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Elevated Oxidative Stress in Patients with Ataxia Telangiectasia

JANINE REICHENBACH,[1] RALF SCHUBERT,[1] DETLEF SCHINDLER,[2]
KLAUS MÜLLER,[1] HANSJOSEF BÖHLES,[1] and STEFAN ZIELEN[1]

ABSTRACT

Ataxia telangiectasia (AT) is a pleiotropic genetic disorder characterized by progressive neurodegeneration, especially of cerebellar Purkinje cells, immunodeficiency, increased incidence of cancer, and premature aging. The disease is caused by functional inactivation of the ATM (AT-mutated) gene product, which is thought to act as a sensor of reactive oxygen species and oxidative damage of cellular macromolecules and DNA. The compound phenotype of AT might thus be linked to a continuous state of oxidative stress leading to an increase of programmed cell death (apoptosis). To assess this hypothesis, we analyzed lipid peroxidation products and the oxidative stress associated DNA base damage 8-hydroxy-2-deoxyguanosine in patients with AT. Oxidative damage to lipids and DNA was found to be markedly increased in AT patients. These results indicate that ATM might play an important role in the maintenance of cell homeostasis in response to oxidative damage. In this context, a better control of levels of reactive oxygen species could be a rational foundation of therapeutic intervention to help alleviate some of the symptoms associated with AT. *Antioxid. Redox Signal.* 4, 465–469.

INTRODUCTION

Ataxia telangiectasia (AT) is an autosomal recessive genetic disorder characterized by neurodegeneration with progressive cerebellar ataxia, oculocutaneous telangiectasia, variable cellular and humoral immunodeficiency, premature aging, and an increased predisposition to lymphoreticular malignancies. The gene responsible for the pleiotropic phenotype of the disease, *ATM* (AT-mutated), is a member of a family of large proteins that have a phosphatidylinositol 3-kinase catalytic domain at the C-terminus (33). These proteins are involved to different extents in cell-cycle control, cellular responses to DNA damage, and chromosomal maintenance (17, 19, 23, 25, 42). However, the roles for ATM in programmed cell death (apoptosis), an important cellular response to DNA damage, are less clear (2, 8, 9, 26, 39, 40). Especially, the mechanisms underlying the apoptotic loss of naive T cells as well as postmitotic cerebellar Purkinje cells in AT patients have remained elusive (37). A hypothesis explaining the sensitivity of AT cells to apoptosis postulates that AT cells are unable to counteract the effects of reactive oxygen species (ROS), which damage macromolecules and induce cell death (4, 20). In this perspective, ATM would act as a sensor for oxidative stress, caused by an excess of ROS (32). ATM abnormalities may thus lead to apoptotic cell death due to oxidative damage (30). In earlier studies, we showed diminished antioxidant competence and a decreased ability to counteract oxidative stress in patients with AT (29). To study further the roles played by ATM in the cellular reaction to oxidative damage, we analyzed lipid peroxidation products and the oxidative stress-associated DNA base damage 8-hydroxy-2-deoxyguanosine (8-OHdG) in patients with AT. Oxidative damage to lipids and DNA was found to be markedly increased in AT patients. These results indicate that ATM might play an important role in the maintenance of cell homeostasis in response to oxidative damage.

[1]Department of Pediatrics, Johann-Wolfgang Goethe Universität Frankfurt, Frankfurt, Germany.
[2]Institute for Human Genetics, Bayerische Julius-Maximilians-Universität, Würzburg, Germany.

MATERIALS AND METHODS

AT patients

Thirty-three patients with AT (median age 16, range 3–27 years; 15 male, 18 female) were studied. Diagnosis was established in accordance with the World Health Organization recommendations (1). All patients showed increased levels of α-fetoprotein (data not shown) and atypical G2 arrest measured by flow cytometry. EDTA blood was collected from patients and healthy age-matched controls.

The study was approved by the local Human Committee of Ethics, and informed consent was obtained from patients and parents before entering the study.

Detection of apoptosis

Flow cytometry was used to measure the percentage of apoptosis after 7-amino actinomycin D (7AAD) staining (28, 34). In brief, cells were incubated with 20 μg/ml 7AAD (in phosphate-buffered saline/azide) for 20 min at 4°C in the dark; afterwards cells were analyzed within 30 min in their staining solution. Samples were analyzed by a flow cytometer (FACScan; Becton–Dickinson, San Jose, CA, U.S.A.) equipped with an air-cooled 488-nm argon-ion laser. On each sample, 10 000 events were collected and data analysis was performed with Lysis II software (Becton–Dickinson).

Lipid peroxidation

Total lipid peroxidation was measured from plasma samples of 33 patients and 19 controls by the commercial Per-Ox assay (ImmunDiagnostik, Bensheim, Germany). Measurement was performed according to the manufacturer's instructions.

8-OHdG

Oxidative DNA damage was analyzed by measurement of 8-OHdG in aqueous solution of DNA samples derived from peripheral blood mononuclear cells (PBMC). DNA was isolated using a blood and cell culture DNA maxi kit (Qiagen, Valencia, CA, U.S.A.). DNA was recovered by spooling, washed once with 70% ethanol, and then air-dried. After DNA was dissolved in 10 m*M* phosphate buffer (pH 7.4), DNA concentration was determined by UV spectroscopy. DNA concentration was adjusted to 0.3 mg/ml. Hydrolysis of DNA was accomplished by the combined use of four enzymes as described (6). Measurement was then performed by high-performance liquid chromatography–mass spectrometry as described (7).

Statistical analysis

Results were expressed as means ± SD and analyzed using the unpaired Student's *t* test (SPSS Inc., Chicago, IL, U.S.A.). $p < 0.05$ was regarded as significant.

RESULTS

Spontaneous apoptosis is increased in AT PBMC

To examine spontaneous programmed cell death *in vitro*, membrane permeabilization was detected by 7AAD staining (28) after 24 and 72 h (early/late apoptotic and dead cells, respectively) of incubation of cells in culture medium. In accordance with prior investigations (8, 35, 38), AT lymphocytes displayed a higher rate of spontaneous apoptosis compared with controls: (a) at 24 h: $p < 0.01$; patients, 13.55 ± 5.77% (range 7.76–24.61%), controls, 7.27 ± 1.72% (range 4.15–9.73%); (b) at 72 h: patients, 25.94 ± 7.58% (range 15.02–41.56%), controls, 12.96 ± 6.82% (range 5.92–20.09%) (Fig. 1). Given the high level of spontaneous apoptosis together with the diminished capacity to counteract oxidative stress (29), we addressed the question whether apoptosis might be accompanied by oxidative damage of macromolecules in AT cells.

AT lymphocytes show evidence of oxidative damage to lipids

To study the role of oxidative stress *in vivo*, markers of oxidative damage to lipids were measured in plasma of 33 patients with AT by the Per-Ox assay (ImmunDiagnostik). We quantified levels of lipid hydrogen peroxide (H_2O_2) equivalents in plasma (Fig. 2). Lipid H_2O_2 equivalents were found to be significantly elevated in patients with AT [$p < 0.005$; patients, 334.03 ± 140.27 (range 89.13–571.02); controls (n= 19), 199.84 ± 119.08 (range 103.79–516.48)]. However, there was no correlation of levels of lipid hydroperoxides and spontaneous apoptosis as measured by 7AAD staining.

8-OHdG

Oxidative stress has been reported to cause DNA damage, mainly in the form of oxidized nucleic bases (10). A common

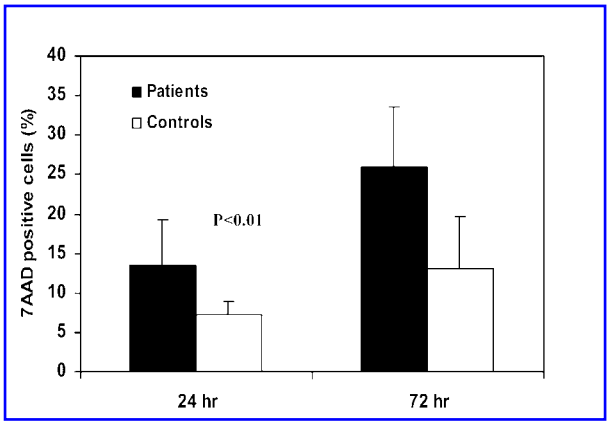

FIG. 1. Spontaneous apoptosis of AT lymphocytes and lymphocytes derived from controls were measured by 7AAD incorporation. Cells were harvested after 24 and 72 h of incubation in culture medium and after staining with 7AAD 7AAD+ cells (early/late apoptotic and dead cells, respectively) were detected by flow cytometry. Results are shown as means ± SD. p value is based on Student's t test.

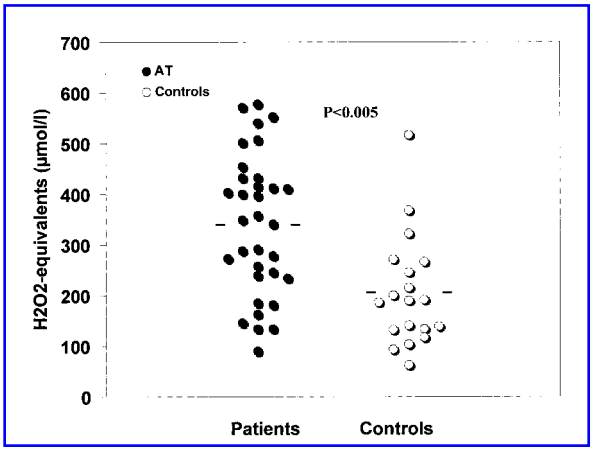

FIG. 2. Total lipid peroxides from plasma samples of 33 patients and 19 controls were measured by a photometric Per-Ox assay. H_2O_2 equivalents (µmol/H_2O_2) single values are presented as dots, and means are shown as horizontal bars. *p* value is based on Student's *t* test.

and extensively studied DNA modification caused by oxidation occurs by addition of OH˙ to the C-8 position of guanine leading to the formation of 8-oxodeoxyguanosine (21, 27). We analyzed the ratio of 8-OHdG to 2-deoxyguanosine (dG) in DNA samples of six patients with AT (Fig. 3). This ratio was markedly lower in AT patients ($p < 0.005$), *i.e.,* oxidative DNA damage the form of 8-OHdG was increased. There was a trend toward higher levels of spontaneous apoptosis in patients with a very low 8-OHdG to dG ratio, *i.e.,* with an increase of oxidative DNA damage. However, due to the small number of patients, the correlation was not analyzed. The ratio of 8-OHdG to dG was as follows: patients, 22,200 ± 6,671 (range 15,000–31,100); controls, 46,020 ± 21,523 (range 24,000–90,000).

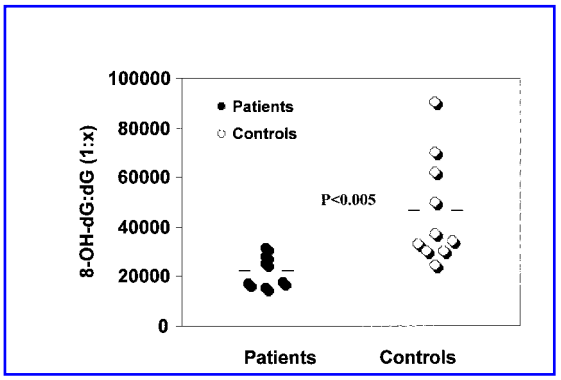

FIG. 3. Oxidative DNA damage was detected by the amount of 8-OHdG as described in Materials and Methods. DNA was isolated from PBMC of six AT patients and 10 controls. Single values (dots) are shown as the ratio of 8-OHdG to unoxidized guanosine (dG), and means are presented as horizontal bars. *p* value is based on Student's *t* test.

DISCUSSION

At nontoxic concentrations, ROS may play a role in signal transduction (11, 36), but at higher levels they can cause oxidative damage to macromolecules, resulting in lipid peroxidation, strand breaks, and oxidation of guanosine residues in DNA (20, 41). To prevent oxidative damage in cells, the generation and elimination of free radicals have to be in balance (15). Our previous work demonstrated that the cellular antioxidative capacity is overwhelmed in patients with AT (29). However, it was not clear whether the diminished antioxidant competence has consequences on the cellular redox homeostasis in patients with AT. Our present results provide further evidence that macromolecules and DNA from patients with AT are indeed under oxidative stress and suffer oxidative damage.

It has been suggested that oxidative stress might play a role in the clinical and cellular manifestations of AT (30, 31), and several reports have implicated ATM as a serine protein kinase that senses and controls cellular reactions to this form of stress (31, 37). According to the hypothesis that dysfunctional ATM leads to inefficient control of redox homeostasis, we found a significant increase of lipid peroxidation products in plasma of patients with AT. This finding might be linked to the detected increase of spontaneous and oxidative stress-induced apoptosis in lymphocytes of our patients with AT, because free oxygen radicals can kill cells via the oxidation of membrane polyunsaturated fatty acids to fatty acid hydroperoxides (15). By extracting a hydrogen atom from adjacent fatty acyl chains, lipid hydroperoxides can initiate an autocatalytic chain reaction ultimately leading to membrane destruction and cell death (15). In addition, hydroperoxy fatty acids can also be synthesized intracellularly by the action of cellular lipoxygenases in association with increased levels of ROS (13, 22). The neurodegeneration in patients with AT might be linked to the observed oxidation of membrane lipids, because brain contains large amounts of polyunsaturated fatty acids, which are particularly vulnerable to free radical attack (14). In this context, it is interesting to note that other groups have detected increased levels of oxidant-modified proteins and lipids in tissues of ATM-knockout mice, particularly in cerebellar Purkinje cells (3, 18). In mice, high levels of ATM protein expression are found in the brain, suggesting an important, yet nonelucidated, role for functional ATM in the nervous system (5). As the preferential loss of Purkinje and granule cells in the cerebellum occurs primarily during the development of the nervous system, it has been speculated that intensive signaling activity in developing cerebellar neurons would lead to a rise in ROS (24). Cellular defense mechanisms responsible for protecting cells from ROS being possibly impaired in ATM-deficient cells, a greater susceptibility to cumulative damage and inappropriate apoptosis might be the consequence (24, 30). However, in our experimental setting, there was no correlation of levels of lipid hydroperoxides and spontaneous apoptosis. This might be due to the fact that measurement of oxidized lipids in plasma represents only a fraction of lipid hydroperoxides, and thus allows only a rough estimate of the overall oxidative damage to cellular lipids. In addition, other mechanisms than oxidative damage to lipids are likely to be involved in the increase of sponta-

neous and oxidative stress-induced programmed cell death in patients with AT.

Oxygen radicals can also attack deoxynucleic acids, and the resulting oxidative DNA damage is implicated in mutagenesis, carcinogenesis, and aging (16). The DNA base damage 8-OHdG is a prominent indicator of oxidative stress and has been well characterized as a premutagenic lesion in mammalian cells and putative initiator of the carcinogenic process (12). As 8-OHdG does not effectively block the progression of DNA replication, it has a high probability of read-through and mutation fixation (12). The analysis of the ratio of oxidized 8-OHdG to dG revealed an increase of oxidative DNA damage in patients with AT. It remains to be tested on a larger number of patients whether this finding may be linked to the increase of cellular degeneration in AT and whether it may explain some of the diverse aspects of this pleiotropic disorder, such as immunodeficiency, neurodegeneration, and premature aging. Nevertheless, our findings of reduced antioxidant capacity and increased oxidative damage to lipids and DNA suggest that a better control of ROS levels should be part of future therapy of patients with AT.

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ABBREVIATIONS

7AAD, 7-aminoactinomycin D; AT, ataxia telangiectasia; ATM, ataxia telangiectasia-mutated; dG, 2-deoxyguanosine; H_2O_2, hydrogen peroxide; 8-OHdG, 8-hydroxy-2-deoxyguanosine; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species.

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Address reprint requests to:
Prof. S. Zielen
Kinderklinik der
Johann Wolfgang Goethe-Universität Frankfurt
Theodor Stern Kai 7
60590 Frankfurt
Germany

E-mail: Stefan.Zielen@kgu.de

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