic effect of AGEs via RAGE is still missing. The first description of AGEs in AD dates back to 1994 when it was demonstrated that plaque fractions of AD brain contain higher levels of AGEs than age-matched control brains (Vitek et al., 1994). Although some authors have suggested that AGEs are very late markers of AD, it is now widely accepted that early AGEs are active participants in the progression of AD by rendering modified protein deposits insoluble and by enhancing the inflammatory response (Smith et al., 1995). Here, we hypothesize that AGEs exert their mitogenic effect in vivo in apolipoprotein E (ApoE) deficient (*ApoE−/−*) mice, a well-established mouse model of neurodegeneration. These mice show AD-typical synaptic and cytoskeletal alterations (Masliah et al., 1995) and an increased oxidative stress status (Ramassamy et al., 1999). We studied whether elevated oxidative stress in this mouse model supports the generation of AGEs, among them AGE-modified β-amyloid cross-links. If true, then suppression of AGE formations by an α-lipoic acid (LA)-based antioxidative therapy would be expected to prevent mitogenic signaling and polyploidy.

2. Methods

2.1. Preparation of AGE (HSA-AGE) and control proteins

To a sterile nonpyrogenic infusion solution containing 20% human serum albumin (HSA-inf) (Baxter, Unterschleißheim, Germany), 1 M glucose, and 200 mM Na_2HPO_4 were added, and the pH was adjusted to 8.0 using solid NaOH. The solution was sterile filtered and incubated at 50 °C for 8 weeks. Afterward, HSA-AGEs are generated. As a control, an HSA infusion solution was prepared analogously but without glucose and incubated under the same conditions (HSA-inc). HSA-inf, HSA-inc, and HSA-AGE were intensively dialyzed against 0.9% NaCl at 4 °C, and the protein content was assayed by the BCA method (Pierce Biotechnology, Rockford, IL, USA). Both HSA and HSA-AGE samples were tested for endotoxin using the Limulus amebocyte assay (E-Toxate, Sigma, Taufkirchen, Germany). Stock samples were made to 120 μM and stored at −20 °C until use.

2.2. Animal model

Male C57BL/6 (ApoE competent; wild-type mice) and male apolipoprotein (Apo) E deficient mice with identical genetic background (*ApoE−/−*) (Charles River Wiga, Sulzfeld, Germany) at the age of 8, 24, and 78 weeks (n = 48) were used for this study. Mice were housed in standard cages in a temperature-controlled room (22 °C ± 2 °C) on a 12 hour light/dark cycle (light on at 6 AM) with free access to food (4.2% fat) and water under specific pathogen-free conditions. For reduction of oxidative stress, an additional set of *ApoE−/−* mice received a daily dose of LA (0.4 mg/kg body weight) at the age of 4 weeks for 74 weeks (n = 7). The experimental protocol was approved by the local Animal Research Committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei of the state Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-1.1-031/12), and all animals received humane care according to the German legislation on protection of animals and the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23 revised 1985).

2.3. Sampling and assays

All animals were exsanguinated by puncture of the vena cava inferior for immediate separation of plasma, followed by harvest of brain tissue. Measurement of plasma malondialdehyde (MDA), serving as an indicator of lipid peroxidation and oxidative stress, was performed using the MDA-586 method according to the manufacturer's instructions (OxisResearch, Portland, OR, USA). One hemisphere of harvested brain was processed for Western blot analysis and the other for immunohistochemistry as well as for fluorescent in situ hybridization (FISH).

2.4. Human brain tissue

Brains used in this study were obtained from nondemented controls (n = 4) and from a patient with AD who died without a history of neuropsychiatric disorder, mental impairment or, diabetes mellitus. There had to be clear evidence that the patient was alert, well oriented, and capable of functioning relatively independent shortly before death. The neuropathologic diagnosis of AD (score A3 and B3 [former Braak V and VI], 78 years old [mean ± 2.8], n = 4) was examined with the thioflavin S method and the Gallyas staining for demonstration of neuritic plaques, neurofibrillary tangles, and neuropil threads in the entorhinal cortex, hippocampus, frontal cortex, temporal cortex, and occipital cortex according to the guidelines of National Institute on Aging-Alzheimer's Association (Montine et al., 2012) and to the suggestions of Braak and Braak (Braak and Braak, 1991). Case recruitment, acquisition of patients' personal data, performing the autopsy, and handling the autoptic material were performed in accordance with the Helsinki Declaration and the convention of the council of Europe on Human Rights and Biomedicine and had been approved by the responsible Ethical Committee of Leipzig University.

2.5. Chromogenic in situ hybridization (CISH) and FISH

2.5.1. Human probe

Chromosome 11 probe was made from overlapping bacterial artificial chromosomes obtained from RPCI11 library (reference number 407N16). This probe comes from the region of human chromosome 11 that encodes the β-site amyloid precursor protein (APP)-cleaving enzyme 1, located on chromosome 11 (q23.3-q24.1). Human BAC sequence was labeled by standard nick-translation protocols using digoxygenin-labeled dUTP for at least 90 minutes at 15 °C and stored at −20 °C. After digoxygenin labeling, the probe was concentrated with human Cot-1 (Invitrogen, San Diego, CA, USA) for at least 2 hours at −80 °C to block repeat human sequences.

2.5.2. Mouse probes

The mouse-specific direct labeled satellite DNA probe was commercially purchased (StarFISH, Cambio, Cambridge, UK).

2.5.3. Immunohistochemistry

For the double staining, namely CISH and immunohistochemistry of AGE, human brain tissue sections were deparaffinized, pretreated with 30% Pretreatment Powder (Ventana, Tucson, AZ, USA) for 15 minutes at 45 °C, and then digested with protease (Ventana; 0.25 mg/mL) for 25 minutes at 45 °C. After rinsing, slides were dehydrated through alcohols and allowed to air dry. The probe was applied to the section, and a glass coverslip was overlaid to prevent evaporation. Slides were heated to 90 °C for 12 minutes to denature the DNA and then incubated overnight at 37 °C. Slides were soaked in 50% formamide/2× SSC for 5 minutes to remove the coverslip. Then they were rinsed twice in the same buffer for 15 minutes at 37 °C and once in 0.1× SSC for 30 minutes and transferred to phosphate buffer at room temperature (RT). After blocking, the sections were incubated with a directly labeled primary antibody (anti-DIG-AP, Fab fragments, Roche, Penzberg, Germany) at a dilution of 1:100 (in PBS and 1% GNS). The detection was performed with Vector Red Alkaline