Increased poly(ADP-ribosyl)ation of nuclear proteins in Alzheimer’s disease

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Summary

Experimental studies indicate that overactivation of the DNA repair protein poly(ADP-ribose) polymerase (PARP) in response to oxidative damage to DNA can cause cell death due to depletion of NAD+. Oxidative damage to DNA and other macromolecules has been reported to be increased in the brains of patients with Alzheimer’s disease. In the present study we sought evidence of PARP activation in Alzheimer’s disease by immunostaining sections of frontal and temporal lobe from autopsy material of 20 patients and 10 controls, both for PARP itself and for its end-product, poly(ADP-ribose). All of the brains had previously been subjected to detailed neuropathological examination to confirm the diagnosis of Alzheimer’s disease or, in the controls, to exclude Alzheimer’s disease-type pathology. Double immunolabelling for poly(ADP-ribose) and microtubule-associated protein 2 (MAP2), glial fibrillary-acidic protein (GFAP), CD68, Aβ-protein or tau was used to assess the identity of the cells with poly(ADP-ribose) accumulation and their relationship to plaques and neurofibrillary tangles. Both PARP- and poly(ADP-ribose)-immunolabelled cells were detected in a much higher proportion of Alzheimer’s disease (20 out of 20) brains than of control brains (5 out of 10) (P = 0.0018). Double-immunolabelling for poly(ADP-ribose) and markers of neuronal, astrocytic and microglial differentiation (MAP2, GFAP and CD68, respectively) showed many of the cells containing poly(ADP-ribose) to be neurons. Most of these were small pyramidal neurons in cortical laminae 3 and 5. A few of the cells containing poly(ADP-ribose) were astrocytes. No poly(ADP-ribose) accumulation was detected in microglia. Double-immunolabelling for poly(ADP-ribose) and tau or Aβ-protein indicated that the cells with accumulation of poly(ADP-ribose) did not contain tangles and relatively few occurred within plaques. Our findings indicate that there is enhanced PARP activity in Alzheimer’s disease and suggest that pharmacological interventions aimed at inhibiting PARP may have a role in slowing the progression of the disease.

Keywords: Alzheimer’s disease; oxidative stress; DNA damage; poly(ADP-ribose) polymerase; NAD+-ADP-ribosyltransferase

Abbreviations: ADP-ribose = adenosine 5′-diphosphoribose; AGE = advanced glycation end-product; Aβ-protein = amyloid-β protein; CD68 = cluster-of-differentiation antigen 68; GFAP = glial fibrillary acidic protein; ISEL = in situ end-labelling; MAP2 = microtubule-associated protein 2; NAD = nicotinamide adenine dinucleotide; PARP = poly(ADP-ribose) polymerase (or NAD+-ADP-ribosyltransferase); PBS = phosphate-buffered saline

Introduction

Alzheimer’s disease has long been known to cause loss of neurons from several regions of the brain, although the mechanisms of cell loss have not been established. Neuropathological studies have shown fragmentation of nuclear DNA, as demonstrated by in situ end-labelling (ISEL), in a much higher proportion of neurons, oligodendrocytes, astrocytes and microglia in the brains of patients with Alzheimer’s disease than in age-matched controls (Su et al., 1994; Dragunow et al., 1995; Lassmann et al., 1995; Troncoso et al., 1996). These observations have been interpreted as evidence that the loss of neurons is due to apoptosis, possibly initiated by Aβ-protein or other inducers of oxidative stress. The extent of morphologically demonstrable apoptosis in Alzheimer’s disease is, however, very limited and difficult to reconcile with the high density of ISEL-positive cells. It remains unproven that the fragmentation of nuclear DNA in Alzheimer’s disease signifies apoptosis, particularly in the context of non-mitotic cells.

The DNA damage associated with oxidative stress is known to activate DNA repair proteins, including poly(ADP-ribose) polymerase (PARP). PARP is a zinc-finger DNA-binding protein that is activated by single- or double-strand...
Table 1: Cases of Alzheimer’s disease and controls

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex</th>
<th>Autopsy delay (h)</th>
<th>Autopsy diagnoses</th>
<th>PARP labelling</th>
<th>Poly(ADP-ribose) labelling</th>
<th>Aβ-protein deposition*</th>
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*Numerous neuritic and variable numbers of diffuse plaques of Aβ-protein were present in the cerebral cortex in all of the cases of Alzheimer’s disease. In the single control with Aβ-protein deposits, these were almost exclusively in the form of diffuse plaques and were not associated with tangles in the neocortex.

Breaks in DNA (Lautier et al., 1990; Szabó et al., 1996; Weinfeld et al., 1997). PARP catalyses the cleavage of NAD⁺ into adenosine 5'-diphosphoribose (ADP-ribose) and nicotinamide, and the covalent attachment of polymers of ADP-ribose to histones and other nuclear proteins, including PARP itself (Satoh and Lindahl, 1992; de Murcia et al., 1997; Szabó and Ohshima, 1997). Recently we showed striking upregulation of PARP and accumulation of poly(ADP-ribose) in neurons and glia of patients who had suffered a cardiac arrest (Love et al., 1998a, b), a finding in keeping with experimental studies showing that PARP is activated in response to free radical-mediated injury to DNA after brain ischaemia and reperfusion (Zhang et al., 1994). Overactivation of PARP in injured cells can cause massive consumption of NAD⁺, with resulting cell death due to energy depletion (Zhang et al., 1994; Eliasson et al., 1997; Endres et al., 1997; Takahashi et al., 1997).

In the present study, increased immunohistochemical expression of PARP and intranuclear accumulation of its end-product poly(ADP-ribose) have been shown to occur in a much greater proportion of brains from patients with Alzheimer’s disease than of control brains. Most of the accumulation of poly(ADP-ribose) occurs in neurons. Our findings raise the possibility that overactivation of PARP may contribute to neuronal loss in Alzheimer’s disease.

Method
We examined sections of the frontal and temporal lobes from 20 patients (age range 64–95 years, mean = 79, SD = 8) who had neuropathologically confirmed Alzheimer’s disease that conformed to the criteria for a diagnosis of ‘definite’ Alzheimer’s disease according to the Consortium to Establish a Registry for Alzheimer’s Disease (Mirra et al., 1991, 1993). We also examined frontal and temporal lobe sections from 10 neurologically normal controls (age range 63–83 years, mean = 73, SD = 7) (Table 1). Most of the cases were obtained from the Frenchay Hospital Dementia Brain Bank. Other diagnoses made at autopsy are included in Table 1.
Single immunolabelling for PARP or poly(ADP-ribose)

Immunohistochemistry for PARP and poly(ADP-ribose) was performed with the appropriate mouse monoclonal antibodies, obtained from Serotec, Oxford, UK (catalogue numbers MCA1522 and MCA1480) [see Aoufouchi (1997) and Kawamitsu et al. (1984) for information concerning the characterization of these antibodies]. Sections were dewaxed in xylene and rehydrated through graded alcohols to water. Those for PARP immunohistochemistry were then microwaved in 0.01 M sodium citrate buffer (pH 6.0), the buffer being brought to boiling point twice over a 5-min period. Non-specific peroxidase activity was quenched by immersing the sections for 30 min in methanol containing 3% \text{H}_2\text{O}_2. The sections were then incubated first in 20% normal goat serum and subsequently overnight at room temperature in anti-PARP diluted 1 : 500 in phosphate-buffered saline (PBS). Bound antibody was visualized by incubation with biotinylated goat anti-mouse/rabbit immunoglobulin (Duet Kit, Dako, Cambridge, UK) and streptavidin–biotin–horseradish peroxidase, and reaction with 0.01% \text{H}_2\text{O}_2 and dianaminobenzidine. The sections for poly(ADP-ribose) immunohistochemistry were digested with trypsin (0.05 g/100 ml) for 10 min at 37°C, incubated in 20% normal goat serum and subsequently overnight at room temperature in anti-poly(ADP-ribose) diluted 1 : 200 in PBS. Bound antibody was visualized as described for PARP. Negative controls comprised sections immunostained as above apart from omission of the primary antibody.

Double immunolabelling for poly(ADP-ribose) and MAP2, GFAP, CD68, Aβ-protein or tau

Sections were immunostained for poly(ADP-ribose) as described above, apart from an initial preincubation in 100% formic acid for 30 min at room temperature of the sections that were also to be immunostained for Aβ-protein. The sections were washed in water and PBS, again incubated for 20 min in 20% normal goat serum and then incubated at room temperature in monoclonal antibody to MAP2 (obtained prediluted from Zymed, San Francisco, Calif., USA), CD68 (Dako; diluted 1 : 160 000 in PBS, overnight incubation), glial filibrillary acidic protein (GFAP) (Dako; diluted 1 : 400 in PBS, overnight incubation), Aβ-protein (Dako; diluted 1 : 2000, overnight incubation) or tau (Sigma, UK; diluted 1 : 2000, 20 min incubation). The sections were washed in PBS, incubated with biotinylated goat anti-mouse/rabbit immunoglobulin (Duet Kit, Dako) and streptavidin–biotin–horseradish peroxidase, and bound second antibody–peroxidase was visualized by reaction with Vector VIP peroxidase according to the manufacturer’s instructions (Vector, UK). Control sections, immunostained as above apart from the omission of one or both primary antibodies, were included in each batch of reactions.

Statistical analysis

Fisher’s exact test was used for comparison of the relative numbers of Alzheimer’s disease and control brains containing cells that could be immunolabelled for PARP or poly(ADP-ribose).

Results

Single immunolabelling for PARP or poly(ADP-ribose)

Sparse to moderate numbers of cells in the neocortex and hippocampus (see below) were immunolabelled for PARP in all 20 of the Alzheimer’s disease brains but also in some (5 out of 10) of the controls (Table 1; Fig. 1). Poly(ADP-ribose) was detected in at least occasional cells in the neocortex (Fig. 2) and hippocampus in all 20 of the Alzheimer’s disease cases and in the same proportion of controls as showed PARP immunolabelling (5 out of 10), although not in all of the same cases (Fig. 2A and B). Statistical analysis showed the difference between the proportions of Alzheimer’s disease and control brains that were immunolabelled with the two antibodies to be highly significant (\(P = 0.0018\)).
The immunostaining for both antigens was predominantly nuclear but combined cytoplasmic and nuclear PARP immunoreactivity was seen in some of the cells (Fig. 1). The affected cells were predominantly scattered pyramidal neurons in the subiculum and neurons and glia in the cerebral neocortex. The density of immunostained neurons varied from case to case: in some only occasional cells were labelled, in others as many as 10% of the cells were labelled in some parts of the cortex (Fig. 2). Subjective assessment did not reveal a consistent relationship between the density and distribution of PARP- or poly(ADP-ribose)-immunolabelled nuclei and the extent of Aβ-protein deposition or tangle formation. Many of the labelled cells were in laminae 3 and 5 and were clearly recognizable as pyramidal neurons. Some of the smaller labelled nuclei appeared to be glial. No PARP or poly(ADP-ribose) immunoreactivity was evident in the granule cells of the dentate fascia and there was no labelling of vascular endothelial cells in the grey matter. Cases positive for PARP contained scattered immunolabelled cells in the white matter. Only sparse cells in the white matter were immunolabelled for poly(ADP-ribose) in any of the brains.

Double immunolabelling for poly(ADP-ribose) and MAP2, GFAP or CD68

Double immunolabelling confirmed that most of the cells with nuclear accumulation of poly(ADP-ribose) also showed the cytoplasmic MAP2 immunoreactivity of neurons (Fig. 3). The shape of the neurons was clearly demonstrated by the distribution of cytoplasmic labelling for MAP2, and this revealed that many of the neurons with accumulation of poly(ADP-ribose) were pyramidal cells of small to medium size. Sections immunostained for both poly(ADP-ribose) and GFAP included occasional GFAP-immunoreactive astrocytes that contained nuclear poly(ADP-ribose) (Fig. 4). Most of the astrocytes did not, however, show poly(ADP-ribose) immunoreactivity. Although sections immunostained for both CD68 and poly(ADP-ribose) did contain scattered cells that
cytoplasmic immunoreactivity for tau protein. -protein and none showed β incorporated within deposits of A. relatively few of the poly(ADP-ribose)-containing cells were tangles or neuropil threads (Figs 5 and 6). Despite this, A-protein deposits and tau-immunoreactive neurofibrillary β containing poly(ADP-ribose) were in close proximity to disease cases. Inevitably, therefore, many of the cells abundant within the sections of brain from the Alzheimer’s disease cases. As expected, both A-protein or tau reacted with one or other antibody, none of the cells was double-labelled with both antibodies.

**Double immunolabelling for poly(ADP-ribose) and Aβ-protein or tau**

As expected, both Aβ-protein and tau were relatively abundant within the sections of brain from the Alzheimer’s disease cases. Inevitably, therefore, many of the cells containing poly(ADP-ribose) were in close proximity to Aβ-protein deposits and tau-immunoreactive neurofibrillary tangles or neuropil threads (Figs 5 and 6). Despite this, relatively few of the poly(ADP-ribose)-containing cells were incorporated within deposits of Aβ-protein and none showed cytoplasmic immunoreactivity for tau protein.

**Discussion**

In the present study we have shown that both PARP and poly(ADP-ribose) can be detected immunohistochemically in the frontal and temporal cortex much more commonly in Alzheimer’s disease than control brains. The demonstration that brains from patients with Alzheimer’s disease accumulate poly(ADP-ribose) indicates that there are increased levels of functional PARP enzyme and implies an elevated consumption of NAD⁺ by the affected cells. PARP is activated by single- or double-strand breaks in DNA (Lautier et al., 1990; Szabó et al., 1996; Weinfeld et al., 1997) and the finding that this DNA repair enzyme is overexpressed in Alzheimer’s disease is in keeping with many previous reports of oxidative damage to DNA and other macromolecules in this disease, and with data from studies in which damage to DNA was demonstrated by ISEL (Su et al., 1994; Benzi and Moretti, 1995; Dragunow et al., 1995; Lassmann et al., 1995; Smith et al., 1995; Troncoso et al., 1996; Lyras et al., 1997).

The precise pathogenesis of the damage to DNA in Alzheimer’s disease is still unclear but probably reflects a general increase in the production of reactive oxygen species in the affected brains. Other evidence of oxidative stress in this disease includes the demonstration of increased levels of protein carbonyls and nitrotyrosine (Hensley et al., 1995; Good et al., 1996; Lyras et al., 1996; Smith et al., 1997), accumulation of products of lipid peroxidation (Sayre et al., 1997) and the expression of haem oxygenase-1 in association with neurofibrillary tangles, dystrophic neurites, granulovacuolar degeneration and reactive astrocytes in Alzheimer’s disease (Smith et al., 1994; Schipper et al., 1995). Several studies have shown Aβ-protein to induce lipid peroxidation and carbonyl formation in vitro (Harris et al., 1995; Mark et al., 1996) and, possibly as a result, to impair glutamate transporter-mediated uptake of glutamate (Harris et al., 1995b; Keller et al., 1997). The resulting accumulation of glutamate may itself lead to the generation of reactive oxygen species by its binding to NMDA (N-methyl-D-aspartate) receptors, causing influx of calcium ions, activation of neuronal nitric oxide synthase and production of nitric oxide (Choi, 1994). A further potential source of oxidative stress in Alzheimer’s disease is the accumulation of tau protein modified by advanced glycation end-products (AGEs) (Yan et al., 1994). AGE-recombinant tau protein was shown to cause the production of reactive oxygen species when it was introduced into the cytoplasm of neuroblastoma cells in vitro (Yan et al., 1994).

There is good evidence that overactivation of PARP, with resulting energy depletion due to consumption of NAD⁺, plays a major role in cell death in brain ischaemia. The use of PARP inhibitors greatly reduces the extent of brain infarcts, and PARP knock-out mice are highly resistant to ischaemic brain damage (Eliasson et al., 1997; Endres et al., 1997; Takahashi et al., 1997). PARP inhibitors have been used as adjunctive therapy for several other diseases in which oxidative damage to DNA and overactivity of PARP have...
been implicated. Nicotinamide (niacinamide), in particular, has been found to be of benefit in delaying the progression of recent-onset insulin-dependent diabetes mellitus (Pozzilli et al., 1995, 1996; Elliott et al., 1996; Gale, 1996; Pozzilli et al., 1996), in bullous pemphigoid (Poskitt and Wojnarowska, 1995; Hornschuh et al., 1997), lichen planus pemphigoides (Fivenson and Kimbrough, 1997) and osteoarthritis (Jonas et al., 1996). The fact that PARP expression is elevated in Alzheimer’s disease suggests that PARP inhibitors may have a therapeutic role in this disorder too. It may be relevant that the administration of NADH, which would be expected to counteract PARP-mediated deoxidization of the oxidized form of this coenzyme, was found to be of benefit in Alzheimer’s disease (Birkmayer, 1996).

An important confounding factor in interpreting the significance of oxidative damage in Alzheimer’s disease in the present study, as in all of the previous autopsy studies of this disorder, is the potential contribution of agonal disease processes, such as septicemia and hypoxia, that are themselves inducers of oxidative stress. Differences in the time course of expression of PARP and poly(ADP-ribose) in the context of a stimulus that is only agonal or transitory may indeed explain the disparity in the labelling of these two antigens in a few of the control brains. Although the possible influence of agonal processes cannot be ignored in an autopsy study, in the present series terminal illnesses such as bronchopneumonia were as common in the controls as in the cases of Alzheimer’s disease, despite which significantly fewer of the control brains had evidence of PARP activation. More information about the role of oxidative stress in Alzheimer’s disease may, in time, be obtained from studies of transgenic mouse models, in which the complication of agonal disease processes can be avoided. Although to date none of these models reproduces all aspects of the human disease, it is of interest that the immunohistochemical expression of Cu-Zn-superoxide dismutase and haem oxygenase-1, two markers of oxidative stress, was recently reported to be greatly increased in aged mice overexpressing a mutant amyloid precursor protein transgene and not in wild-type controls of the same age (Pappolla et al., 1998).

In conclusion, we have shown that a much higher proportion of Alzheimer’s disease than of control brains show evidence of increased activity of the DNA repair protein PARP. This finding is consistent with previous observations that Alzheimer’s disease brains also show more DNA damage and suggests that pharmacological interventions aimed at reducing the activity of PARP may be of benefit in this disorder. Further studies are needed to determine the time-course of PARP activity in relation to the stage of disease and whether or not the distribution of PARP activity in the early stages correlates with that of the later neuronal loss.

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