Increased Levels of DNA Breaks in Cerebral Cortex of Alzheimer's Disease Patients

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Received 30 May 1989; Accepted 21 November 1989

MULLAART, E., M. E. T. I. BOERRIGTER, R. RAVID, D. F. SWAAB AND J. VIJG. *Increased levels of DNA breaks in cerebral cortex of Alzheimer's disease patients.* NEUROBIOL AGING 11(3) 169–173, 1990. —It has been hypothesized that Alzheimer's disease (AD) is caused by an accumulation of damage in DNA due to defective DNA-repair (21). Attempts to test this hypothesis by determining the activity of DNA-repair systems in nonneuronal cells from AD patients and controls so far provided conflicting results. An alternative approach is the direct comparison of DNA-damage levels in neuronal tissue of AD patients and controls. In the present study we assayed the level of DNA breaks and alkali-labile sites in cerebral cortex tissue samples from AD patients and controls obtained from rapid autopsies. Our data on 11 AD patients and 8 control subjects indicate an at least two-fold higher level of DNA damage in cortex of AD patients as compared to controls.

DNA breaks Alzheimer's disease Cerebral cortex Alkaline elution

ALZHEIMER'S DISEASE (AD), the most common cause of dementia, is generally characterized by brain atrophy caused by neuronal shrinkage and/or death, neuritic plaques and neurofibrillary tangles (28,29). It has been suggested that the degradation and death of neurons in AD brains is caused by an accumulation of DNA damage, as a consequence of a general DNA repair defect (21). This hypothesis was derived from the observation that nonneuronal cells such as fibroblasts and lymphocytes isolated from AD patients are more sensitive to gamma rays and certain alkylating agents as compared to cells of the same types derived from normal controls; no defect was observed in the survival of such cells after UV-irradiation (22, 23, 26). These observations suggest that AD cells are less able to remove lesions induced by gamma rays and alkylating agents, such as single-strand breaks (SSB) and various small base damages, which are normally removed via short-patch repair (20). Indeed, both Li and Kamin-skas (14) and Robison *et al.* (24) reported a decreased capacity to remove DNA breaks and alkali-labile sites (damage that is converted into breaks in alkali) induced by alkylating agents in fibroblasts from AD patients, when compared to cells from control individuals. However, Kinsella *et al.* (10,11), using the same fibroblast lines, did not observe such a difference. Preliminary results from our own laboratory also indicate proficient repair of alkylating agent-induced alkali-labile sites in fibroblast cell lines from familial AD patients.

Indications for an increased level of DNA damage in the brains of AD patients are the observed alterations in the extent of DNA supercoiling (1) and chromatin structure (6,13) in the cortex. Such changes, which might well be due to an increased level of DNA breaks, are important since they could be the underlying cause of the reduced level of total poly(A)⁺ RNA (25) and the loss of a number of messenger RNAs (5) observed in the cortex of AD patients.

A direct way of testing the hypothesis that AD is associated with the occurrence of excessive amounts of DNA damage in the brain, is by assaying the level of DNA breaks and alkali-labile sites in neuronal tissue of AD patients and controls. In the present study the highly sensitive alkaline elution assay was used to quantitate the amount of DNA breaks and alkali-labile sites in brain samples from AD patients and controls of about the same age. The results presented here indicate an about two-fold increase in the level of DNA breaks in cortex of AD patients as compared to control subjects.

METHOD

Brain Material

Human brain material was obtained from rapid autopsies (1.5–6 hr postmortem delay) and diagnoses were made on the basis of clinical records and neuropathological confirmations (Table 1) (neuropathological protocol by F. C. Stam, W. Kamp-

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FIG. 1. The effect of postmortem delay on the level of breaks detected in DNA from human cerebral cortex of control individuals. (A) Alkaline elution curves of DNA from control human occipital cortex with a postmortem delay of 2.5 hr (○) (87–340.4) and 6 hr (●) (88–9.3). Each point is the mean ($\pm$ SD) of 3 independent elution experiments with the same cortex sample. (B) Relationship between the amount of DNA breaks detected in human occipital (□) or frontal cortex (■) and the postmortem delay. Lines were drawn according to linear regression analysis; slopes 1212 ± 371 and 1645 ± 304 breaks/nucleus/hr for occipital and frontal cortex, respectively. Each point is the mean ($\pm$ SD) of independent elutions with 3 samples from each cortex.

horst, Free University of Amsterdam, the Netherlands and D. Troost, Academic Medical Center, Amsterdam, the Netherlands). The AD group showed extensive neocortical and hippocampal neuropathological changes (senile plaques and tangles) typical for AD. The areas examined are cortical areas: 4, 10, 17, 18, 20, 38 and 42, corpus striatum, gyrus brevis, hippocampus, mesencephalon including substantia nigra, medulla oblongata, thalamus, locus coeruleus and cerebellum. The control brains displayed no neuropathological abnormalities.

Isolation of Brain Nuclei

Cerebral cortex was cut into small pieces and washed twice with 10 ml PBS/EDTA (8.1 mM Na_2HPO_4 , 15 mM KH_2PO_4 , 0.14 M NaCl, 2.6 mM KCl and 5 mM EDTA pH 6.7). A suspension was made by gently pipetting up and down, using a pipet with a 1-mm diameter opening. The suspension was filtered through a cotton gauze and centrifuged (10 min at 1300 rpm), after which the pellet was resuspended in about 10 ml PBS/EDTA. This isolation method resulted in a suspension of nuclei, 70% of which could be microscopically identified as neuronal nuclei (9).

Determination of the Number of DNA Breaks

The amount of DNA damage was determined by using the alkaline elution assay as described previously (18,19), but with some modifications. About 1.10^6 nuclei, suspended in 0.5 ml PBS/EDTA, were added to 0.5 ml sarkosyl lysis buffer (0.2% sarkosyl, 2 M NaCl and 0.02 M EDTA, pH 10.0) layered onto a 25-mm diameter polycarbonate membrane filter (Nuclepore; pore size 5 μm). The lysis buffer was allowed to pass through the filter by gravity and replaced by 3 ml sarkosyl lysis buffer containing proteinase K (0.5 mg/ml). After a 1/2-hr lysis period at 20°C the filter was drained by gravity and the DNA on the filter was washed twice with 5 ml 0.02 M EDTA, pH 10. The DNA was finally

eluted through the filter with 0.06 M NaOH, 0.02 M EDTA, pH 12.6 (calculated) at 30 $\mu\text{l}/\text{min}$. Six 4.5-ml fractions were collected. After the elution, the filter with the residual DNA was transferred to a glass vial and, after addition of 4.5-ml elution buffer, irradiated with 100 Gy of ^{60}Co -gamma rays (Gamma-cell 100, Atomic Energy of Canada) in order to release the DNA. The 6 eluate fractions and the irradiated membrane fraction were neutralized with 0.8 ml 4 M NaCl, 0.6 M NaOH, 1.0 M NaH_2PO_4 , which further contained Hoechst 33258 dye (0.5 mg/l). The fluorescence of the Hoechst/DNA complex was measured at 430 nm (filters 3-73, 4-76 and 5-58) by excitation at 370 nm (filters 7-54 and 7-60) in a Pye Unicam LC-FL detector. The elution results were plotted as the log percentage of DNA remaining on the filter as a function of the fraction number. Mean slopes of the linear initial part of three independent elution curves from each sample were used to calculate the number of DNA breaks. Since many elution curves were too steep to allow accurate determination of the initial slope, a set of calibration graphs was prepared by performing elutions with gamma-irradiated human lymphocytes (range: 0–15 Gy gamma rays). The steep experimental curves were fitted to these graphs, the number of lesions was expressed in Gy-equivalents and converted into breaks on basis of the assumption that 1 Gy corresponds to 1,000 breaks per nucleus (30). Beyond 10,000 breaks per nucleus (about 10 Gy) no further discrimination is possible.

RESULTS

We have used a highly sensitive alkaline elution assay to quantify the amount of DNA damage present in brain samples from AD patients and controls. The DNA breaks detected with this assay are either single-strand breaks (SSB) already present in the DNA or originate from DNA lesions which are converted into breaks during alkali treatment (alkali-labile sites) (12). The method is based on elution of DNA through membrane filters, which

TABLE 1
HUMAN BRAIN SAMPLES

Donor	Age (years)	Sex	Brain Weight (g)	PM Delay (hr)*	PH	Cause of Death	Damages per Nucleus† ($\times 10^{-3}$)			
							Uncorrected		Corrected‡	
							Occipital	Frontal	Occipital	Frontal
(A) Controls§										
87-290.0	51	f	1190	2.75	6.50	Broncho alveolar carcinoma; no metastases in brain.	5.1 ± 0.4	3.7 ± 0.5	5.0 ± 0.4	3.6 ± 0.5
87-340.4	76	m	1245	2.5	6.89	Lung carcinoma; no metastases in brain.	6.3 ± 0.2	6.4 ± 0.6	6.5 ± 0.2	6.7 ± 0.6
88-9.3	69	f	nd	6.0	6.10	Septic shock in Non-Hodgkins Lymphoma following treatment with cytostatics.	≥ 10	9.7 ± 0.7	≥ 6.0	4.2 ± 1.4
88-29.4	60	m	1350	5.0	6.45	Septic shock following aorta valve implantation.	8.1 ± 0.7	9.6 ± 0.9	5.4 ± 0.9	5.8 ± 1.3
88-194.3	65	m	1310	4.25	6.18	Heart failure.	≥ 10	8.7 ± 1.0	≥ 8.1	6.1 ± 1.2
88-231.2	73	m	1410	3.50	6.93	Postoperative heart failure.	7.7 ± 0.7	4.9 ± 0.2	6.7 ± 0.8	3.6 ± 0.4
88-328.1	66	f	1100	5.5	6.24	Postoperative heart failure.	9.7 ± 2.2	≥ 10	6.3 ± 2.3	≥ 5.4
88-366.0	71	f	1240	4.25	6.28	Sepsis and cardiogenic shock.	≥ 10	7.2 ± 0.3	≥ 8.1	4.7 ± 0.7
mean	66		1263	4.22	6.45				6.5	5.0
SEM	(3)		(42)	(0.44)	(0.11)				(0.4)	(0.4)
(B) AD patients§										
87-318	88	f	1070	2.75	6.93	Pneumonia.	≥ 10	≥ 10	≥ 9.9	≥ 9.9
87-329	88	f	960	2.25	6.30	Non-Hodgkins lymphoma, broncho pneumonia, heart failure.	≥ 10	≥ 10	≥ 10.5	≥ 10.7
88-082	81	f	990	2.50	6.32	Heart failure.	≥ 10	8.5 ± 0.9	≥ 10.2	8.8 ± 0.9
88-090	70	m	865	2.50	6.14	Subdural hematoma.	9.4 ± 3.7	6.0 ± 0.6	9.7 ± 3.7	6.3 ± 0.6
88-109	85	f	1020	1.50	6.32	Intestinal bleeding.	≥ 10	9.3 ± 0.4	≥ 11.5	11.3 ± 0.6
88-252	66	m	1250	2.25	6.50	Cachexia and presumable sepsis.	9.3 ± 1.1	7.1 ± 0.7	9.8 ± 1.1	7.9 ± 0.7
88-255	82	f	980	3.50	6.40	Sepsis; Pneumonia.	≥ 10	≥ 10	≥ 9.0	≥ 8.7
88-272	84	f	1000	3.75	6.42	Septic shock; cachexia; basilar artery insufficiency.	9.9 ± 0.7	≥ 10	8.7 ± 0.8	≥ 8.3
88-296	88	f	940	2.5	6.42	Myocard infarct.	≥ 10	≥ 10	≥ 10.2	≥ 10.3
88-303	79	f	1190	4.0	6.44	Cachexia and internal bleeding as result of chronic myeloid leukemia.	9.9 ± 0.4	9.7 ± 0.2	8.3 ± 0.6	7.6 ± 0.5
88-325	64	f	800	2.5	6.44	Cachexia and dehydration.	8.6 ± 0.2	8.2 ± 0.2	8.9 ± 0.2	8.5 ± 0.2
mean	80		1006	2.73	6.42				9.7	8.9
SEM	(3)		(39)	(0.22)	(0.06)				(0.3)	(0.4)
(C) Parkinson's patients§										
87-235	80	f	1180	3.0	6.99	Pneumonia.	5.7 ± 0.3	6.8 ± 0.7	5.3 ± 0.3	6.3 ± 0.7
88-072¶	92	f	1020	3.5	6.09	Heart failure.	>10	8.3 ± 0.8	>9.0	7.0 ± 0.9

*The PM delay (postmortem delay) is defined as the period between death and the moment the brains were placed on ice.

†The number of damages per nucleus is given as the mean ($\pm$ SD) of 3 different elutions performed on the same brain sample.

‡Amount of damages per nucleus after normalization to 2.7-hr postmortem delay.

§The diagnosis of the subjects as controls, AD patients or Parkinson disease patients was made as described in the Method section.

¶Patient having the neuropathological signs of both Parkinson and AD.

nd = not determined.

requires the unwinding into single strands. Breaks form initiation points of unwinding; consequently, the more breaks the higher the elution rate. In principle, the method can be used to study various forms of DNA lesions in autopsy material. However, a serious problem that became apparent during the course of the present study, is the postmortem induction of DNA breaks.

The adverse effect of the postmortem interval on the DNA integrity of human cerebral cortex is illustrated by comparing elution curves of nuclei isolated from occipital cortex after short

and long postmortem intervals (Fig. 1A). The slope of such curves represents the elution rate of the DNA through the filter. From Fig. 1A it is clear that the 6-hr postmortem sample with the steeper slope contains more DNA breaks than the 2.5-hr postmortem sample. In order to estimate the level of DNA breaks present, these elution curves were compared with those obtained using gamma-irradiated cell samples. When the level of DNA breaks in occipital and frontal cortex of 8 human control subjects (as judged by histopathological analysis; see Table 1) was plotted against the

FIG. 2. Typical alkaline elution curves obtained with DNA from a control individual (87–290.0) (○) and an AD patient (87–318) (●). Each point is the mean ($\pm$ SD) of 3 independent elution experiments with the same cortex sample.

postmortem delay, a fairly linear increase was observed (Fig. 1B). In DNA from cerebral cortex of both young and old rats break levels were found to increase as well with prolonged postmortem delay, albeit at a slower rate (16,19).

Typical elution curves obtained with DNA from the cerebral cortex of a control individual and of an AD patient, both with short postmortem intervals (2.75 hr), are shown in Fig. 2. The slopes are clearly different, the steeper slope belonging to DNA isolated from the AD patient. When comparing the level of DNA breaks in cerebral cortex of 11 AD patients with that of 3 control subjects at short postmortem delays (i.e., ≤ 4 hr) an approximately two-fold increase was observed in AD patients; 6400 ± 1300 and 5000 ± 1400 breaks per cell for the occipital and frontal cortex of the control subjects compared to 9700 ± 500 and 9000 ± 1300 breaks per cell for the occipital and frontal cortex of the AD patients (Table 1).

In order to compare all brain samples studied, the linear relationship between postmortem delay and the level of DNA breaks (Fig. 1B) was used to normalize all values to a postmortem delay of 2.7 hr (mean postmortem delay of the AD group). The results confirmed the difference observed after selection for short postmortem delays: the level of DNA breaks in both occipital and frontal lobes of cerebral cortex of the AD group was significantly higher than that in the control group ($p < 0.0005$, as tested by Student's *t*-test; Table 1).

During the course of this study, brain samples from 2 patients having Parkinson's disease became available. One of these had the neuropathological features of both Parkinson and AD (88-072). Interestingly, the cortex from the other patient who had Parkinson's disease without dementia (87-235), had low levels of DNA breaks, whereas the DNA of the Parkinson/AD patient DNA contained about twice as many lesions per nucleus (Table 1).

DISCUSSION

A highly sensitive alkaline elution assay was used to quantitate

the level of "spontaneous" DNA breaks and/or alkali-labile sites in the cerebral cortex from AD patients and controls. Our data on 11 AD patients and 8 control individuals, corrected for postmortem delay, indicate an approximately two-fold higher level of DNA breaks in cortex of AD patients as compared to controls. Comparison of the uncorrected number of breaks in AD and controls selected for short postmortem intervals, revealed the same difference.

The possibility that the difference in breakage level between AD and control is related to the difference in mean age (66 ± 3 and 80 ± 3 for controls and AD patients, respectively), is unlikely since the levels of DNA breaks in the two youngest AD patients (64 and 66 years of age) are about twice as high as the levels in the two oldest control subjects (73 and 76 years of age). In rodents there seems to be no age-related increase in the level of DNA breaks (7,19). However, it should be realized that Chetsanga *et al.* (3) did observe an increased level of breaks in the brains of old mice.

It should be noted that the number of breaks in the cortex DNA of many AD patients, and some controls, was so high that very steep elution curves were obtained, which allowed only the estimation of the minimal levels of DNA breaks ($\geq 10,000$ per nucleus). On the average, this phenomenon very likely has resulted in an underestimation of the amount of lesions present in most of the AD patients relative to controls. The question may arise whether in these samples still lesions in intact DNA were determined, or that strongly degraded DNA was assayed predominantly originating from necrotic cells. Microscopic examination of the brain samples did not favor the latter explanation, however; the nuclei had the normal appearance. Moreover, when alkaline sucrose gradient centrifugation was applied, a less sensitive method for detection of DNA breaks (17,31), extensively degraded DNA was not observed; the levels remained below 60,000 breaks per nucleus (results not shown). This result was confirmed by using field inversion electrophoresis (2) experiments, which demonstrated that the DNA of the AD and control samples tested was of high molecular weight (longer than 10^6 basepairs) (results not shown).

Nevertheless, it should be realized that the increased level of DNA damage observed in the cortex of AD patients could still be an effect rather than the cause of the disease. For example, even moderately increased DNA breakage could be a side effect of neuronal degeneration or could result from an extended agonal state. Although this possibility can not be ruled out completely, several points argue against it. In the first place, the level of DNA breaks in AD brains was found to be slightly higher in occipital than in frontal cortex, while cell degeneration and cell death is generally more severe in the frontal cortex (4,27). Secondly, the pH of the brain, which is a good indicator for the agonal state of the patients (8), did not differ between AD patients and control individuals (Table 1; $p > 0.5$ as tested by Student's *t*-test). The fact that the cerebral cortex of a Parkinson's patient did not contain elevated levels of DNA damage, might suggest that an increased level of DNA breaks in cortex is specific for AD and not a general phenomenon associated with neuronal degenerative diseases.

On the basis of the results presented above we interpret our data as evidence of a disturbed DNA-damage metabolism in AD patients. In this respect it is tempting to seek the cause of this disease in a general defect in a DNA-repair pathway via which brain-specific DNA lesions are removed as proposed by Robbins (21). However, the elevated level of DNA damage in AD brains could just as well result from a higher rate of damage induction as a consequence of alterations in cellular metabolism. For example, there is evidence for a higher rate of lipid peroxidation in AD brains (15), which might lead to an increase in DNA-damage induction.

ACKNOWLEDGEMENTS

We thank Drs. F. Berends, J. A. Hardy, D. L. Knook and P. H. M. Lohman for critically reading the manuscript and Mr. M. Boermans for

preparing the figures. This study was supported by Senetek PLC, the Dutch Ministry of Welfare and Health Affairs and the Sandoz Foundation for Gerontological Research. Brain samples were provided by The Netherlands brain bank, Amsterdam.

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