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Identification of a mutant-like conformation of p53 in fibroblasts from sporadic Alzheimer's disease patients

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Abstract

Here we show that fibroblasts from sporadic Alzheimer's disease (AD) patients specifically express an anomalous and detectable conformational state of p53 that makes these cells distinct from fibroblasts of age-matched non-AD subjects. In particular, we found that, in contrast to non-AD fibroblasts, p53 in AD fibroblasts is expressed at higher levels in resting condition, and presents a significant impairment of its DNA binding and transcriptional activity. All together, these findings figured out the presence of a mutant-like p53 phenotype. However, gene sequencing of the entire p53 gene from either AD or non-AD did not unravel point mutations. Based on immunoprecipitation studies with conformation-specific p53 antibodies (PAb1620 and PAb240), which discriminated folded versus unfolded p53 tertiary structure, we found that a significant amount of p53 assumed an unfolded tertiary structure in fibroblasts from AD patients. This conformational mutant-like p53 form was virtually undetectable in fibroblasts from non-AD patients.

These data, independently from their relevance in understanding the etiopathogenesis of AD, might be useful for supporting AD diagnosis.

Keywords: Protein tertiary structure; Human; Cell cycle; Reactive oxygen species; p21; EMSA

1. Introduction

Alzheimer disease (AD) is a progressive neurodegenerative disorder characterized by the presence in the brain of senile plaques and neurofibrillary tangles. To date, recognition and quantification of these neuropathological lesions remains the only valid criteria for an unequivocal diagnosis of AD. Neurologists, taking advantage to validated clinical criteria, can diagnose probable or possible AD, if the dementia is atypical in onset and progression, with an accuracy of nearly 90% and 50%, respectively [32]. For these reasons, the identification of biological markers of AD, in addition to increasing our understanding of the pathogenesis of the disease, can be extremely useful to improve diagnostic accuracy and/or to monitor the efficacy of putative therapies. In this regard, peripheral cells may be of great importance, because of their easy accessibility.

Among extra-neuronal tissue, cultured skin fibroblasts derived from AD patients (AD-fibroblasts) have gained particular attention since they show a number of abnormalities in metabolic and biochemical processes, with some of them mirroring events that occur in the AD brain [9,15]. For example, in AD fibroblasts, changes have found to occur in signal transduction systems, including ion channels, cyclic AMP and phosphatidylinositide cascade, as well as in β-amyloid (Aβ) protein regulation [11,26,27]. Dysfunction in mitogen-activated protein kinases signalling cascade was also found in fibroblasts from both familiar and sporadic AD, but not in age-matched controls [37]. Abnormalities in ERK1/2 phosphorylation were also specifically found in AD fibroblasts since they were not present in fibroblasts from Huntington's disease patients with dementia [37]. Furthermore, reduced and abnormal glyceraldehyde-3-phosphate dehydrogenase

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(GAPDH) activity [21] and impaired bradykinin B2 receptor modulation by phorbol ester [16] were detected in AD fibroblasts in comparison with age-matched controls. Finally, recent evidences obtained in our laboratory, demonstrated an impairment of p53 signalling pathway following an oxidative injury that resulted in a less vulnerability of AD fibroblasts [35].

p53 is a multifunctional protein whose main role is to maintain genomic integrity [18]. Exposure to multiple signals, including radiation, genotoxic chemicals, hypoxia, oxidative stress, and depletion of ribonucleotides, induces p53 to accumulate in the nucleus, to bind to specific DNA sequences and to transactivate several genes most of them involved in cell-cycle control, DNA repair, and apoptosis [1]. Lack of p53 activity could be due essentially to gene mutations, as often occurs in tumours, or post-transcriptional modification both altering p53 tertiary structure and preventing binding to specific DNA sequences [4,20,22,23]. Recently, p53 has also been found to be involved in neurodegenerative processes [5,13,19,25,34].

In this study, we investigated the expression pattern, the activity, and the conformational structure of p53 in cultured skin fibroblasts derived from sporadic AD patients and age-matched non-AD subjects, in resting conditions and after experimental cytotoxic insults.

Based on immunoprecipitation studies with conformation-specific p53 antibodies, we found that in fibroblasts from AD patients a significant amount of total p53 assume an unfolded tertiary structure and this alteration can compromise p53 response to different cytotoxic injuries. These results, independently from their relevance in understanding the etiopathogenesis of AD, might be useful for supporting AD diagnosis.

2. Methods

2.1. Patients

Fibroblasts from six non-AD controls (six females, mean age 67.0 + 6.1 years) and seven AD patients (five females, two males, mean age 73.8 ± 9.4 years) were selected from the cell lines present in our cell repository originally established in 1993 [14].

The diagnosis of probable AD was made 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 1–4-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, 11.0 ± 7.75; non-AD, 27.5 ± 2.1. Three AD patients were non testable at the MMSE because of severe disease. The ApoE genotypes were as follows: four controls were hetherozygous E3/E4 and the others homozygous E3/E3; five AD patients were hetherozygous E3/E4 and two homozygous E3/E3. Non-AD were patients without established cognitive disorders. Neither AD nor non-AD patients presented tumour at the time of tissue biopsy.

2.2. Skin fibroblast cultures

Fibroblast cultures were established as previously described [14]. All cell lines were frozen at passage 2–4 in a modified growth medium containing 20% foetal calf serum and 10% dimethylsulfoxide. For the experiments, cell lines were simultaneously thawed and grown up to passages 9–12. Cells were cultured as previously described [35]. Each set of experiments was done using cells at the same passage (ranging from 10 to 12), carefully matching AD and non-AD samples. Culture conditions were kept constant throughout the experiments.

2.3. Cell treatment

Cells were used at 80% confluent monolayers. For H_2O_2 exposure, culture cells were washed with phosphate buffer saline (PBS) and treated with 1 mM H_2O_2 for 15 min. After washing, cells returned to fresh medium for additional time according to the experiments. Doxorubicin and *cis*-platinum (CDDP), were added to the medium at the concentration of 0.5 and 50 μg/ml, respectively, and then cells were cultured for additional 48 h. For each cell line the experiments were repeated at least three times.

2.4. Western blot analysis

Proteins were extracted from the cells as previously described [35] and protein content determined by a conventional method (BCA protein assay kit, Pierce, Rockford, IL). Thirty micrograms 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 antibody (1:200) (Ab8 NeoMarkers) anti-p21 (1:50) (Ab6 Oncogene), or anti-β-tubulin (1:1.500) (Ab3, Neo Markers) antibody in 3% non-fat dried milk (Sigma). The secondary antibodies (Dako) 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.

2.5. Immunoprecipitation analysis

To analyse p53 conformation, cells were lysed in immunoprecipitation buffer (10 mM Tris, pH 7.6; 140 mM NaCl; 0.5% NP40 including protease inhibitors) for 20 min on ice, and cell debris was cleared by centrifugation. Protein content was determined by a conventional method (BCA protein assay Kit, Pierce, Rockford, IL). Before immunoprecipitation

experiments, an aliquot of 10 μg of protein extracts from each individual sample were processed for Western blot analysis and probed with anti-β-tubulin antibody to validate protein content measurements (data not shown). Based on the previous results, 100 μg of protein extracts were used for immunoprecipitation experiments performed in a volume of 500 μl. To prevent non-specific binding, the supernatant of immunoprecipitated samples was pre-cleared with 10% (w/v) protein A/G (50 μl) for 20 min on ice, followed by centrifugation. For immunoprecipitation of p53, 1 μg of the conformation-specific antibodies PAb1620 (wild-type specific), PAb240 (mutant specific) or PAbBP53.12 (recognizing both wild-type and mutant p53) were added to the samples overnight at 4 °C. Immuno complexes were collected by using protein A/G suspension and washed five times with immunoprecipitation buffer. Immunoprecipitated p53 was recovered by resuspending the pellets in Laemmli sample buffer. p53 was detected by Western blotting using as primary antibody the polyclonal anti-p53 antibody CM1 (Novocastra) at a 1:1000 dilution.

2.6. Immunofluorescence analysis

Cells were fixed in 1:1 (v/v) methanol and acetic acid for 5 min at −20 °C. After washing in PBS, cells were labelled with the anti-p53 monoclonal antibody Ab8 (NeoMarkers clone DO7 + BP53-12) in PBS containing 1% bovine serum albumin (BSA). Fluorescein-conjugated secondary antibody was used. Slides were mounted with moviol and examined using a fluorescence microscope. Quantitative analysis of immunofluorecence was performed using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

2.7. DNA extraction and purification

About 5×10^5 cells from eight samples, four control subjects and four AD patients, were harvested and processed for genomic DNA extraction and purification using GenElute™ Mammalian Genomic DNA Purification Miniprep (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Genomic DNA was quantified and the integrity of DNA was verified by agarose gel electrophoresis.

2.8. p53 gene mutation analysis

The exons 1–11 of the p53 gene were amplified in a 50 μl volume reaction containing 200 ng of genomic DNA, 2 μM of each primer, 200 μM of dNTPs, 1.5 U of Pfu Polymerase (Promega, Madison, WI, USA) and 1× buffer supplied with the enzyme. PCR reactions were carried out on a Perkin-Elmer Cetus Thermal Cycler 480 (PE Applied Biosystems, Foster City, CA, USA) as follows: 1 cycle consisting of 5 min at 94 °C, 35 cycles each consisting of 30 s at 94 °C, 30 s at the annealing temperature as reported in Table 1 for each exon, and 1 min at 72 °C, and a final cycle of 5 min at 72 °C. Sequences of p53 primers used for PCR reactions were selected in the flanking region of each exon as described by Duddy et al. [7], and reported in Table 1. PCR products were analysed by agarose gel electrophoresis, purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's protocol, quantified both by spettrophotometer and by agarose gel electrophoresis. Purified fragments were used for direct cycle

Table 1
Primers used for PCR amplification and sequencing of p53 exons

Exon	Primers	Sequences	Annealing temperature (°C)	Product size (bp)
1	Forward	5′-GAGAATCCTGACTCTGCACC-3′	60	351
	Reverse	5′AGCCGAGAGCCCGTGACTCA-3′		
2/3	Forward	5′-CAGGGTTGGAAGCGTCTCATGC-3′	66	363
	Reverse	5′-GAGCAGTCAGAGGACCAGGTCC-3′		
4	Forward	5′-GACCTGGTCCTCTGACTGCT-3′	56	359
	Reverse	5′-GCATTGAAGTCTCATGGAAG-3′		
5	Forward	5′-ACTTGTGCCCTGACTTTCAACT-3′	60	303
	Reverse	5′-CAATCAGTGAGGAATCAGAGGC-3′		
6	Forward	5′-TCAGATAGCGATGGTGAGCAG-3′	60	261
	Reverse	5′-TCAGATAGCGATGGTGAGCAG-3′		
7	Forward	5′-AGGCGCACTGGCCTCATCTT-3′	60	240
	Reverse	5′-GAAATCGGTAAGAGGTGGGC-3′		
8	Forward	5′-GGAGTAGATGGAGCCTGGTTT-3′	60	298
	Reverse	5′-GGTGATAAAAGTGAATCTGAGGC-3′		
9	Forward	5′-AGACCAAGGGTGCAGTTAT-3′	61	294
	Reverse	5′-GTTAGTTAGCTACAACCAGGAGCC-3′		
10	Forward	5′-CAATTGTAACTTGAACCATC-3′	55	258
	Reverse	5′-ATGAGAATGGAATCCTATGG-3′		
11	Forward	5′-GCACAGACCCTCTCACTCATGT-3′	61	229
	Reverse	5′-CAAGGGTTCAAAGACCCAAAAC-3′		

sequencing with ABI PRISM BigDye® Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) and analyzed by capillary electrophoresis on an ABI PRISM 3700 DNA Analyser (PE Applied Biosystems, Foster City, CA, USA). Both forward sequence and reverse sequence for each exon were run. Electropherograms of the coding sequences and splice junctions were checked manually for the presence of any mutations.

2.9. Electrophoretic gel mobility-shift assay

Double-stranded p53 consensus binding-sequence $p53^{cs}$ (GCTTGGACATGCCCGGGCATGTCC) and an oligonucleotide containing the Oct-1 binding sequence [24] were synthesized and end-labelled with [γ-^{32}P]ATP. Nuclear protein fraction was prepared according to Uberti et al. [33]. Binding reaction mixtures containing 0.5 ng ^{32}P-labelled oligonucleotide, 10 μg nuclear extract, 0.5 μg poly(dI–dC), 1 mM DTT and 100 ng monoclonal antibody PAb421, was incubated for 30 min at 20 °C. PAb421 antibody binds to the C-terminus of p53, supershifts and stabilizes p53-DNA complexes; it is required for the detection of high-affinity stable complexes under the conditions of this assay. Thirty microliters of each reaction mixture were loaded onto a 5% non-denaturating polyacrylamide gel and run in TBE buffer at 120 V for 2–3 h. The gels were then fixed, vacuum dried, and exposed to X-ray film at −80 °C for 1 day.

2.10. 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 min at room temperature, stained with 400 μl of propidium iodide (50 μg/ml) and analysed by flow cytometry using 488 nm excitation.

3. Results

3.1. p53 accumulation in AD fibroblasts

Using immunofluorescence analysis we found that p53 levels in resting condition were very low in fibroblasts from non-AD subjects. This is frequently observed in many cell phenotypes harbouring a wild-type p53 because of its very fast turnover [18]. In contrast, fibroblasts from AD patients displayed an intense p53 staining both in the nucleus and cytoplasm. Representative micrographs of p53 staining in fibroblasts from AD patients and age-matched controls are shown in Fig. 1, panel A. Quantitative analysis of immunostainings performed on six non-AD and six AD fibroblast cell lines indicated a significant increase of p53 levels in AD fibroblasts in comparison to non-AD cells (Fig. 1A, right panel). Similar results were obtained when p53 content was measured by Western blot analysis. As shown in a representative blot in Fig. 1B, p53 was virtually undetectable in protein extracts from non-AD cells (lanes 1 and 2), while an intense signal was found in AD fibroblasts (lanes 3 and 4). Western blot and densitometric analysis of cell lines derived from six AD patients and six age-matched non-AD subjects indicated a three-fold increase of p53 expression in AD fibroblasts (Fig. 1B, right panel).

3.2. Lack of p53 functional activity in AD fibroblasts

Induction of p53 in response to stress occurs essentially by post-transcriptional modifications resulting in protein

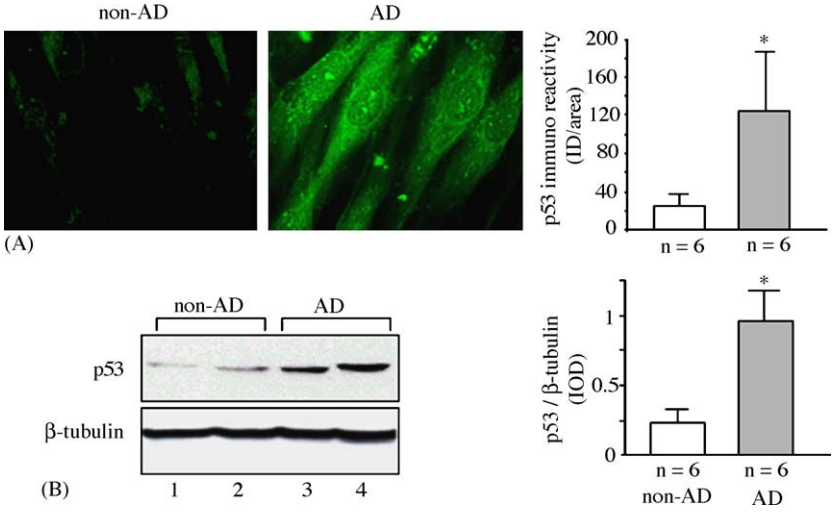

Fig. 1. p53 protein expression in fibroblasts from AD and non-AD patients. Left panels show the results from representative experiments of immunofluorescence (A) and Western blot analysis (B) on non-AD and AD fibroblasts, as indicated, in resting conditions. Numbers in panel B indicate individual samples from non-AD (lanes 1 and 2) and AD (lanes 3 and 4). Right panels report quantitative analysis of immunostainings (upper) and immunoblots (lower) performed on six non-AD and six AD different fibroblast cell lines. Bars represent the mean values ± S.E.M. $^{*}p < 0.01$ vs. the corresponding non-AD values.

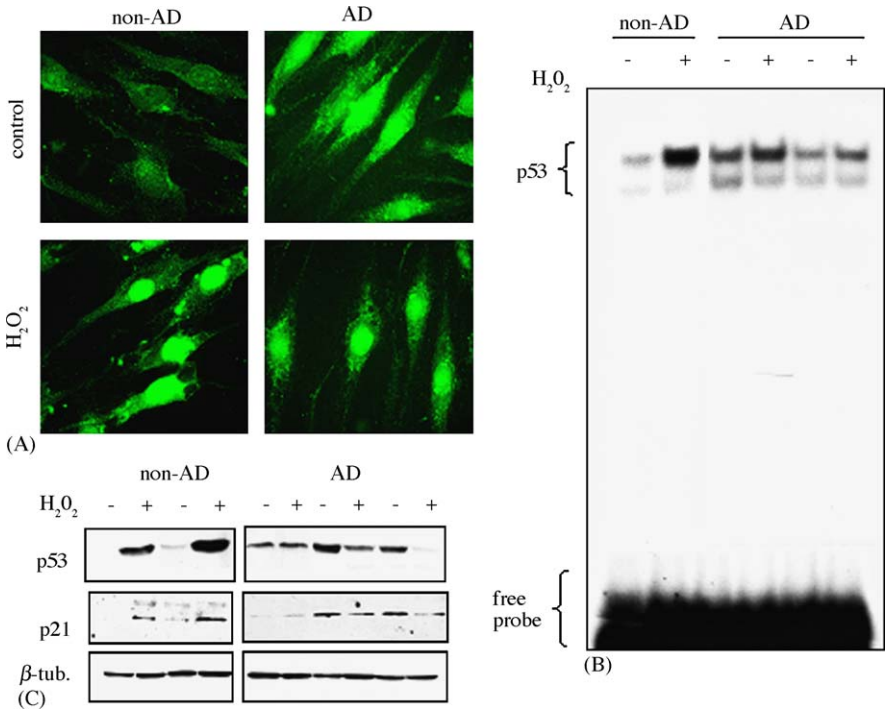

Fig. 2. p53 functional activity in fibroblasts from AD and non-AD patients. Cells were treated with H_2O_2 (1 mM) for 15 min and processed after 2 h for immunofluorescence (A), EMSA (B) or Western blot analysis (C). (A) Immunofluorescence experiments performed on one non-AD and one AD fibroblast cell line were carried out with a monoclonal anti-p53 antibody (see Section 2). (B) Nuclear extracts derived from one non-AD and two AD cell lines were incubated with ^{32}P-labeled p53 consensus DNA-binding sequence in the presence of PAb421, and analysed by EMSA. (C) Total extracts derived from two non-AD and three AD cell lines were loaded onto 12% SDS-PAGE gel and immunobloted with anti-p53, anti-p21 and anti-β-tubulin antibodies.

stabilization, by escaping from proteosome-mediated degradation, and in conformational changes that increase the affinity of the protein for specific DNA sequences. This active form of p53 regulates the transcription of a number of target genes, including genes that mediate negative regulation of cell cycle [8]. Among the latter is p21/waf1, a cyclin kinase inhibitor acting at both G1 and G2 phases. The treatment of non-AD fibroblasts with a brief pulse (15 min) of 1 mM H_2O_2 induced a rapid accumulation of p53 protein (Fig. 2C, left panels), that translocated into the nucleus as shown by immunofluorescence experiments (Fig. 2A, left panels). This event was accompanied by an enhancement of p53 DNA binding activity and the induction of p21/waf1 protein expression as measured by EMSA (Fig. 2B, lanes 1 and 2) and Western blot analysis, respectively (Fig. 2C, left panel). In fibroblasts from AD patients, p53 staining was already high and detectable in both nucleus and cytoplasm of untreated cells. The nuclear component slightly increased after the H_2O_2 insult (Fig. 2A and C, right panels). EMSA experiments shown an intense band corresponding to p53-probe complex in basal condition of AD fibroblasts that only barely increased following H_2O_2 pulse (Fig. 2B). In AD cell lines, p53 DNA binding activity was not supported by evidence of transcriptional activity, as demonstrated by measuring p21 expression (Fig. 2C, right panels). This data are in line with our previous findings demonstrating, in a different set of AD fibroblast cell lines, an anomalous expression of p53 and of its target gene products [35]. In all, these data suggest that p53 signalling pathway is impaired in fibroblasts from sporadic AD patients.

3.3. AD fibroblasts result less sensitive to different cytotoxic stimuli

To further elucidate the significance of an impairment of p53 in AD fibroblasts, AD and non-AD cell lines were challenged with different cytotoxic agents including doxorubicin and CDDP. Flow cytometric analysis of cell cycle distribution showed the presence of a sub-Go cell fraction following H_2O_2, doxorubicin and CDDP in non-AD fibroblasts, suggesting that these cells underwent apoptosis. Differently these three different cytotoxic agents did not modified the representative pattern of cell distribution of AD fibroblast cell line. Representative data are shown in Fig. 3A. The quantitative analysis of cells distribution in sub-Go phase was performed on fibroblasts derived from five AD and five non-AD patients. H_2O_2, doxorubicin and CDDP induced a significant increased of sub-Go fraction in non-AD cells, while AD fibroblasts resulted more resistant to H_2O_2, doxorubicin and CDDP induced apoptosis (Fig. 3B)

3.4. Evidence for p53 conformational changes in AD fibroblasts

High p53 content and impaired p53 transcriptional activity is a common feature of many solid tumours carrying out

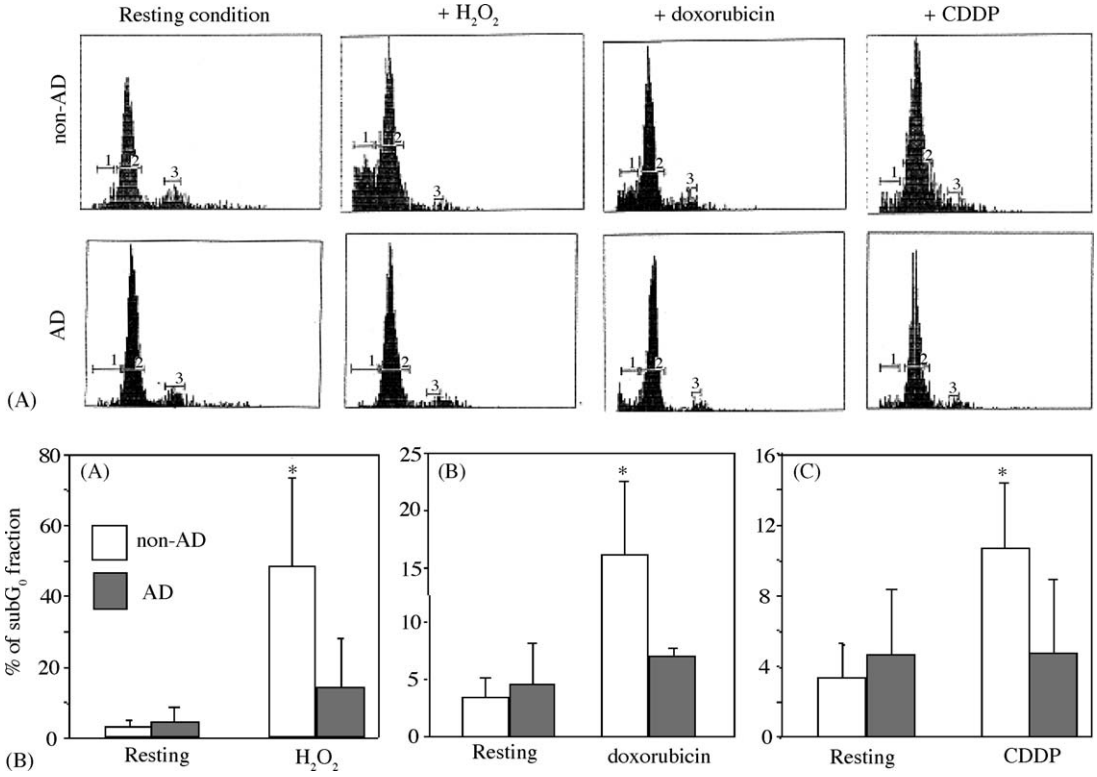

Fig. 3. Sensitivity to different cytotoxic stimuli of fibroblasts from AD and non-AD patients. (A) Representative patterns of cell cycle distribution by flow cytometric analysis of fibroblasts from AD and non-AD samples in resting condition and after treatment with H_2O_2, doxorubicin and CDDP. (B) Distribution of cells in sub-Go phase of fibroblasts derived from five AD and five non-AD patients. Bars represent the mean values ± S.E.M. $^*p < 0.01$ vs. the corresponding non-AD values.

mutations in p53 gene. Thus, we investigated in fibroblasts from sporadic AD patients the presence of point mutations of p53 gene.

For gene analysis, exons 1–11, codifying the entire p53 gene, were took into consideration. To this end, genomic DNA from fibroblasts of four AD and four non-AD patients was extracted, exons and intron–exon boundaries of exons 1–11 were PCR-amplified, and sequenced. Analysis of the amplified products revealed no points mutation in the p53 gene sequences from both AD and non-AD fibroblasts (data not shown).

High p53 content and impaired p53 transcriptional activity is also the result of a change in the tertiary structure of the protein [36]. This alteration confers on p53 a mutant phenotype [24]. We thus investigated on the conformational state of p53 in fibroblasts from AD patients.

The conformational status of p53 in fibroblasts derived from AD and non-AD subjects were studied by immunoprecipitation technique using two conformational-specific antibodies, PAb1620 and PAb240, which discriminate folded versus unfolded p53 tertiary structure [23]. In particular, the PAb1620 epitope is exposed in the wild-type p53 form that is competent for DNA binding and depends on the tertiary folding of the protein. In contrast, the PAb240 epitope is cryptic in the protein that is competent for DNA binding but is exposed by denaturation. Many p53 mutants have been shown to lose reactivity to PAb1620 and to react to PAb240, leading to the identification of two alternative "wild-type" (PAb1620+; PAb240−) and "mutant" (PAb1620−; PAb240+) protein phenotypes [23]. PAbBP53.12, that recognizes an epitope localized in the N-terminus domain of the molecule, was used to immunoprecipitate both folded "wild-type" and unfolded "mutant like" p53 phenotypes. Immunoprecipitates were then analysed by Western blot analysis with a rabbit polyclonal p53 antibody. Fig. 4, panel A shows a representative experiment performed on one non-AD and one AD fibroblast cell line in resting condition. As expected, non-AD fibroblasts expressed exclusively wild-type p53 as demonstrated by the reactivity with PAb1620, and the absence of reactivity with PAb240. In AD cells although the PAb1620 positive phenotype was presented, an unfolded p53 isoform appeared, as identified by reactivity to PAb240. When more non-AD ($n = 6$) and AD ($n = 6$) cell lines were tested for immunoprecipitation experiment, two well separate p53 phenotype patterns were identified. The calculated ratio between the intensity of the bands immunoreactive to PAb1620 and PAb240 was significantly lower in AD fibroblasts in comparison with that derived from non-AD subjects (Fig. 4, panel B).

Furthermore, the analysis of PAb240 (+) isoforms performed on four non-AD and five AD cell lines clearly underscored a conformational mutant-like p53 that was a peculiar

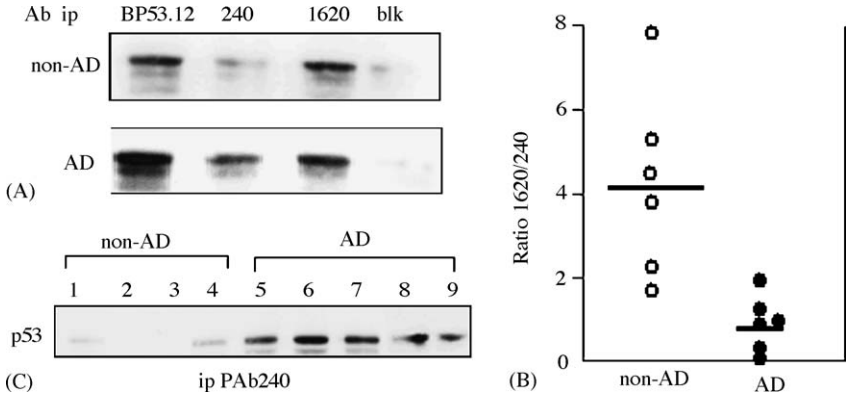

Fig. 4. p53 conformational state in fibroblasts from AD and non-AD patients. Protein extracts derived from six non-AD and six AD fibroblast cell line were immunoprecipitated with the following antibodies: PAb240 (specific for "mutant" unfolded conformation); PAb1620 (specific for wild-type, folded conformation) and BP53.12 (which reacts with both conformers). Immunoprecipitates were analysed by Western blot with the CM1 polyclonal anti-p53 antibody. (A) Representative experiment performed on one non-AD and one AD fibroblast cell line. Immunoprecipitated antibodies were omitted in control (blk) samples. (B) Calculated ratio between the intensity of the PAb1620 and PAb240 immunoreactive bands obtained from six non-AD (white circle) and six AD (black circle) fibroblast cell lines. (C) Four non-AD and five AD cell lines were processed in simultaneity for immunoprecipitation experiment only with PAb240.

feature of AD fibroblasts and absent in non-AD cell lines (Fig. 4, panel C).

4. Discussion

Although there have been vast advances in AD research, this field still faces a great number of serious impediments to progress in translating basic science discoveries into effective treatments and evidence-based clinical practices for dementia. One of the many challenges that remains to be addressed is the availability of appropriate biological markers of the disease. In this regard, a growing body of evidence suggests that peripheral cells can be considered a good model to study in vitro the dynamic alterations of metabolic and biochemical processes that may mirror events occurring in the AD brain. For example, alterations of protein kinase C activity and APP metabolism described in AD brain have been consistently found in cultured skin fibroblasts [2,29]. Further abnormalities in AD peripheral cells have also been described in the calcium homeostasis, with an increase of cytosolic free-calcium in platelets and lymphocytes derived from AD patients [27], and in oxidative metabolism, with alterations in mitochondrial oxidative pathway in fibroblasts from sporadic AD patients [9,10].

We recently found in fibroblasts from sporadic AD patients a specific alteration of the p53 intracellular pathway involved in sensing and repairing DNA damage [35]. Here we show that fibroblasts from sporadic AD patients specifically express an anomalous and detectable conformational state of p53 that makes these cells distinct from fibroblasts of age-matched non-AD subjects.

p53 may be expressed in the cells in at least two different conformational forms, referred to as "wild type" and "mutant", which differ in terms of stability, transcriptional activity, and sensitivity to specific antibodies [23]. We found that p53 in fibroblasts from AD patients, contrary to that present in fibroblasts from non-AD subjects: (1) is expressed at higher levels in resting conditions, (2) only barely increases into the nucleus after H_2O_2 treatment, (3) presents impaired transcriptional activity, and (4) is distinguishably reactive to the PAb240 (mutant specific) p53 antibody. These results clearly show that AD fibroblasts express a certain amount of p53 endowed with functional and structural features to be defined as having a "mutant-like" conformation. It should be underlined that wild type p53, as recognized by the PAb1620 antibody, was still present in AD fibroblasts and only a fraction of the total p53 showed immunoreactivity to the PAb240 (mutant specific) p53 antibody. Since, at the present, we have no record of any kind of skin tumour in all the sporadic AD patients studied so far it may be inferred that the impairment of p53 in AD fibroblasts is compensated by the activity of other tumour suppressor pathways.

We cannot speculate at this time whether mutant p53 is also present in the brain of AD patients and eventually the relevance of such impairment in terms of neuronal function. Studies in this direction are now in progress in our laboratory. There are however, previous post-mortem studies suggesting an involvement of p53 in degenerating neurons in AD. These include de la Monte et al. [6] showing increased p53 and Fas expression in specific populations of cortical neurons; Kitamura et al. [17] showing increased amount of p53 in temporal cortex, mainly localized in glial cells; Seidl et al. [30] showing higher levels of p53 in frontal and temporal lobe from Down syndrome patients. If p53 levels are increased in specific neurons in AD brain, one could infer that these are degenerating neurons; alternatively, the increased level might reflect the expression of a conformational mutant isoform of p53, as we detected in AD fibroblasts. It could also be of interest to verify whether other peripheral, and more accessible, cells express mutant p53. This information will allow the availability of a large number of biological samples to validate the

presence of mutant p53 as an appropriate biological marker of AD.

Mutant isoforms of p53 are indeed typically present in tumours carrying p53 gene mutation. However, sequence analysis of all exons of p53 gene suggests that the PAb240 reactive p53 found in AD fibroblasts is not the result of gene mutation. Thus, our data suggest that one of the peripheral events associated to the disease is responsible for generating such p53 conformation isoform.

One of the causes for modifying p53 tertiary structure to acquire the PAb240 reactive phenotype is an abnormally high oxidative state [23,28,31,36] and a number of evidence suggests that peripheral cells, including fibroblasts, of AD patients are exposed to high levels of oxygen radicals [3,9,12,29]. Although this condition has been associated with neuronal loss in the brain, no data are available, at least up to now, of a clear sign of pathology in peripheral organs. Since PAb240 reactive p53 may be generated in vitro by exposing various types of cells to experimental oxidative stress, we may speculate that PAb240 reactive p53 in AD fibroblasts is the result of an elevated redox status of these cells.

Finally, it is intriguing that the PAb240 reactive p53 identified in AD fibroblasts has many biochemical and functional features of the mutant p53 found in tumours. This apparent link can be added to the growing list of evidence accumulated in the very recent years that suggests an involvement of cancer-related mechanisms in the development of AD.

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References

[1] Almog N, Rotter V. Involvement of p53 in cell differentiation and development. Biochim Biophys Acta 1997;1333:F1–27.
[2] Bergamaschi S, Binetti G, Govoni S, Wetsel WC, Battaini F, Trabucchi M, et al. Defective phorbol ester-stimulated secretion of beta-amyloid precursor protein from Alzheimer's disease fibroblasts. Neurosci Lett 1995;201:1–5.
[3] Cecchi C, Fiorillo C, Sorbi S, Latorraca S, Nacmias B, Bagnoli S, et al. Oxidative stress and reduced antioxidant defenses in peripheral cells from familial Alzheimer's patients. Free Radic Biol Med 2002;33:1372–9.
[4] Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor–DNA complex: understanding tumorigenic mutations. Science 1994;265:346–55.
[5] Culmsee C, Zhu X, Yu QS, Chan SL, Camandola S, Guo Z, et al. A synthetic inhibitor of p53 protects neurons against death induced by ischemic and excitotoxic insults, and amyloid beta-peptide. J Neurochem 2001;77:220–8.
[6] de la Monte SM, Sohn YK, Wands JR. Correlates of p53- and Fas (CD95)-mediated apoptosis in Alzheimer's disease. J Neurol Sci 1997;152:73–83.
[7] Duddy PM, Hanby AM, Barnes DM, Camplejohn RS. Improving the detection of p53 mutations in breast cancer by use of the FASAY, a functional assay. J Mol Diag 2000;2:139–44.
[8] El-Deiry WS. Regulation of p53 downstream genes. Semin Cancer Biol 1998;8:345–57.
[9] Gasparini L, Racchi M, Binetti G, Trabucchi M, Solerte SB, Alkon D, et al. Peripheral markers in testing pathophysiological hypotheses and diagnosing Alzheimer's disease. FASEB J 1998;12:17–34.
[10] Gibson GE, Huang HM. Oxidative processes in the brain and non-neuronal tissues as biomarkers of Alzheimer's disease. Front Biosci 2002;7:d1007–15.
[11] Gibson G, Martins R, Blass J, Gandy S. Altered oxidation and signal transduction systems in fibroblasts from Alzheimer patients. Life Sci 1996;59:477–89.
[12] Gibson GE. Interactions of oxidative stress with cellular calcium dynamics and glucose metabolism in Alzheimer's disease. Free Radic Biol Med 2002;32:1061–70.
[13] Gilman CP, Chan SL, Guo Z, Zhu X, Greig N, Mattson MP. p53 is present in synapses where it mediates mitochondrial dysfunction and synaptic degeneration in response to DNA damage, and oxidative and excitotoxic insults. Neuromol Med 2003;3:159–72.
[14] Govoni S, Bergamaschi S, Racchi M, Battaini F, Binetti G, Bianchetti A, et al. Cytosol protein kinase c downregulation in fibroblasts from Alzheimer's disease patients. Neurology 1993;43:2581–6.
[15] Govoni S, Gasparini L, Racchi M, Trabucchi M. Peripheral cells as an investigational tool for Alzheimer's disease. Life Sci 1996;59:461–8.
[16] Jong YJ, Dalemar LR, Seehra K, Baenziger NL. Bradykinin receptor modulation in cellular models of aging and Alzheimer's disease. Int Immunopharmacol 2002;2:1833–40.
[17] Kitamura Y, Shimohama S, Kamoshima W, Matsuoka Y, Nomura Y, Taniguchi T. Changes of p53 in the brains of patients with Alzheimer's disease. Biochem Biophys Res Commun 1997;232:418–21.
[18] Ko LJ, Prives C. p53: puzzle and paradigm. Genes Dev 1996;10:1054–72.
[19] Leker RR, Aharonowiz M, Greig NH, Ovadia H. The role of p53-induced apoptosis in cerebral ischemia: effects of the p53 inhibitor pifithrin alpha. Exp Neurol 2004;187:478–86.
[20] Levine AJ, Perry ME, Chang A, Silver A, Dittmer D, Wu M, et al. The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumorigenesis. Br J Cancer 1994;69:409–16.
[21] Mazzola JL, Sirover MA. Subcellular alteration of glyceraldehyde-3-phosphate dehydrogenase in Alzheimer's disease fibroblasts. J Neurosci Res 2003;71:279–85.
[22] McLure KG, Lee PW. p53 DNA binding can be modulated by factors that alter the conformational equilibrium. EMBO J 1999;18:763–70.
[23] Meplan C, Richard MJ, Hainaut P. Redox signalling and transition metals in the control of the p53 pathway. Biochem Pharmacol 2000;59:25–33.
[24] Milner J. Flexibility: the key to p53 function? Trends Biochem Sci 1995;20:49–51.
[25] Morrison RS, Kinoshita Y, Johnson MD, Guo W, Garden GA. p53-dependent cell death signaling in neurons. Neurochem Res 2003;28:15–27.
[26] Palotas A, Kalman J, Laskay G, Juhasz A, Janka Z, Penke B. Comparative studies on $[Ca2+]_i$-level of fibroblasts from Alzheimer patients and control individuals. Neurochem Res 2001;26:817–20.
[27] Palotas A, Kalman J, Palotas M, Juhasz A, Janka Z, Penke B. Fibroblasts and lymphocytes from Alzheimer patients are resistant to beta-amyloid-induced increase in the intracellular calcium concentration. Prog Neuropsychopharmacol Biol Psychiatry 2002;26:971–4.
[28] Parks D, Bolinger R, Mann K. Redox state regulates binding of p53 to sequence-specific DNA, but not to non-specific or mismatched DNA. Nucl Acids Res 1997;25:1289–95.
[29] Racchi M, Bergamaschi S, Govoni S, Wetsel WC, Bianchetti A, Binetti G, et al. Characterization and distribution of protein kinase C isoforms in human skin fibroblasts. Arch Biochem Biophys 1994;314:107–11.

[30] Seidl R, Fang-Kircher S, Bidmon B, Cairns N, Lubec G. Apoptosis-associated proteins p53 and APO-1/Fas (CD95) in brains of adult patients with down syndrome. Neurosci Lett 1999;260:9–12.
[31] Sun Y, Oberley LW. Redox regulation of transcriptional activators. Free Radic Biol Med 1996;21:335–48.
[32] Turner RS. Biomarkers of Alzheimer's disease and mild cognitive impairment: are we there yet? Exp Neurol 2003;183:7–10.
[33] Uberti D, Yavin E, Gil S, Ayasola KR, Goldfinger N, Rotter V. Hydrogen peroxide induces nuclear translocation of p53 and apoptosis in cells of oligodendroglia origin. Brain Res Mol Brain Res 1999;65:167–75.
[34] Uberti D, Belloni M, Grilli M, Spano P, Memo M. Induction of tumour-suppressor phosphoprotein p53 in the apoptosis of cultured rat cerebellar neurones triggered by excitatory amino acids. Eur J Neurosci 1998;10:246–54.
[35] Uberti D, Carsana T, Bernardi E, Rodella L, Grigolato P, Lanni C, et al. Selective impairment of p53-mediated cell death in fibroblasts from sporadic Alzheimer's disease patients. J Cell Sci 2002;115:3131–8.
[36] Verhaegh GW, Parat MO, Richard MJ, Hainaut P. Modulation of p53 protein conformation and DNA-binding activity by intracellular chelation of zinc. Mol Carcinog 1998;21:205–14.
[37] Zhao WQ, Ravindranath L, Mohamed AS, Zohar O, Chen GH, Lyketsos CG, et al. MAP kinase signaling cascade dysfunction specific to Alzheimer's disease in fibroblasts. Neurobiol Dis 2002;11:166–83.