T-CELL restricted intracellular antigen related protein (TIAR) is an RNA-binding protein that is supposed to be involved in the process of stress-induced apoptosis. TIAR triggers DNA fragmentation in permeabilized thymocytes and its expression diminishes in the cell nucleus and rises simultaneously in the cytoplasm during Fas-induced cell death. Using a monoclonal antibody against TIAR, we stained different areas of the hippocampus from seven controls and 14 patients with Alzheimer's disease (AD). There was a clear expression of TIAR in the hippocampus of non-demented controls. Surprisingly, a significant increase was found in the expression of TIAR in the hippocampal area in AD. The increased expression of TIAR in AD may be related to the process of neurodegeneration in the hippocampus. *NeuroReport* 9: 1451-1454 © 1998 Rapid Science Ltd.

Key words: Alzheimer's disease; Apoptosis; Hippocampus; Human; Immunocytochemistry; TIAR

Increased expression of the TIAR protein in the hippocampus of Alzheimer patients

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Introduction

T-cell restricted intracellular antigen related protein (TIAR) is an RNA-binding protein related to the T cell-associated protein, TIA-1. There is about 90% similarity between TIAR and TIA-1 proteins.¹ TIAR, which has two isoforms, 42 kDa and 50 kDa, is involved in triggering DNA fragmentation in permeabilized thymocytes suggesting a role in the process of stress-induced apoptosis.¹

TIAR is generally concentrated in the nucleus where it decorates RNA tracts, the sites of active RNA transcription. In Jurkat cells (human acute T-cell leukemia) undergoing apoptosis, TIAR was found to accumulate in the cytoplasm, while exogenous stimuli, such as an anti-CD3 antibody, which triggers cellular activation but not apoptosis did not induce redistribution of TIAR.^{1,2} Interestingly, this redistribution of TIAR seems to be relatively specific to this protein, since the expression of most nuclear proteins does not change during apoptosis.¹ It has been shown that redistribution of TIAR during Fas-mediated apoptosis precedes DNA fragmentation and is not a general consequence of nuclear disintegration.¹

The TIAR gene was cloned in 1992³ and its protein was found to be concentrated in the nucleus of hematopoietic and non-hematopoietic cells.¹ The present study was carried out to study for the first time the expression of TIAR in the human hippocampus, and possible changes in the expression of this molecule in Alzheimer's disease (AD).

Materials and Methods

Human tissue: Human tissue was provided by the Netherlands Brain Bank (coordinator Dr R. Ravid). Seven controls and 14 patients with AD were studied (Table 1). The AD patients were clinically assessed and diagnosed as probable AD by excluding other possible causes of dementia by history, physical examination and laboratory tests according to the NINCDS-ADRDA criteria.⁴ The clinical diagnosis of AD and the integrity of the controls were confirmed by neuropathological examination. The AD brains showed extensive neocortical and hippocampal senile plaques, neurofibrillary tangles and dystrophic neurites in a variety of areas. Unlike age, brain weight was found to be significantly different between controls and AD patients (Table 1).

Western blot: Using 0.1 g of protein extracted from the hippocampus of two subjects, one control (89 years old) and one AD patient (90 years old), a Western blot was performed on 11.5% SDS gels. As a positive control, an extract of mouse macrophages was used, prepared from the RAW 264.7 cell line (Transduction Labs, USA). The gel was blotted on nitrocellulose paper for 2.5 h. This was followed by blocking the staining in supermix (0.5 M Tris, 1.5 M NaCl, 2.5% gelatin and 5% Triton X-100; pH 7.6; Sigma, USA) and by staining with the antibody against TIAR (1/5000).

Table 1. Clinical data on control and Alzheimer brains used

Group	number	age (yrs)	PMD	BW	pH-CSF	GDS
Control	5m/2f	73.7 ± 0.7	5.24 ± 0.11*	1303 ± 19.6*	6.73 ± 0.32	–
Alzheimer	9m/5f	74.3 ± 1.15	3.98 ± 0.08	1174 ± 17.9	6.72 ± 0.08	6.64 ± 0.23

PMD: postmortem delay (in hours); GDS: global deterioration scale (Reisberg *et al.* 1982); BW: brain weight (in grams); m, male; f: female); Data expressed as mean ± standard error of the mean; * $P < 0.01$. After excluding the three subjects with the highest postmortem delay in the control group, the difference in postmortem delay was not significant any more. And the percentage of TIAR positive neurons was still significant, for the different areas of the hippocampus, when AD patients were compared to controls.

Immunocytochemistry: The monoclonal antibody against human TIAR (Transduction Labs, USA) was raised against a 22.9 kDa protein fragment corresponding to amino acid 161–365 of human TIAR. After rehydration in a series of ethanols, endogenous peroxidase was blocked by 2% hydrogen peroxide in methanol. Sections were treated by microwaving⁵ in citrate buffer twice for 5 min at 80°C. After cooling, the sections were pre-incubated in a solution of 1% bovine serum albumin (BSA), 2% horse serum in phosphate buffered saline (PBS; pH 7.4) for 30 min, followed by overnight incubation with a monoclonal antibody against TIAR in PBS at 4°C (1:5000). Sections were then incubated for 60 min with biotinylated anti-mouse IgE (Vector, USA) in 1% BSA in PBS solution (1:200). Subsequently, they were incubated with ABC-Elite (Vector, USA) in 1% BSA in PBS solution (1:500) for 60 min. Finally, they were washed in tris-buffered saline (TBS; pH 7.6) and stained with diaminobenzidine (Sigma, USA) containing ammonium nickel sulphate and hydrogen peroxide in TBS for 15 min. The reaction was terminated by washing the sections in TBS.

Quantification: An IBAS-KAT image analysis system (Kontron KAT Based system) was used to quantify the TIAR stained neurons. The image analysis system was connected to a CCD video camera (Sony XC-77CE: for details see Ref. 6). For each subject two to three sections were used in order to count the number of neurons stained by TIAR. Each section was analyzed as follows. First, an image was loaded with a ×2.5 objective, and CA1, CA2-3 and CA4, the dentate gyrus and subiculum were outlined separately. A grid of rectangular areas was then superimposed over the image and viewed through a ×40 objective. Coordinates of all grids covered by the areas of the hippocampal outline were stored. There was a systematic random selection of 15–25 fields per area by the computer, the images of which were automatically loaded into the image analyzer with a ×40 objective. Each neuronal profile containing a nucleolus was manually indicated as either positive or negative, and the total number of stained and non-stained neural profiles was calculated

(a total of 30,134 neurons was analyzed). Double nuclei were not observed.

Statistical methods: The differences in mean percentage of TIAR-positive neurons in controls and AD patients were tested using the Mann-Whitney U-test. The Pearson's test was used to study the correlation between different parameters. Two-way ANOVA was used to study the interactions between the disease and the brain area on the number of cells expressing TIAR.

Results

Western blot: The Western blots of control and AD hippocampi showed two clear bands at 46–48 kDa, confirming the expression of two different isoforms

FIG. 1. Western blot of homogenates from the hippocampus of a control and an AD patient. Note the clear increase in the intensity of the two bands at 46–48 kDa in the AD brain.

FIG. 2. Immunocytochemical staining of TIAR in the CA1 area of a representative control (#91120; **a**) and in the CA1 area of a representative AD patient (#95113; **b**). Note the clear increase in the number of TIAR-stained neurons in AD. Scale bar: 56 μ m.

FIG. 3. Graph showing the percentage of TIAR-stained neurons in controls and AD patients. Note that the greatest increase in percentage of TIAR-positive neurons occurs in the CA1 area of the hippocampus.

of TIAR in the human brain. The mouse macrophage extract also showed a band at 46 kDa. As shown in Fig. 1, unlike the protein extract from the control subject, the hippocampus of the AD patient showed two clear bands around 46 kDa.

Immunocytochemistry: Microscopically, a considerable number of neurons was stained in AD cases and in controls. The staining was almost exclusively nuclear. Both in controls and in AD patients only a few neurons (not more than one or two per section) showed cytoplasmic staining when the entire hippocampal area was screened (Fig. 2a,b).

Quantification: There was a higher percentage of TIAR-positive neurons in the different areas of the hippocampus in AD patients than in controls (Fig. 3). The CA2-3 area showed the highest percentage of TIAR-positive neurons in both controls and AD patients. The two-way ANOVA did not show any significant effect of brain area on the number of neurons expressing TIAR ($p = 0.105$; $F = 1.97$). No significant correlation was found between the number of TIAR-positive neurons and age of the subjects.

Discussion

In the present study we showed for the first time that TIAR is expressed in the hippocampus of the human brain. Furthermore, a clearly increased expression of TIAR was found in the AD brain compared with that of controls. It has been reported in other cellular systems that TIAR, which is normally a nuclear protein, migrates to the cytoplasm in the process of apoptosis.^{1,2} Increased cytoplasmic staining was, however, not observed in AD. We did not observe many neurons with cytoplasmic TIAR in either controls or in AD patients, suggesting that these neurons are not in the process of apoptosis or that neurons that had undergone apoptosis were already phagocytized.

Because of massive shrinkage in the hippocampal area in AD, one may ask whether the increased intensity of nuclear TIAR staining in the hippocampus of AD patients is due to high condensation of nuclei. However, this can be ruled out by the observation that the remaining neurons in the hippocampus do not seem to shrink significantly in AD.⁷

Our previous data obtained using TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labelling) suggests that apoptosis in the human hypothalamus is extremely rare.⁸ However, numerous neurons in the hippocampus of the AD brain are TUNEL positive.⁸⁻¹² This is very much in agreement with the

increased expression of TIAR molecule being related to apoptosis. The TIAR molecule is expressed at a very low level in the hypothalamus, a brain area that shows little cell death in AD¹³ (our own unpublished observations), which also agrees with the idea that TIAR is involved in the process of neurodegeneration.

Conclusion

Our study shows a clear difference in the expression of TIAR in the hippocampus of control and AD brains. Furthermore, we found a clear increased immunoreactivity with TIAR in the hippocampus of AD patients.

References

1. Taupin JL, Tian Q, Kedersha N *et al.* *Proc Natl Acad Sci USA* **92**, 1629–1633 (1995).
2. Anderson P. *Micro Mol Biol Rev* **61**, 33–46 (1997).
3. Kawakami A, Tian Q, Duan X *et al.* *Proc Natl Acad Sci USA* **89**, 8681–8685 (1992).
4. McKhann G, Drachman D, Folstein M *et al.* *Neurology* **34**, 939–944 (1984).
5. Shi SR, Key ME and Kalra KL. *J Histochem Cytochem* **39**, 741–748 (1991).
6. Salehi A, Verhaagen J, Dijkhuizen PA *et al.* *Neuroscience* **75**, 373–387 (1996).
7. Salehi A, Ravid Eh Gonatas NK *et al.* *J Neuropathol Exp Neurol* **54**, 704–709 (1995).
8. Lucassen PJ, Chung WCJ, Kamphorst W *et al.* *J Neuropathol Exp Neurol* **56**, 887–900 (1997).
9. Lassmann H, Bancher C, Breitschopf H *et al.* *Acta Neuropathol* **89**, 35–41 (1995).
10. Smale G, Nichols NR, Brady DR *et al.* *Exp Neurol* **133**, 225–230 (1995).
11. Li W, Chan W, Lai H *et al.* *J Mol Neurosci* **8**, 75–82 (1997).
12. Cotman CW and Anderson AJ. *Mol Neurobiol* **10**, 19–45 (1995).
13. Swaab D, Grundke-Iqbal K, Iqbal K *et al.* *Brain Res* **550**, 239–249 (1992).
14. Reisberg B, Ferris SH, De Leon MJ *et al.* *Am J Psychiat* **139**, 1136–1139 (1982).

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