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Detection of oxidative DNA damage in lymphocytes of patients with Alzheimer's disease

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Oxidative damage to DNA may play an important role in both normal ageing and in neurodegenerative diseases. The deleterious consequences of excessive oxidations and the pathophysiological role of reactive oxygen species have been intensively studied in Alzheimer's disease. Although the role of oxidative stress in the aetiology of Alzheimer's disease is still not clear, the detection of an increased damage status in the cells of patients could have important therapeutic implications. The levels of oxidative damage in peripheral lymphocytes of 24 Alzheimer's disease patients and of 21 age-matched controls were determined by comet assay applied to freshly isolated blood samples with oxidative lesion-specific DNA repair endonucleases (endonuclease III for oxidized pyrimidines, formamidopyrimidine glycosylase for oxidized purines). It was demonstrated that Alzheimer's disease is associated with elevated levels of oxidized pyrimidines and purines ($p < 0.0001$) as compared with age-matched control subjects. It was also demonstrated that the comet assay is useful as a biomarker of oxidative DNA damage when used with oxidative lesion-specific enzymes.

Keywords: Alzheimer's disease, oxidative stress, comet assay, lesion-specific endonucleases.

Introduction

The brain can be particularly vulnerable to oxidative damage because it has high energy requirements and a high oxygen consumption rate, and there is a growing body of evidence suggesting that this oxidative injury is involved in the pathogenesis of Alzheimer's disease (AD). This concept is originally derived from the free radical hypothesis of ageing, which states that an age-related excess of reactive oxygen species (ROS) results in an accumulation of damage to major cell components (Pratico and Delanty 2000). AD is the most frequent form of dementia and affects >5% of the population over the age of 65 years. Numerous hypotheses have been proposed to explain the neurodegenerative mechanisms that occur in AD. One hypothesis is that oxidative stress plays an important role in degenerative neuronal death and disease progression. Several studies have demonstrated enhanced levels of lipid peroxidation, protein oxidation, DNA oxidation and mitochondrial oxidation in AD (Smith *et al.* 1991, Mecocci *et al.* 1994, Sayre *et al.* 1997, Mecocci *et al.* 1998). The important role of oxidative stress in other neurodegen-

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erative disorders such as multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis and muscular dystrophy has also been reported (McCord 2000).

Oxidative damage can originate from endogenous sources as a result of leakage of ROS from mitochondria during respiration. ROS can attack almost any cellular structure or molecule. Oxidative damage to DNA occurs as a result of interaction of DNA with ROS such as DNA–protein cross-links, damage to the deoxyribose-phosphate backbone as well as specific chemical modifications of purine and pyrimidine bases (Zwart *et al.* 1999, Scandalios 2002). Oxidative base modifications can result in mutations, whereas oxidation of deoxyribose moieties can induce base release or strand breaks. *In vivo* damaged DNA is repaired by endonucleases and glycosylases liberating deoxynucleotides and bases, respectively. Major DNA oxidation products of purines are recognized by the specialized repair enzyme formamidopyrimidine-glycosylase (Fpg) and the oxidized pyrimidines are recognized by endonuclease III (Endo III) (Zwart *et al.* 1999, Cadet *et al.* 2000). These DNA repair enzymes recognize DNA modifications that are typically induced by ROS and can be used as specific tools for identification of oxidative lesions of nuclei.

Single-cell gel electrophoresis (SCGE) or 'comet assay' is a rapid and very sensitive method to examine DNA damage and repair at the individual cell level (Singh *et al.* 1988). Comet assay is developed with some modifications to detect specific classes of DNA damage such as oxidative DNA damage. This method makes use of repair DNA glycosylases/endonucleases with a specificity for oxidative base damage to create breaks in DNA at sites of damage, and by comparing the DNA migration in enzyme-treated and -untreated slides, quantitation can easily be made (Collins *et al.* 1993). The present paper aimed to assess the oxidative DNA damage in peripheral lymphocytes of patients with AD and control individuals by using the modified comet assay.

Materials and methods

Subjects

Twenty-four patients with AD and 21 age-matched controls were used with the approval of the local ethics committee. Informed consent was obtained from all control subjects and family members of all patients with AD. Patients were diagnosed by the Psychiatry Department, Faculty of Medicine, Gazi University. The clinical diagnosis of AD was based on DSM-IV criteria (American Psychiatric Association 1994). The practical method for grading the cognitive state of patients was their Mini Mental State Examination (MMSE). MMSE scores <23 were accepted as moderate and <18 as severe dementia (Folstein *et al.* 1975). Among the patients with AD, 18 had moderate dementia and six had severe dementia.

The mean ages of the patients with AD and controls were 72.16 and 68.38 years, respectively. The control subjects were selected within healthy elderly people who admitted to the check-up centre of the Faculty of Medicine and none had verifiable symptoms of dementia or other neurological disorders. Standardized health questionnaires from all patients and controls relating to their occupational exposure and lifestyle factors such as smoking, drug consumption, viral diseases, recent vaccinations, radio-diagnostic examinations and dietary habits were obtained and none of the participated subjects had the confounding variable that might play a role in the induction or expression of oxidative DNA damage, except for one AD patient who was a smoker and therefore an age-matched control was selected with matching smoking habits.

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical (Steinheim, Germany) unless otherwise stated. Roswell Park Memorial Institute (RPMI)-1640 medium was supplied from Biological Industries (Kibbutz Beit Haemek, Israel). Freeze-dried Endo III and Fpg were kindly provided by Dr Andrew Collins, Rowett Research Institute, Aberdeen, UK. Enzymes were reconstituted with 100 µl enzyme buffer (40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 0.1 M KCl, 0.5 mM ethylenediamine tetra-acetic acid [EDTA], 0.2 g ml^{-1} bovine serum albumin, pH 8.0, with KOH), and 10-µl aliquots stored frozen at −80°C. Before use, 990 µl enzyme buffer were added to the 10-µl aliquot.

Blood sampling and lymphocyte preparations

Lymphocytes were obtained from venous blood sample and supplemented with anticoagulant heparin (Liquemine, F. Hoffmann-La Roche Ltd., Genzacherstrasse 124, CH 4070, Basel, Switzerland). Each analysis was done in duplicate and carried out immediately after sample collection without freezing or storing.

A total of 30 µl blood were added to phosphate buffer saline (PBS) in an Eppendorf and underlayed with Histopaque 1077. Cells were immediately centrifuged at 200*g* for 3 min at 4°C. Lymphocytes were retrieved from just above the boundary between PBS and Histopaque and were added to PBS and centrifuged at 200*g* for 3 min at 4°C.

Comet assay

The basic alkaline technique was followed with modifications for the detection of oxidized bases by using bacterial repair endonucleases as described by Collins *et al.* (1993).

Slide scoring

A number of 100 cells per subject were analysed at 400× magnification under a fluorescence microscope (Zeiss, Axioscope 2, Göttingen, Germany) equipped with an excitation filter of 546 nm and barrier filter of 590 nm. Cells can be classified by eye into four categories based on the extent of migration, i.e. undamaged (no migration), low, high and complete migration. In this study, there was no complete migration in any of the cells and each of 100 cells was assigned into three categories as no migration/undamaged cell (NM), low migration (LM) and high migration (HM) depending on the fraction of DNA pulled out into the tail under the influence of the electric field. The total comet score (TCS) per subject was calculated as follows to determine the DNA damage.

$$\mathrm{TCS} = 0 \times \mathrm{NM\ (number\ of\ comets\ in\ category\ NM)} + 1 \times \mathrm{LM\ (number\ of\ comets\ in\ category\ LM)} + 2 \times \mathrm{HM\ (number\ of\ comets\ in\ category\ HM)}.$$

The overall score for each slide was therefore between 0 (undamaged) and 200 (maximally damaged). Analysis was performed blindly by one slide reader.

Statistical analysis

Data were analysed for statistical significance using an unpaired *t*-test. $p < 0.05$ was considered as being statistically significant for comparison between data sets.

Results

The comet assay was run with or without Fpg or Endo III digestion before electrophoresis. Fpg and Endo III were used to detect oxidized pyrimidines and purines, respectively. After incubation with these specific repair endonucleases, additional breaks are formed at sites of such lesions, and the relative amount of DNA in the tail of the comet was increased.

Means of total comet scores of all individuals were calculated and presented as mean ± SD in table 1. Levels of single-strand breaks (SSB), SSB + Fpg-sensitive sites and SSB + Endo III-sensitive sites in the DNA of lymphocytes are shown in figure 1.

Table 1. Oxidative DNA damage assessed in lymphocyte samples from patients with Alzheimer's disease (AD) and from controls in terms of mean total comet score ± SD*.

Groups	Digestion conditions		
	Buffer	Endonuclease III	Formamidopyrimidine-glycosylase (Fpg)
Controls ($n=21$)	12.95 ± 2.71	14.57 ± 2.56	14.43 ± 2.50
Patients with AD ($n=24$)	16.75 ± 4.55	36.13 ± 6.91	31.33 ± 7.27

* Total comet score = 0 × (number of comets in category no migration) + 1 × (number of comets in category low migration) + 2 × (number of comets in category high migration).

Significant elevation ($p < 0.01$) of basal levels of SSB and alkali-labile sites in the peripheral lymphocytes of patients with AD (16.75 ± 4.55) were observed as compared with controls (12.95 ± 2.71) (figure 1A).

Inclusion of Endo III or Fpg in the comet assay revealed extra sites of damage (oxidized pyrimidines and altered purines, respectively) in both patients and controls. To determine the basal level of oxidation damage of pyrimidines in patients with AD, the nuclei of lymphocytes were treated with Endo III, a pyrimidine oxidation-specific endonuclease. Significant elevation ($p < 0.0001$) in the mean total comet score (reflecting oxidized pyrimidines) was found in patients with AD (36.13 ± 6.91) as compared with controls (14.57 ± 2.56) (Figure 1B). Lymphocytes from patients were also treated with Fpg, a purine oxidation-specific endonuclease, and a significant elevation ($p < 0.0001$) in the mean total comet score (reflecting oxidized purines) was found in patients with AD (31.33 ± 7.27) as compared with controls (14.43 ± 2.50) (figure 1C).

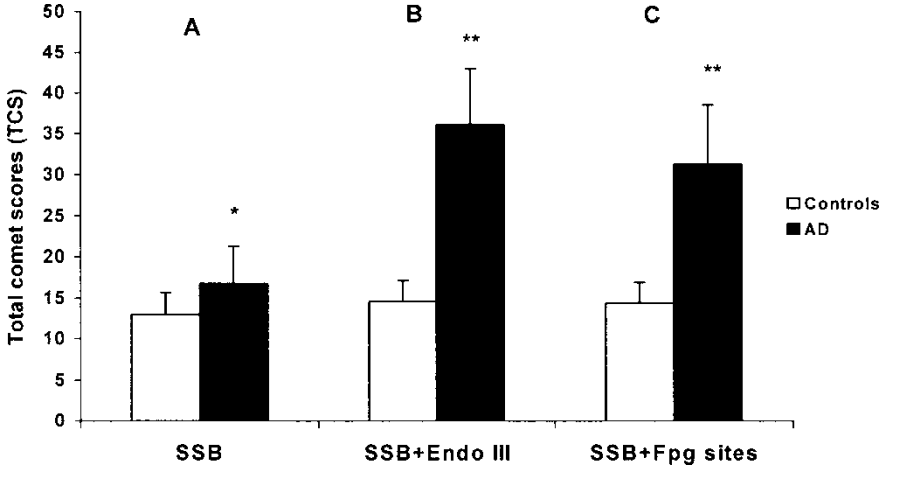

Figure 1. Levels of single-strand breaks (SSB) (A), single-strand breaks + endonuclease III sites (SSB + Endo III) (B) and single-strand breaks + formamidopyrimidine-glycosylase sites (SSB + Fpg) (C) in lymphocytes of patients with Alzheimer's disease (■) and in control individuals (□). Results are means(SD. *$p < 0.01$, **$p < 0.0001$ (unpaired t-test).

Discussion

Although there are limited studies about the oxidative DNA damage in living patients with AD, recently reported ones (as discussed below) seem to confirm the elevated levels of DNA damage. Oxidative stress to the central nervous system predominantly manifests as lipid peroxidation, which may promote the formation of additional ROS, and enhancement of protein and DNA oxidative damage. 8-hydroxyguanosine (8OHG) or 8-hydroxy-2′-deoxyguanosine (8-OHdG) and DNA strand breaks have been evaluated as the quantitative markers of oxidant stress in AD (Migliore and Coppede 2002). Nunomura *et al.* (2001) reported that a significant increase of 8OHG and an oxidized amino acid (nitrotyrosine) were detected in neurons of patients with AD, and noted that the increased oxidative damage is an early event in AD that decreases with the progression of the disease. Lyras *et al.* (1997) also showed an increased oxidative damage to protein and DNA in the brains of patients with AD by measuring products of damage to four DNA bases with gas chromatography-mass spectrometry. Moreover, two recent studies by Mecocci *et al.* (1998, 2002) report increased levels of 8-OHdG and decreased levels of plasma antioxidants in lymphocytes of patients with AD as compared with controls, suggesting that oxidative stress in AD is detectable not only in the central nervous system, but also in peripheral cells. According to increasing experimental evidence, the impairment in cellular total antioxidant capacity plays a central role in AD. The activities of the antioxidant enzymes such as Cu/Zn superoxide dismutase and catalase are reported to reduce significantly in the frontal and temporal cortex of patients with AD (Marcus *et al.* 1998, Cecchi *et al.* 2002). On the other hand, Boerrigter *et al.* (1992) reported evidence for decreased capacity for DNA repair in fibroblasts or lymphocytes derived from some patients with familial AD. Davydov *et al.* (2003) investigated the alterations in DNA repair proteins in patients with AD by immunohistochemical analysis to show if DNA repair compromised in patients with AD and suggested that the absence of sufficiently efficient DNA repair mechanisms involved in the removal of small base damages in the brain could result in the accumulation of mis- or non-repaired DNA damage. There is also a strong evidence of oxidative damage in the AD brain and many biochemical alterations are known to occur in peripheral tissues including glucose metabolism, abnormal Aβ processing, mitochondrial function and calcium dynamics (Peterson and Goldman 1986, Huang *et al.* 1994).

The present findings also support the idea that there is a significant increase in oxidative damage to DNA in AD by demonstrating the association between the disease and the elevated levels of oxidized DNA bases in peripheral lymphocytes. This association is supported with increased levels of oxidized purines and pyrimidines by using the comet assay modified with oxidative lesion-specific endonucleases (Fpg and Endo III). Similar to our assessment, Morocz *et al.* (2002) also found statistically significant elevations of oxidized purines in lymphocytes of patients with AD. However, they reported slightly but not significantly higher levels of oxidized pyrimidines by using the modified comet assay.

In conclusion, the findings of increased oxidative damage to DNA as observed by comet assay with lesion-specific endonucleases strengthens the possibility that AD is associated with increased free radical damage to DNA and further studies on

patients with AD could clarify the role of oxidative stress in the pathogenic mechanisms of AD. The study also demonstrates the usefulness of the comet assay with oxidative lesion-specific enzymes as a biomarker of oxidative DNA damage.

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