Selective impairment of p53-mediated cell death in fibroblasts from sporadic Alzheimer's disease patients

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Summary

In this study, we evaluated the response of different human skin fibroblast cultures obtained from eight probable Alzheimer's disease patients and eight non-Alzheimer's disease subjects to an acute oxidative injury elicited by H_2O_2. This treatment generates reactive oxygen species, which are responsible for DNA damage and apoptosis. To compare the sensitivity of fibroblasts from Alzheimer's disease or non-Alzheimer's disease patients to H_2O_2 exposure, we evaluated different parameters, including cell viability, the extension of DNA damage and the ability of the cells to arrest proliferation and to activate an apoptotic program. We found that fibroblasts from Alzheimer's disease patients were more resistant that those from control subjects to H_2O_2 treatment, although the extent of DNA damage induced by the oxidative injury was similar in both experimental groups. The protective mechanism of Alzheimer's disease fibroblasts was related to an impairment of H_2O_2-induced cell cycle arrest and characterized by an accelerated re-entry into the cell cycle and a diminished induction of apoptosis. Fibroblasts from Alzheimer's disease patients also have a profound impairment in the H_2O_2-activated, p53-dependent pathway, which results in a lack of activation of p53 or p53-target genes, including *p21*, *GADD45* and *bax*. This study demonstrates a specific alteration of an intracellular pathway involved in sensing and repairing DNA damage in peripheral cells from Alzheimer's disease patients.

Key words: DNA damage, Human, Cell cycle, Reactive oxygen species, p21, GADD45, Bax

Introduction

Free radicals are believed to be important intermediary risk factors involved in initiation and progression of neurodegeneration in Alzheimer's disease (AD) (for a review, see Prasad et al., 1999). Free radicals, such as O^{-}*, HO_2*, R*, RO_2* and H_2O_2, are highly unstable and reactive molecules that are generated during normal aerobic respiration, during bacterial or viral infection and during normal oxidative metabolism of several substances. When the levels of such reactive oxygen species (ROS) exceed the antioxidant capacity of the cell, they became toxic and cause oxidative injury. Mitochondria, membrane lipids, proteins and nuclear genes of highly metabolic cells are the primary targets for the action of ROS.

The contribution of oxidative injury and ROS generation to AD is supported by a number of neurochemical and neuropathological studies in the brain of AD patients showing accumulation of iron and aluminium (Savory et al., 1999; Campbell and Bondy, 2000), high levels of peroxynitrite (Hensley et al., 1995; Hensley et al., 1998; Aksenov et al., 2001), high expression of heme oxygenase (Takahashi et al., 2000; Takeda et al., 2000) and high levels of markers of lipid peroxidase activity, such as thiobarbituric acid-reactive substance (Lovell et al., 1995; Marcus et al., 1998) and 4-hydroxynonenal (Lovell et al., 1997; Markesbery and Lovell, 1998). Moreover, neurotoxicity associated both with Ca^{2+}-mediated activation of N-Methyl-D-Aspartate-type glutamate receptor and with β-amyloid plaques deposition involve free radical generation (Ciani et al., 1996; Hensley et al., 1996; Mark et al., 1996; Weber, 1999). Finally, clinical studies show that oral vitamin E intake or selegiline treatment delays the progression of the disease in patients with moderate to severe cognitive impairment (Sano et al., 1997). Signs of oxidative stress in AD are also detectable in peripheral cells. Indeed, fibroblasts from sporadic AD patients show impaired oxidative metabolism (Gibson et al., 1996; Gasparini et al., 1998), lymphoblasts from familial AD patients carrying a mutation in the *presenilin-1* gene have low levels of reduced glutathione (Cecchi et al., 1999), and lymphocytes from mice carrying multiple presenilin-1 mutations show an accumulation of ROS (Eckert et al., 2001). All together, these data suggest that brain and peripheral cells of AD patients are abnormally exposed to ROS. However, although this condition in the brain may be associated with a neuronal cell loss, no evidence is available, at least up to now, of a clear sign of pathology in peripheral organs.

In this study, we evaluated the response of different human skin fibroblast cultures obtained from probable AD patients and non-AD subjects to an acute oxidative injury elicited by H_2O_2. It is well recognized that one of the main effects following H_2O_2 exposure is damage to DNA molecules. Cells respond to this event by arresting the cell cycle to allow repair of damaged DNA. The damage and the ability to repair it address the cell fate in terms of re-entry into the cell cycle or

inducing apoptosis. Thus, to compare the sensitivity of fibroblasts of AD or non-AD patients to H_2O_2 exposure, we took into consideration different parameters, including cell viability, the extension of DNA damage and the ability to arrest proliferation and to activate an apoptotic program. We found that fibroblasts from AD patients are more resistant that those from non-AD subjects to H_2O_2. The protective mechanism involves an impairment of ROS-activated, p53-dependent cell death.

Materials and Methods

Patients

Fibroblasts from eight non-AD controls (five females, three males; mean age 70.7±9.2 years) and eight AD patients (six females, two males; mean age 74.0±8.2 years) were selected from the cell lines present in our cell repository originally established in 1993 (Govoni et al., 1993) and further expanded within MURST (Ministry of University and Research) research programs.

The diagnosis of probable AD was made by senior neurologists according to the criteria developed by National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA). All AD patients presented a 1 to 4 year history of progressive cognitive impairment predominantly affecting memory. Cognitive status was quantified using the Mini-Mental State Examination (MMSE); the scores were as follows: AD, 3.6±2.4; non-AD, 25.1±1.7. Three AD patients were non-testable using the MMSE because of severe disease. The ApoE genotypes were as follows: two controls were respectively heterozygous E3/E4 and E3/E2 and the others homozygous E3/E3; four AD patients were heterozygous E3/E4 and four homozygous E3/E3. Thus the overall distribution of E4 alleles was consistent with the allele frequency indicated by the literature (Frisoni et al., 1994). A summary of demographic characteristics of all subjects enrolled in the study is reported in Table 1.

Skin fibroblast cultures

Fibroblast cultures were established as previously described (Govoni et al., 1993). All cell lines were frozen at passage two-four in a modified growth medium containing 90% fetal calf serum (FCS) and 10% dimethylsulfoxide. For the experiments, cell lines were simultaneously thawed and grown up to passages nine-twelve. Cells were grown in Eagle's Minimum Essential Medium (GIBCO, Madison, WI), supplemented with 10% FCS, penicillin (100 U/ml) and streptomicin (100 μg/ml), non-essential amino acid solution (1% v/v) and Tricine buffer (GIBCO) (20 mM, pH 7.4) at 37°C in 5% CO_2/95% air. The medium was changed every 3 days. As aging affects the apoptotic cell response to genotoxic stress (Suh et al., 2002), each set of experiments was done using cells from the same passage, carefully matching AD and control cultures. Culture conditions were kept constant throughout the experiments.

Table 1. Demographic characteristics of all subjects enrolled in the study

	Non-AD (*n*=8)	AD (*n*=8)
Age (years)	70.7±9.2	74.0±8.2
Gender (F/M)	5/3	6/2
MMSE	25.1±1.7	3.6±2.4
Duration of dementia (months)	–	48.9±9.1

AD, patients with a diagnosis of probable AD. This diagnosis was made according to the criteria developed by the National Institutes of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ARDA); non-AD, patients with no sign of cognitive disorders; MMSE, Mini-Mental State Examination; *n*, number of patients; F, female; M, male. Values are expressed as means±s.e.m.

H_2O_2 treatment

80% confluent monolayers of cells were exposed to H_2O_2. Briefly, culture cells were washed with phosphate buffer saline (PBS) and treated with 1 mM H_2O_2 for 15 minutes. After washing, cells were returned to full fresh medium for variable times according to the experiments. For each cell line the experiments were repeated at least three times.

Cell viability

Cell viability was evaluated 24 hours after the addition of the cytotoxic agent to the media by measuring lactate dehydrogenase (LDH) activity using the Cytoxicity Detection Kit (Boehringer Mannheim) and an ELISA reader (340 ATC, SLT LabInstruments, NC). Cytotoxicity was evaluated as a percentage of the maximum amount of releasable LDH enzyme activity, which is determined by lysing the cells with 1% of TritonX-100.

Chromosomal condensation and DNA fragmentation were determined using the chromatin dye Hoechst 33258. After treatments, cells were stained with 1 μM Hoechst 33258 for 5 minutes. After three rinses with PBS buffer, cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. Coverglasses were mounted and analysed under fluorescence microscope.

Western blot analysis

Cells were harvested in 80 μl of lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 0.5 μg/μl leupeptin, 5 μg/μl aprotinin and 1 μg/ml pepstatin. Samples were sonicated and centrifuged at 15,000 ***g*** for 30 minutes at 4°C. The resulting supernatants were isolated and protein content determined by a conventional method (BCA protein assay Kit, Pierce, Rockford, IL). 30 μg of protein extracts were electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Germany). Filters were incubated at room temperature overnight with anti-p53 (1:500) (Ab240, Neo Markers), anti-p21 (1:200) (F5, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax (1:200) (B9, Santa Cruz Biotechnology, Santa Cruz, CA), anti-GADD45 (1:200) (C4, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-tubulin (1:1,500) (Ab3, Neo Markers) antibodies in 3% non-fat dried milk (Sigma). The secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and a chemiluminescence blotting substrate kit (Boehringer, Mannheim, Germany) were used for immunodetection. For immunoprecipitation experiments, 150 μg of protein extracts were resuspended in 500 μl RIPA buffer (140 mM NaCl, 10 mM Tris-HCl, pH 8.1% TritonX-100, 0.1% SDS, 1 mM Na-ortho-vanadate and 1 mM PMSF) and then incubated with 2 μg/ml of mouse p53 antibody (Ab8, Neo Markers) at 4°C overnight. Immunocomplexes were collected with *Staphylococcus aureus* Protein A suspension and washed five times with RIPA buffer. Immunoprecipitated p53 was recovered by resuspending the pellets in loading buffer, and protein was detected by western blotting with rabbit antibodies against p53 phospho-serine-15 (1:500) (Oncogene) or p53 phospho-serine392 (1:500) (Oncogene). Peroxidase-conjugated goat anti-rabbit immunoglogulin G and a chemiluminescence blotting substrate kit (Boehringer, Mannheim, Germany) were used for immunodetection. Evaluation of immunoreactivity was performed on immunoblots by densitometric analysis using a KLB 2222-020 Ultra Scan XL laser densitometer.

Immunocytochemistry analysis for 8-hydroxy-deoxyguanosine (8OH-dG)

The cells were fixed in 75% ethanol at –20°C. The cells were then treated with RNase (100 μg/ml) in 10 mM Tris buffer (pH 7.5) containing 1 mM EDTA and 0.4 M NaCl at 37°C for 1 hour, followed by an incubation with proteinase K (10 μg/ml) at room temperature for 7 minutes. After rinsing with PBS, DNA was denaturated by treatment with 4 N HCl for 7 minutes at room temperature. The pH was adjusted with 50 mM trizma base for 5 minutes followed by washing in PBS. Non-specific binding sites were blocked with 10% rabbit serum for 1 hour at 37°C. Cells were incubated with primary anti-8OH-dG antibody (1:30) (1F711, Pharmigen) at 4°C overnight. Anti-rabbit anti-mouse IgG coniugated to biotin and ABC reagents and avidin conjugated to horseradish peroxide were used. To localize peroxidase, cells were treated with diaminobenzidine for 10 minutes. After mounting with Permount, coverglasses were analysed by using a camera adapted to a Nikon microscope with a ×20 objective. The image processing and quantitative analysis of 8OH-dG-positive cells were performed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Data were expressed as a number of labeled positive cells in the examined field. The mean profile was obtained from at least three separate determinations in triplicate for each cell line. The statistical significance of differences between the values was made by one-way analysis of variance followed by a Student's *t*-test.

Measurement of DNA synthesis

Cells were seeded in 24-well culture plates at a density of about 5×10^4 cells per well. 1 μCi/ml [^{3}H]thymidine was added to the cells 6 hours before or immediately after the H_2O_2 pulse. Cells were then incubated for an additional time at 37°C. At the end of the incubation, cells were washed with ice-cold PBS and further incubated for 10 minutes at 4°C with 10% trichloroacetic acid, followed by 1 N NaOH and 1 N HCl for 20 minutes at room temperature. The resulting solution was collected and analysed for radioactivity content.

Flow cytometry

For analysis of cell cycle distribution, both floating and adherent cells were collected and fixed in 70% ethanol in distilled water and stored at –20°C. After washing in PBS, the cells were treated with 100 μl of ribonuclease for 5 minutes at room temperature, stained with 400 μl of propidium iodide (50 μg/ml) and analysed by flow cytometry using 488 nm excitation.

Results

Cell viability

Exposure of human skin fibroblasts to 1 mM H_2O_2 for 15 minutes resulted in cell loss that is detectable 24 hours later by measuring LDH release in the medium (Fig. 1A). At an earlier time, that is, 12 hours after the H_2O_2 pulse, cells exhibited typical signs of apoptosis, such as pyknotic and shrinking nuclei as visualized by Hoechst 33258 staining, whereas the cell nuclei of untreated cells show a normal morphology (Fig. 1B).

The experimental paradigm described above was applied to fibroblasts from probable AD patients. In particular, cell cultures from eight AD patients and eight non-AD subjects at similar number of passages (nine to twelve) were challenged with 15 minutes pulse of 1 mM H_2O_2, cultured for an additional 24 hours and than evaluated for cell viability. As depicted in Fig. 2, cell death, expressed as percentage of H_2O_2-induced LDH release over basal, was significant lower in fibroblasts from AD (26.4±3.8) in comparison with those from non-AD patients (41.7±4.6).

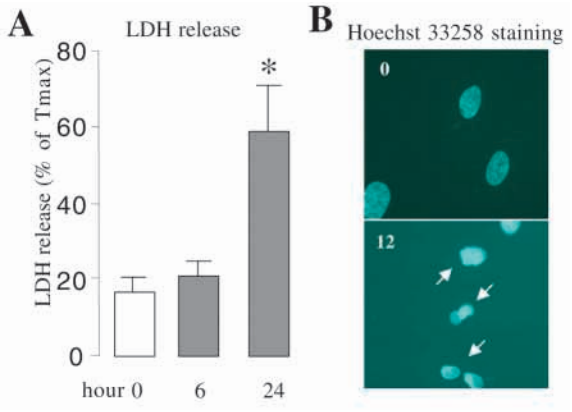

Fig. 1. Biochemical (A) and morphological (B) evidence for H_2O_2-induced loss of human skin fibroblasts. Cell viability was determined by measuring LDH activity in conditioned media (A); cells in apoptosis were determined morphologically by Hoechst 33258 staining (B). Cells were exposed to 1 mM H_2O_2 for 15 minutes, washed and analysed at different times after the lesion, as indicated at the bottom of A and in inserts in B. Bars represent mean±s.e.m. of at least three different experiments and are from three separate cell preparations. Arrows in B indicate cells in apoptosis. *P<0.01 versus 0 time.

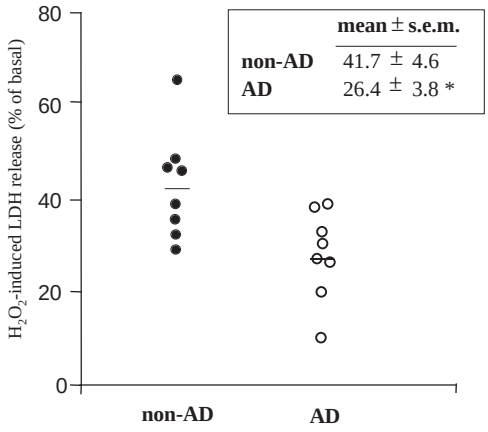

Fig. 2. H_2O_2-induced cell loss of human skin fibroblasts from non-AD and AD patients. Cell viability was determined by measuring LDH activity in the conditioned media 24 hours after H_2O_2 exposure. Each point represents an individual sample. Values are expressed as a percentage increase over basal values. The insert shows the means±s.e.m. from at least three different experiments for each sample using at least three separate cell preparations. *P<0.01 versus non-AD.

Oxidative DNA damage

One of the major events induced by H_2O_2 is the generation of ROS and induction of DNA damage (Gille et al., 1992). We then asked whether the low sensitivity of AD-fibroblasts to the oxidative injury was caused by a diminished capability of ROS to generate oxidative DNA damage. DNA base oxidation was analysed in both cell groups by using a specific antibody

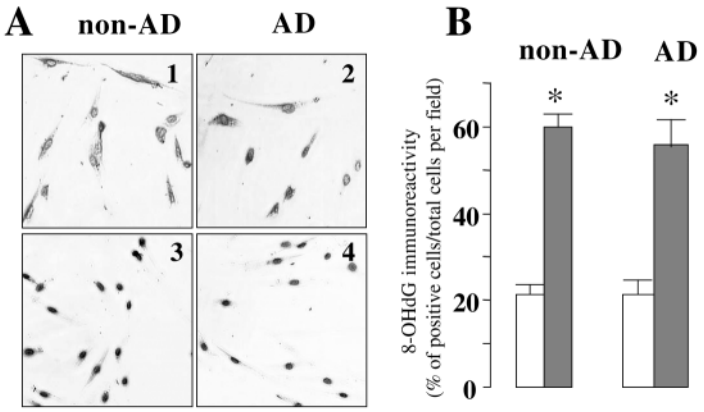

Fig. 3. H_2O_2-induced DNA base damage of human skin fibroblasts from non-AD and AD patients. DNA damage was evaluated immunocytochemically using a specific antibody against 8OH-dG. A shows representative images of non-AD (1,3) and AD (2,4) fibroblasts in basal conditions (1,2) and 2 hours after H_2O_2 treatment (3,4). B shows quantitative analysis of 8OH-dG-positive cells in six non-AD and six AD samples. Bars represent basal (open) and experimental (gray) values and are expressed as a percentage of positive cells per field. Values are the mean±s.e.m. of the results from at least six different fields for each sample. *P<0.01 versus the corresponding basal values.

against 8OH-dG (Shigenaga et al., 1991). Fig. 3A shows a representative immunocytochemistry analysis carried out with 8OH-dG antibody on AD and non-AD fibroblasts after treatment with H_2O_2. Only scattered 8OH-dG-positive nuclei were observed in untreated non-AD fibroblasts. H_2O_2 treatment caused a significant increase in the number of labelled cells within 2 hours. H_2O_2-induced base DNA damage was evident also in AD fibroblasts. Quantitative analysis of DNA damage caused by H_2O_2 exposure was evaluated in six AD and six non-AD cell lines, counting the 8OH-dG-positive cells in at least six different fields of each sample. As shown in Fig. 3B, H_2O_2-induced 8OH-dG generation was similar in AD and non-AD fibroblasts.

Cell cycle and proliferation

As a normal response to DNA damage, cells arrest the cell cycle. The ability of AD and non-AD fibroblasts to arrest the cell cycle and thus to diminish DNA synthesis was evaluated at different time following H_2O_2 treatment by measuring [^{3}H]thymidine uptake. [^{3}H]thymidine was added to the cultures 6 hours before or immediately after H_2O_2 pulse; radioactivity incorporated into the cells was measured at different times (0, 6 and 24 hours) later. No significant difference in the normal cell proliferation rate was observed in untreated AD and non-AD fibroblasts (Fig. 4A,B). Six hours after cytotoxic injury, non-AD cells decreased their proliferation rate, and 24 hours later they completely lost their ability to synthesize DNA (Fig. 4A). In AD fibroblasts, the H_2O_2 insult induced a temporary growth arrest, as visualized by a decreased [^{3}H]thymidine uptake 6 hours after the insult. Then, cells recover their ability to synthesize DNA within 24 hours (Fig. 4B). The different response of AD- and non-AD fibroblasts to H_2O_2 can be easily visualized by comparing the rate of [^{3}H]thymidine uptake in both cell lines between 6 hours and 24 hours (Fig. 4B).

The ability of AD and non-AD fibroblasts to arrest the cell cycle was also evaluated by measuring the distribution of the cells into the different cell cycle phases using standard DNA content analysis by flow cytometry. The cell cycle pattern of non-AD fibroblasts was compared with that of AD either in basal conditions and at different times following the H_2O_2 pulse. Fig. 5 depicts representative DNA histograms generated by flow cytometric analysis of non-AD and AD fibroblast cell lines. No differences were evident in cell cycle distribution of untreated AD and non-AD fibroblasts (Table 2). Six hours following the H_2O_2 pulse, cells from non-AD and AD subjects were enriched in G0/G1 phase. Further, at the same time point, a fraction of cells of both groups were confined in sub-G0 phase, characterizing apoptosis. Twenty hours later, the percentage of non-AD cells in G0/G1 phase further increased showing also an enhancement of cell fraction in sub-G0 phase. On the contrary, at the same time-point, AD fibroblasts shown a pattern of cell cycle distribution comparable with that found in basal conditions. Similar results were obtained in six AD and six non-AD cell lines. As summarized in Table 1, the G0/G1 fraction of non-AD fibroblasts progressively increased from time 0 to 20 hours after H_2O_2 treatment (47.2%, 55.6% and 60.0% at 0, 6 hour and 20 hours, respectively), whereas the percentage of cells in G2/M significantly diminished over time (41.5%, 10.2% and 7.8% at 0, 6 hours and 20 hours, respectively). By contrast, the G0/G1 fraction of H_2O_2-treated AD cells showed a slightly increase 6 hours after injury, which was followed by a decrease at 20 hours. Concomitantly, the

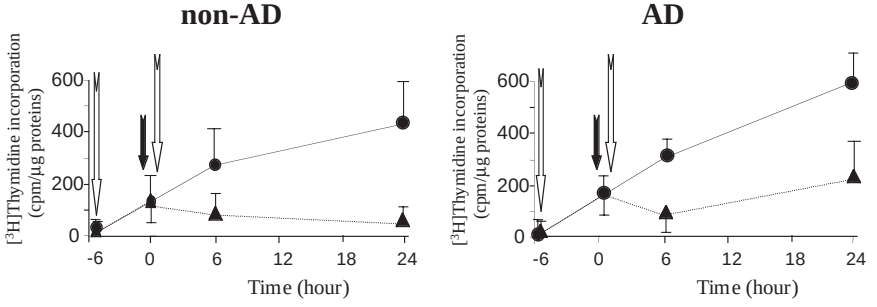

Fig. 4. [^{3}H]Thymidine incorporation in fibroblasts from non-AD and AD patients after H_2O_2 treatment. [^{3}H]Thymidine was added to the culture media at the times indicated by the white arrows. Cells were exposed to H_2O (circles) or 1 mM H_2O_2 (triangles) for 15 minutes at time 0 as indicated by black arrows, then washed and cultured for additional 6 hours or 24 hours. Data are from six non-AD and six AD different cell lines and are expressed as mean±s.e.m. of at least three separate experiments for each sample.

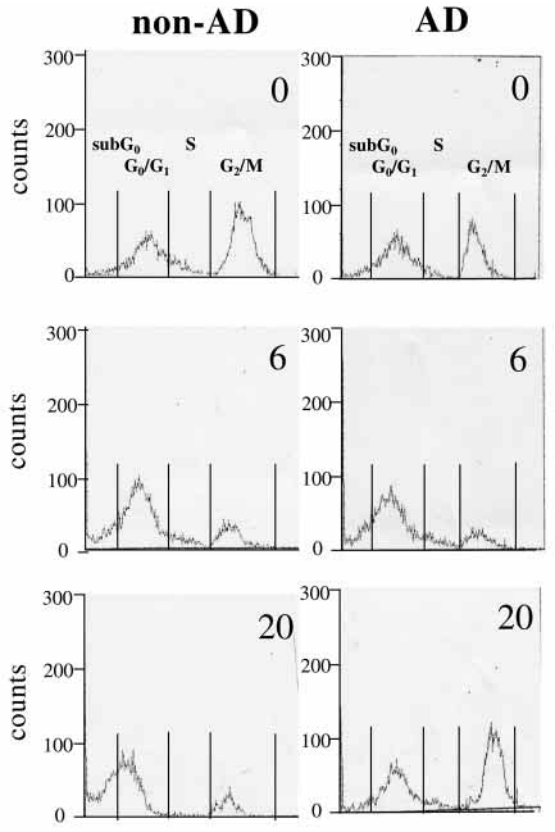

Fig. 5. Representative patterns of cell cycle phase distribution by flow cytometric analysis of fibroblasts from non-AD and AD sample in resting conditions and at different times after H_2O_2 treatment. Cells were exposed to 1 mM H_2O_2 for 15 minutes, washed and cultured for an additional 6 or 20 hours (as indicated by the numbers in the inserts). Time 0 indicates that cells are in the basal condition.

Table 2. Distribution of non-AD and AD fibroblasts in the different cell cycle phases after H_2O_2 exposure

Cell sample	Cell cycle phase	Time after H_2O_2 treatment (hours) 0	6	20
Non-AD	G0/G1	47.2±8.1	55.6±2.6	60.0±2.8
	G2/M	41.5±6.0	10.2±3.9	7.8±2.4
AD	G0/G1	41.8±4.7	47.2±4.6	33.4±6.1*
	G2/M	33.0±6.0	26.2±8.6†	39.4±7.9†

The distribution of cells in different cell cycle phases was evaluated by flow cytometric analysis as reported in the legend of Fig. 5 and in the Materials and Methods. Data are from six non-AD and six AD different cell lines and expressed as means±s.e.m. of at least three separate experiments for each sample.

*P<0.01 in comparison with non-AD G0/G1 values at the same time-point.

†P<0.01 in comparison with non-AD G2/M values at the same time-point.

fraction of G2/M cells decreased after 6 hours and returned to control levels 20 hours after the injury.

p53-dependent apoptotic pathway

The transcription factor p53 is one of the proteins that play an important role in the cellular response to DNA damage by

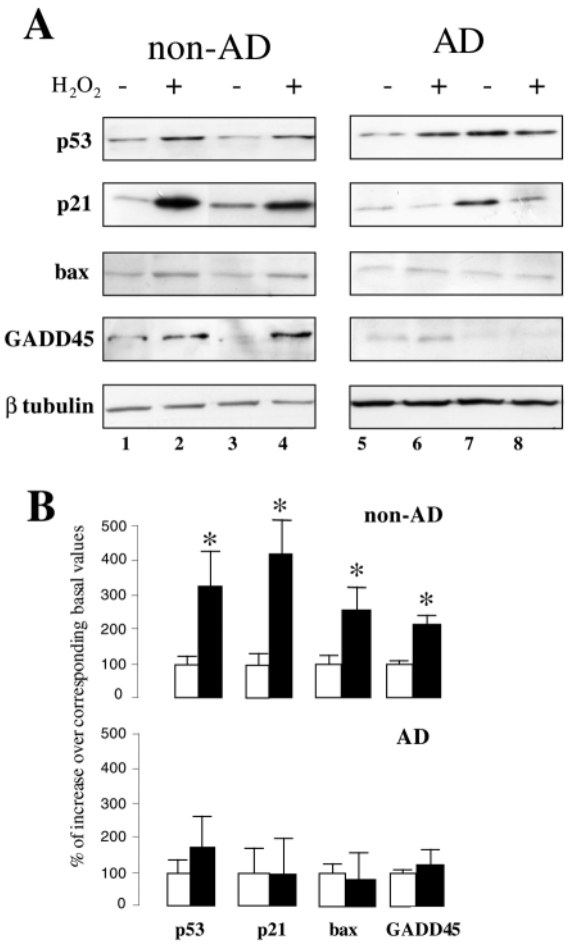

Fig. 6. Expression of p53 and p53-related gene products in fibroblasts from non-AD and AD patients: western blot analysis. (A) Protein extracts from two non-AD (lanes 1-2 and 3-4) and two AD (lanes 5-6 and 7-8) fibroblast cultures in basal conditions (lanes 1, 3, 5 and 7) and 2 hours after the H_2O_2 pulse (lanes 2, 4, 6 and 8) were processed with specific antibodies against p53, p21, bax, GADD45 and β-tubulin, as indicated in the method section. (B) Data obtained in fibroblasts from six non-AD and six AD patients in basal conditions (open bars) and after H_2O_2 pulse (black bars) were calculated by densitometric analysis and normalized as a percentage of basal expression. Values are expressed as means±s.e.m. of at least three separate experiments for each sample. *P<0.01 versus the corresponding basal values.

controlling DNA repair, cell cycle arrest and apoptosis (for a review, see Almog and Rotter, 1997). The effect of H_2O_2 on p53 protein levels and p53-dependent transcriptional activity in non-AD and AD fibroblast was examined by western blot analysis. Protein extracts from cells prior to and 2 hours following 1 mM H_2O_2 pulse were electrophoresed and immunoblotted with antibodies against p53 and the p53 target gene products p21, bax and GADD45. Fig. 6A shows representative results using fibroblasts from two non-AD (lanes 1-4) and two AD subjects (lanes 5-8). 2 hours after the H_2O_2 treatment, levels of p53, p21, bax and GADD45 increased in both non-AD samples. By contrast, samples from AD patients showed anomalous results. One sample showed a H_2O_2-induced increase in p53 levels that was not accompanied by similar changes in p21, bax and GADD45 (Fig. 6A, lanes 5-

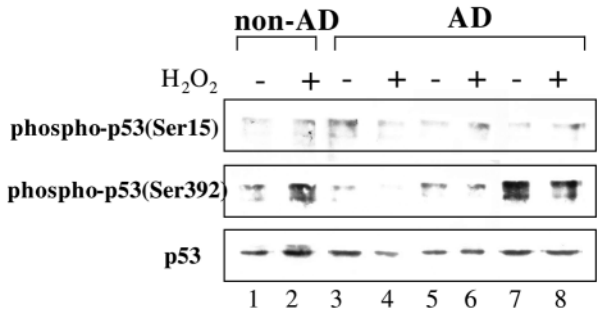

Fig. 7. Phosphorylation status of p53 in fibroblasts from non-AD and AD patients: an immunoprecipitation study. Protein extracts from one non-AD (lanes 1-2) and three AD (lanes 2-4, 5-6 and 7-8) fibroblast cultures in basal conditions (lane 1, 3, 5 and 7) and 2 hours after the H_2O_2 pulse (lane 2, 4, 6 and 8) were immunoprecipitated with antibodies against p53 and immunoblotted with specific antibodies against phospho ser15, phospho ser392 or p53, as indicated.

6). In the other AD sample, H_2O_2 treatment induced a decrease in p53 and p21 levels with no changes in bax and GADD45 protein content (Fig. 6A, lanes 7-8). Data obtained in fibroblasts from six non-AD and six AD patients were evaluated by densitometric analysis and normalized as a percentage of the corresponding basal expression. The results are illustrated in Fig. 6B. All samples from non-AD subjects showed a significant increase in p53, p21, bax and GADD45 protein levels after H_2O_2 treatment. The same treatment did not elicit significant changes in the levels of p53, p21, bax and GADD45 in fibroblasts from AD patients.

To gain further insight into the alteration of p53 pathway in AD fibroblasts, we investigated the possible involvement of upstream regulators of p53 activation by measuring p53 phosphorylation status at ser15 and ser392 in basal conditions and after H_2O_2 treatment. Phosphorylation at N-terminal sites of p53, such as at ser15, releases p53 from MDM2 binding, whereas phosphorylation at C-terminus sites, such as at ser 392, induces p53 DNA binding activity (Lakin and Jackson, 1999; Kapoor et al., 2000). We found that p53 phosphorylation at ser15 and ser392 in non-AD fibroblasts was low in resting conditions and significantly increased after the oxidative pulse (a representative sample is shown in Fig. 7, lines 1-2). Phosphorylation of p53 in AD fibroblasts behaved quite differently. In one AD case (lane 3-4, Fig. 7) phosphorylation at ser15 was high in basal conditions and decreased after H_2O_2. In two other AD samples (lines 5-6 and 7-8), phosphorylation at ser15 was low in basal conditions and increased after H_2O_2. However, phosphorylation at ser392 was high in basal conditions and decreased after H_2O_2. None of the AD samples behaved like their non-AD counterpart, at least in terms of pattern of p53 phosphorylation at ser15 and ser392. These results further underline the heterogeneity of AD fibroblasts and suggest that the impairment of p53 pathway may involve also upstream regulators.

Discussion

In this study we describe and demonstrate an abnormal response of AD fibroblasts to an acute oxidative injury. In particular, fibroblasts from AD patients were found to be less vulnerable to the oxidative injury induced by H_2O_2 in comparison with fibroblasts from non-AD subjects. The protective mechanism involves an impairment of ROS-activated, p53-dependent apoptosis.

The main result of our study is that H_2O_2-induced cell death was less effective in fibroblasts from AD patients. We first asked whether the low sensitivity of AD-fibroblasts to the oxidative injury was due to a diminished capability of ROS to generate oxidative DNA damage. This was suggested by data obtained in similar fibroblast cell lines in which alteration of some components of the mitochondrial oxidative pathways was observed (Curti et al., 1997; Gasparini et al., 1999). To this end, we evaluated the extent of ROS-induced DNA damage, as measured by 8OH-dG formation. When improperly hydroxylated by ROS attack, guanine may become an 8-hydroxyl-guanine residue, which can bind incorrectly to adenine instead of to cytosine and cause a DNA base mismatch (Shigenaga et al., 1991). Post-mortem studies demonstrated that levels of 8OH-dG were elevated in mitochondrial DNA of AD brain relative to controls (Mecocci et al., 1994). Furthermore, lymphocytes isolated by AD patients had higher levels of 8OH-dG than controls (Mecocci et al., 1998). Our results did not show any difference in the ability of H_2O_2 to generate 8OH-dG in fibroblasts from AD and non-AD patients, suggesting that ROS are similarly generated in terms of the amount and genotoxic potency in all fibroblast cell lines.

We then asked whether the lower sensitivity of AD fibroblasts to H_2O_2 was caused by an impairment of the cellular response to a genotoxic insult. As a normal response to DNA damage, cells arrest the cell cycle to allow repair of damaged DNA (Kastan et al., 1992). In the present study, cell cycle and cell cycle phase distribution of fibroblasts was measured by incorporation of [^{3}H]thymidine and flow cytometric analysis. Both techniques gave similar results: fibroblasts from AD patients showed a fast recover from the oxidative lesion. In fact, only 20 hours after the lesion, cell cycle phase distribution of AD fibroblasts was similar to that of controls. Thus, despite the similar extent of DNA damage, fibroblasts from AD patients show an accelerated re-entry into the cell cycle and a diminished induction of apoptosis.

Among the proteins that play an important role in the cellular response to DNA damage is the tumour suppressor p53 (Ko and Prives, 1996; Almong and Rotter, 1997; Grilli and Memo, 1999). p53 is a transcription factor that controls cell cycle arrest, DNA repair and apoptosis (for a review, see Among and Rotter, 1997). Following exposure to DNA damaging agents, p53 is phosphorylated at several sites by different kinases, including casein kinase I and II, cdc2 kinase and the DNA-activated protein kinase (Lakin and Jackson, 1999). These post-transcriptional modifications activate p53, allowing this protein to accumulate into the nucleus, to bind to specific DNA sequences and to transactivate several genes including effectors of the cell cycle, such as p21 (El-Deiry et al., 1993) and GADD45 (Kastan et al., 1992), and cell death, such as bax (Miyashita et al., 1994; Oren, 1994). Lack of functional p53 in different cell phenotypes may shorten DNA-damage-induced cell cycle arrest and results in accelerated proliferation (Gottlieb and Oren, 1996). Moreover, Li-Fraumeni syndrome fibroblasts homozygous for p53 mutation were several fold more resistant to UV cytotoxicity and exhibited much less UV-induced apoptosis

than normal skin fibroblasts expressing wild-type p53 (Ford and Hanawalt, 1997; Delia et al., 1997). In the present study, fibroblasts from AD patients appear to have a profound (and heterogeneous) impairment in the ROS-activated, p53-dependent pathway, which results in abnormal phosphorylation status in the resting condition and a lack of activation of p53 or p53-target genes. It is interesting that one AD sample (line 7-8 in Fig. 6 and line 3-4 in Fig. 7), with high p53 levels in resting conditions, also exhibits signs of phosphorylation at ser15 and an enrichment in G2/M phase. These features are not generalized to all AD samples. For example, another AD cell line shows moderate p53 expression, high phosphorylation at ser392 and low G2/M enrichment. These results further underline the heterogeneity of AD fibroblasts in resting conditions.

p53 belongs to a growing list of transcriptional activators that are post-transcriptionally regulated by redox modulation (Hainaut and Milner, 1993; Sun and Oberley, 1996; Verhaegh et al., 1997). In fact, as a result of protein oxidation, nine of the twelve cysteine residues in p53 localized in the central DNA-binding domain can form disulfide bonds, thus altering the three-dimensional structure of the protein. In this abnormal conformation, p53 loses the capability to transactivate its target genes (Parks et al., 1997). However, at the present we do not know whether the lack of ROS-activated apoptosis is the result of an elevated redox status of these cells (Curti et al., 1997) or whether p53 in these fibroblasts is per se in an oxidative conformational state. In this regard, when cells are exposed repeatedly to low doses of H_2O_2, they became resistant to subsequent higher amount of ROS that would be lethal without pre-treatment (Janssen et al., 1993), suggesting that cells can activate an adaptive genetic program against oxidative stress.

Finally, it should be noted that ROS-activated, p53-dependent pathway in fibroblasts from AD patients was completely lacking, whereas the ROS-induced cell death was decreased only by about 40%. This apparent discrepancy indicates that ROS may induce cell death by activating different pathways, including necrosis and apoptosis, and that p53-dependent apoptosis contributes only partially to the cell death induced by ROS. Preliminary data obtained in our laboratory suggest that non-AD and AD fibroblasts behaved similarly in response to cisplatin-induced apoptosis, suggesting that the p53 impairment of AD fibroblasts may affect specific death pathways (Seluanov et al., 2001).

The brain is particularly vulnerable to oxidative stress because of its high energy requirement and high oxygen consumption rate; it is also rich in peroxidizable fatty acid and has a relative deficit of antioxidant defences compared with other organs (Floyd, 1999). We cannot speculate at this time on whether the lack of ROS-activated, p53-dependent apoptosis is also present in the brain of AD patients. If this is true, it is tempting to suggest that such impairment in sensing and repairing DNA damage could be responsible for generation of malfunctioning neurons, that is cells living with altered gene transcription function. Studies in this direction are now in progress in our laboratory.

In conclusion, this study shows a specific alteration of an intracellular pathway involved in sensing and repairing DNA damage in peripheral cells from AD patients. Whether this is a peripheral sign of the disease requires future investigation.

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