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Spontaneous and induced chromosome damage in somatic cells of sporadic and familial Alzheimer's disease patients

Francesca Trippi, Nicoletta Botto, Roberto Scarpato, Lucia Petrozzi[1], Ubaldo Bonuccelli[1], Stefania Latorraca[2], Sandro Sorbi[2] and Lucia Migliore[3]

Dipartimento di Scienze dell'Uomo e dell'Ambiente, [1]Dipartimento di Neuroscienze, University of Pisa, Pisa and [2]Dipartimento di Scienze Neurologiche e Psichiatriche, University of Florence, Florence, Italy

Alzheimer's disease (AD) is a neurodegenerative disorder of the elderly with a complex etiology due to the interaction between genetic and environmental factors. At least 15% of cases are inherited as an autosomal dominant mutation, but the majority are sporadic. We evaluated cytogenetic alterations, both spontaneous and chemical-induced [aluminium (Al) and griseofulvin (GF)], by means of the micronucleus (MN) test in lymphocytes or skin fibroblasts of 14 patients with sporadic and eight with familial Alzheimer's disease (FAD), respectively. The spontaneous MN frequencies of sporadic (20.8 ± 9.2) and familial (20.7 ± 4.6) AD patients are significantly higher than those of the respective control groups (9.0 ± 6.8 and 6.7 ± 3.4). In all AD patients, GF significantly increased the spontaneous MN frequency of somatic cells to a lesser extent ($P < 0.05$) as compared with the control group. Al treatment did not induce MN in AD patients. The results of the present study indicate that different types of somatic cells from sporadic and familial AD patients show comparable levels of spontaneous cytogenetic anomalies, and MN induction is partially reduced or lacking according to the type of chemical treatments.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the elderly, characterized clinically by progressive impairment of memory and other intellectual functions, with a complex etiology due to the interaction between genetic and environmental factors. At least 15% of cases appear to be due to the inheritance of an autosomal dominant mutation, but the majority are sporadic, showing no clear association with any identifiable genetic or environmental factor (Schellenberg, 1995; Strittmatter and Roses, 1995; Cruts *et al.*, 1996; Blacker *et al.*, 1998). The genetic loci responsible for the familial form are the presenilin 1 (*PS1*), presenilin 2 (*PS2*) and amyloid precursor protein (*APP*) genes (Rosenberg, 2000).

In a previous study we reported that lymphocytes of sporadic AD patients show significant levels of aneuploidy and a high percentage of premature centromere division (Migliore *et al.*, 1997). A lower sensitivity to the treatment *in vitro* with the aneuploidogen griseofulvin (GF) was also observed in AD patients as compared with the control group (Migliore *et al.*, 1997). In light of these findings, we aimed to confirm our results in 14 patients with sporadic and eight with familial Alzheimer's disease (FAD). We studied cytogenetic alterations by means of the micronucleus (MN) assay in peripheral lymphocytes and skin fibroblasts of the sporadic and familial cases, respectively. We assessed *in vitro* MN frequency occurring spontaneously or induced by aluminium (Al), an environmental factor which is thought, although with contradictory results, to increase the risk of developing AD (Martyn *et al.*, 1989; Doll, 1993; McLachlan *et al.*, 1996; Graves *et al.*, 1998; Makjanic *et al.*, 1998; Rogers and Simon, 1999). Al has been reported to affect chromosome segregation, as it induces in human lymphocytes *in vitro* a prevalence of MN containing whole chromosomes (Migliore *et al.*, 1999a).

Materials and methods

Fourteen sporadic AD patients and 11 healthy control subjects were identified. The two groups show comparable mean age, and approximately the same sex and smoking-habit distribution. A diagnosis of probable AD was made by using the criteria of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (McKhann *et al.*, 1984).

Eight FAD patients, identified on the basis of heritable mutations in the 'causative' genes, and six healthy controls were enrolled in the study. Patients were otherwise healthy and had no known previous history of exposure to toxic metals or recent X-ray examination. All patients were drug-free for at least two months before the examination was performed. Only two controls and patients are genetically related. C3 is the sister of F1 and F2; C4 is the first cousin of F4. Informed consent was obtained from each subject or their legal representatives prior to inclusion in the study.

Cell culture, chemical treatment, slide preparation and genotype analysis

Lymphocyte cultures

Blood stimulation, cell harvest and slide preparation were carried out according to the procedure described elsewhere (Migliore *et al.*, 1993), with the exception of cytochalasin B which was added to the cultures at 6 µg/ml final concentration. Treatments were performed in duplicate 24 h after the start of cultures and until harvesting (at 72 h). Treated cultures received 1 mM aluminium sulfate (Al; Sigma, St Louis, MO, USA) diluted in sterile distilled water. As negative control, cultures received sterile distilled water. Treatment was performed in a 100 µl volume.

Fibroblast cultures

Fibroblasts derived from skin biopsies were maintained in Ham F-12 medium (Sigma) supplemented with 7.5% fetal bovine serum (Gibco, Paisley, UK) and antibiotics at 37°C in a humidified atmosphere containing 6% CO_2 in air for 72 h. Confluent cultures were trypsinized and 1×10^5 cells ($\sim17\times10^3$ cell/cm^2) were seeded onto coverslips in 35 mm Petri dishes. Cells were exposed 24 h later and until harvesting to 15 µg/ml griseofulvin (GF; Sigma) dissolved in DMSO, which never exceeded 1% in the medium, and 1 mM Al. Negative controls were set up according to the maximum amount of solvent used to prepare each compound. All treatments were performed in a 100 µl volume. Optimal concentrations and exposure times were chosen according to preliminary cytotoxicity tests. Cyt B was added at 48 h directly to the medium at a final concentration of 6 µg/ml.

Cells were washed once with PBS and fixed twice with methanol/acetic acid (7:1); coverslips were then stained with 3% Giemsa (Merck, Germany) in distilled water for 10 min, air dried and mounted on clean slides.

FAD patients and the related controls were previously characterized for the known mutations by RT–PCR and RFLP methods according to the protocols described elsewhere (Rogaev *et al.*, 1995).

Slide scoring and statistical analysis

Coded slides were analysed using the optical microscope (final magnification ×400). 2000 binucleated cells per experimental point were analysed, following the criteria for MN acceptance listed by Fenech (1993). MN frequency was

[3]To whom correspondence should be addressed. Email: l.migliore@geog.unipi.it

Table I. Demographic characteristics of the population studied (sporadic AD patients group)

	No.	Age[a]	Sex[b]	Smoke[c]
Sporadic AD Patients	14	66.57 ± 9.17	6 m/8 f	3 s/11 ns
Controls	11	63.73 ± 7.16	5 m/6 f	3 s/8 ns

[a]Values represent the mean ± SD of each group.
[b]m, male; f, female.
[c]s, smoker; ns, no smoker.

Table II. Demographic and genetic characteristics of the population studied (familial AD patients group)

Individual code	Age[a]	Sex[b]	Onset	Family	Mutation
FAD patients					
F1	60	m	55	FLO21	PS1 L14392V
F2	45	f	40	FLO21	PS1 L14392V
F3	61	f	45	FLO10	PS2 M1239V
F4	67	f	52	FAD4	PS1 M14146L
F5	47	f	38	FLO11	PS1 M14146L
F6	62	m	48	FLO10	PS2 M1239V
F7	67	m	61	FLO12	APP717
F8	58	f	51	FLO13	APP717
	58.4 ± 8.3				
Controls					
C1	61	m			
C2	46	f			
C3	41	f		FLO21	
C4	64	f		FAD4	
C5	70	m		FLO12	
C6	52	f		FLO13	
	55.7 ± 11.2				

[a]Values represent the mean ± SD of each group.
[b]m, male; f, female.

expressed as the number of micronucleated binucleated cells (containing one or more MN) per 1000 cells**.** The percentage ratio of binucleated to total cells scored (including mono-, bi- and plurinucleated lymphocytes) was used as a parameter of cell toxicity. Multifactor analysis of variance was used to assess differences in spontaneous and induced MN frequency in either group after adjusting for age, sex and smoking habit.

Results

Demographic and genetic characteristics of the study population are reported in Tables I and II for sporadic and FAD patients, respectively. The group with sporadic AD comprised eight females and six males and the control group six females and five males. FAD patient group consisted of five females and three males and the control group four females and two males. The distribution of age is comparable between the two studied groups. With regard to genotype analysis, four FAD patients have mutations in the *PS1* gene, two FAD patients in the *PS2* gene, and two subjects in the *APP* gene. None of these mutations were present in controls.

Tables III–IV and Figure 1 give a summary of the results of the MN test in lymphocytes and fibroblasts of sporadic and FAD patients. The spontaneous MN frequencies of AD patients are significantly higher than those of the respective control groups. Compared to untreated lymphocyte cultures, Al treatment does not increase MN frequency in any sporadic AD patient. In the control group, Al induces MN in most subjects as compared with the respective control value. Similar results

Table III. Results of the MN test in lymphocytes of sporadic AD patients and their controls after treatment with 1mM $Al_2(SO_4)_3$(Al)

	H_2O		Al	
	MN ‰[a]	% BN	MN ‰[a]	% BN
Sporadic AD patients				
S1	11.0	35.0	12.0	20.7
S2	22.0	38.2	23.5	25.5
S3	19.0	39.5	17.0	22.0
S4	20.5	43.5	17.0	26.8
S5	19.5	42.4	18.0	24.5
S6	17.5	39.3	17.5	24.0
S7	12.0	35.4	15.0	27.0
S8	19.5	44.0	24.0	25.2
S9	26.5	36.3	28.5	23.5
S10	14.0	43.2	11.5	20.4
S11	21.0	45.5	15.0	26.0
S12	16.0	32.4	19.0	28.3
S13	23.0	35.5	22.0	27.8
S14	49.0	40.0	45.5	23.5
Mean ± SD	20.8 ± 9.2	39.3 ± 4.0	20.4 ± 8.6	24.7 ± 2.5
Controls				
C7	13.5	43.9	20.5	26.6
C8	7.5	44	13.5	23.5
C9	13	47.4	16.5	28.5
C10	6.0	34.6	8.5	20.9
C11	7.5	39.5	16.0	24.2
C12	7.5	44.6	19.5	21.8
C13	6.0	50.5	6.0	28.2
C14	9.5	48.3	17.5	25.6
C15	5.5	38.5	9.5	26.7
C16	14.0	46.5	31.0	22.4
C17	9.0	35.5	16.0	22.2
Mean ± SD	9.0 ± 6.8	43.0 ± 5.3	15.9 ± 6.8	24.6 ± 2.7

[a]MN frequency is calculated by scoring 2000 binucleated cells.

are observed for FAD fibroblasts treated with Al. MN frequency of FAD patient fibroblasts given Al does not differ significantly from untreated cultures. At variance, Al treatment increases MN level in all control subjects. In the case of GF treatment, all controls and two FAD patients show a significant increase in the basal level of MN. Table V reports the results of the analysis of variance on MN frequencies of the study population according to chemical treatment. In AD patients, GF increased the spontaneous MN frequency of somatic cells to a significantly lesser extent as compared with the control group. No effect of Al treatment was observed in lymphocytes and fibroblasts of AD patients.

Discussion

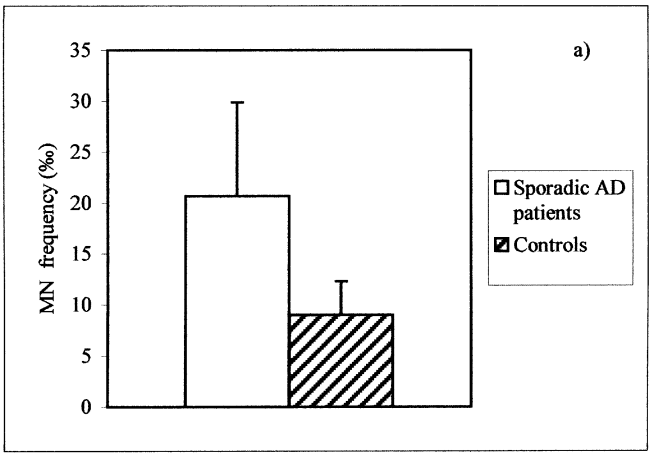
It is well-known that alteration of the structure and/or functioning of several components of the mitotic apparatus (tubulins, topoisomerase II, microtubule- or centromere-associated proteins) (Holloway, 1995) causes mistakes in the distribution of chromosomes to daughter cells thus leading to aneuploidy. It is therefore reasonable to suppose that some of these components, which could be altered in neuronal cells of AD patients (Matsuyama and Jarvik, 1989; Potter, 1991; Strittmatter *et al*., 1994), may be altered also in peripheral cell systems.

In the present study, we assessed *in vitro* the spontaneous and induced frequencies of cytogenetic alteration in somatic cells (e.g. peripheral lymphocytes and skin fibroblasts) of a group of sporadic and familial AD patients. We detected the presence of significantly higher spontaneous MN levels in

Table IV. Results of the MN test in skin fibroblasts of FAD patients and their controls after treatments with 1 mM $Al_2(SO_4)_3$ (Al) and 15 μg/ml griseofulvin (GF)

Individual	H_2O		Al		DMSO (1%)		GF[a]	
code	MN 0[b]	% BN	MN 0[b]	% BN	MN 0[b]	% BN	MN 0[b]	% BN
FAD patients								
F1	22.5	31.0	19.5	20.4	21.0	31.2	22.0	20.5
F2	29.0	38.3	27.0	23.5	24.5	48.0	38.0	21.4
F3	21.0	37.3	15.0	22.5	15.0	40.2	27.5	15.6
F4	23.5	46.3	24.5	26.8	22.0	34.3	36.5	25.1
F5	18.5	30.5	17.0	23.5	20.0	44.9	30.5	23.6
F6	20.5	32.5	15.5	24.0	23.5	29.4	44.0	23.9
F7	13.5	35.4	9.5	28.8	10.5	30.2	13.5	22.3
F8	17.0	33.2	13.5	25.2	16.5	33.0	34.0	25.0
Mean ± SD	20.7 ± 4.6	35.6 ± 5.2	17.7 ± 5.8	24.3 ± 2.6	19.1 ± 4.8	36.4 ± 7.1	30.8 ± 9.7	22.2 ± 3.1
Controls								
C1	3.5	41.9	20	26.7	3.5	38.0	48.5	21.4
C2	6	44.6	11.5	24.6	9	35.6	60	31.4
C3	5	50.5	13.5	28.1	6.5	48.5	44	30.1
C4	7.5	34.6	23	22.4	8	32.3	53	29.0
C5	13	26.0	21	21.2	9.5	26.7	39.5	17.5
C6	5	38.1	20	21.8	6.5	32.1	34	22.4
Mean ± SD	6.7 ± 3.4	39.3 ± 8.5	18.2 ± 4.6	24.1 ± 2.8	7.2 ± 2.2	35.5 ± 7.4	46.5 ± 9.4	25.3 ± 5.6

[a]GF dissolved in 1% DMSO.
[b]MN frequency is calculated by scoring 2000 binucleated cells.



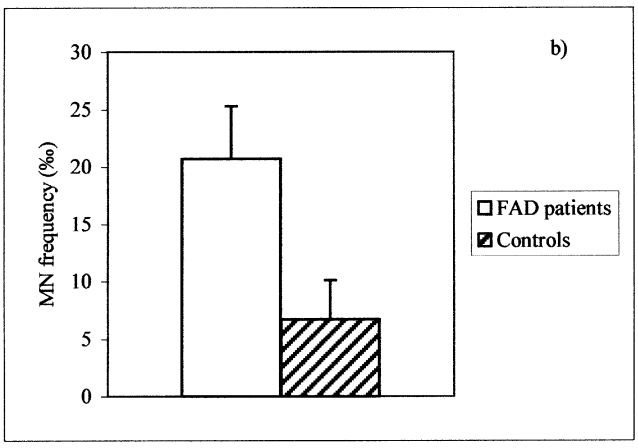


Fig. 1. Average MN frequency in (**a**) lymphocytes of sporadic AD patients and of control group ($P < 0.001$, ANOVA test); (**b**) fibroblasts of FAD patients and of control group ($P < 0.001$, ANOVA test).

Table V. Multifactor analysis of variance in lymphocytes and fibroblasts of the study population according to chemical treatment

Variable	Mean ± SD[a]	F-ratio[b]	*P*
Sporadic AD			
Al treatment		31 920	<0.001
Patients ($n = 14$)	–0.7 ± 2.6		
Controls ($n = 11$)	6.7 ± 3.4		
FAD			
Al treatment		55 514	<<0.001
Patients ($n = 8$)	–3.0 ± 2.2		
Controls ($n = 6$)	11.5 ± 4.7		
GF treatment		37 063	<<0.001
Patients ($n = 8$)	11.6 ± 6.7		
Controls ($n = 6$)	39.3 ± 9.3		

[a]Values represent the difference between treatment-induced and spontaneous MN frequencies.
[b]Sex, smoking habit and age controlled.

both cell types of AD patients as compared with healthy subjects. This confirms the previous findings that lymphocytes of subjects affected by sporadic AD and FAD skin fibroblasts are prone to undergo spontaneously chromosome mal-segregation (Migliore *et al.*, 1997, 1999b; Geller and Potter, 1999). On the other hand, folate deficiency causes increased chromosomal breakage in cultured lymphocytes (Dai *et al.*, 1986, Chen, 1989; Blount *et al.*, 1997), and AD patients show reduced levels of serum folic acid and/or an altered blood folate–homocysteine ratio (Joosten *et al.*, 1997; Clarke *et al.*, 1998).

The chemical treatment of cultured cells showed different patterns of MN induction depending on the substance used. GF treatment significantly increased MN frequency of lymphocytes and fibroblasts of the control group. Both fibroblasts and lymphocytes of AD patients were proved less sensitive to GF treatment. This is further evidence that GF treatment causes reduced MN induction in lymphocytes of sporadic AD patients (Migliore *et al.*, 1997). Griseofulvin, an antimicotic drug,

seems to induce aneuploidy via either nondisjunction or chromosome loss in human lymphocytes (Migliore *et al.*, 1999c). GF is able to bind microtubule associated proteins (MAPs) specifically affecting, therefore, microtubule (MT) polymerization (De Carli and Larizza, 1988). MT inhibitors caused either depolymerization of cytoplasmic MT and formation of neurofibrillary tangles (NFTs) (a peculiar morphological lesion in neuronal cells of AD) in experimental animals and cultured neurons, or a slower rate of MT repolymerization in AD lymphoblasts (Matsuyama and Jarvik, 1989). The decrease, at group level, of a significant response to GF suggests that both lymphocytes and fibroblasts of AD patients may have an impairment of the MAP apparatus.

The cultures receiving Al did not show an increase in the background level of MN for any sporadic and familial AD patients. On the contrary, Al treatment caused MN induction in peripheral lymphocytes of the control groups. Recently, the possibility that dietary aluminium intake could increase the risk of developing the disease was suggested (Rogers and Simon, 1999). Animals treated with Al developed both symptoms and brain lesions that are similar to those found in AD (Swegert *et al.*, 1999; Tsunoda and Sharma, 1999). Human lymphocytes given 1 mM aluminium sulfate showed a significant increase in DNA damage (MN formation) via both chromosome breakage and chromosome loss (Migliore *et al*., 1999a). The molecular mechanism of action of Al is at present not well elucidated, even though it seems to affect the MT system by binding to tubulins competing with Mg^{2+} ion which is the mediator of the polymerization process (Macdonald *et al*., 1987). The potential influence of aluminium ions on the phosphorylation of tubulins and brain microtubular proteins could be of great interest because NFTs contain hyperphosphorylated microtubule-associated protein tau. In addition, Al may interfere with the genetic material, particularly with gene transcription in the brain leading to impairment of cell physiology (Rao *et al.*, 1993; Lukiw *et al.*, 1998). Despite the fact that we do not know exactly what is the intracellular target of Al, either a saturation or an altered functioning of this target could explain the lack of sensitivity to Al treatment by lymphocytes and skin fibroblasts of AD patients.

In conclusion, the results of the present study indicate that different types of somatic cells from sporadic and familial AD patients show comparable levels of spontaneous cytogenetic alterations, and an MN induction partially reduced or lacking according to the type of chemical treatments. It is therefore conceivable to suppose that cells of peripheral systems of AD patients, irrespective of the form of the disease, share similar features leading to the occurrence, at the same extent, of cytogenetic anomalies.

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